MEK/ERK Dependent Activation of STAT1 Mediates Dasatinib-Induced Differentiation of Acute Myeloid Leukemia

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Abstract
Dasatinib (BMS-354825) is a FDA-approved multitargeted kinase inhibitor of BCR/ABL and Src kinases. It is now used in the treatment of chronic myelogenous leukemia (CML) with resistance or intolerance to prior therapies, including imatinib. Here we report a novel effect of dasatinib on inducing the differentiation of acute myeloid leukemia (AML) cells through MEK/ERK-dependent activation of signal transducer and activator of transcription 1 (STAT1). We found that dasatinib could induce the differentiation of AML cells as demonstrated by the expression of differentiation marker CD11b, G0/G1 phase arrest and decreased ratio of nucleus to cytoplasm. Of note, dasatinib induced robust phosphorylation of STAT1 both at Tyr701 and Ser727 as well as the redistribution of STAT1 from the cytoplasm to the nucleus, thus leading to the transcription of STAT1-targeted genes. Knocking down STAT1 expression by shRNA significantly attenuated dasatinib-induced differentiation, indicating an important role of STAT1 in myeloid maturation. We further found that dasatinib-induced activation of STAT1 was regulated by the MEK/ERK kinases. The phosphorylation of MEK and ERK occurred rapidly upon dasatinib treatment and increased progressively as differentiation was induced. MEK inhibitors PD98059 and U0216 not only inhibited the phosphorylation of STAT1, but also abrogated dasatinib-induced myeloid differentiation, suggesting that MEK/ERK dependent phosphorylation of STAT1 might be indispensable for the differentiating effect of dasatinib in AML cells. Taken together, our study suggests that STAT1 is an important mediator in dasatinib-induced differentiation of AML cells, whose activation requires the activation of MEK/ERK cascades.

Citation: Fang Y, Zhong L, Lin M, Zhou X, Jing H, et al. (2013) MEK/ERK Dependent Activation of STAT1 Mediates Dasatinib-Induced Differentiation of Acute Myeloid Leukemia. PLoS ONE 8(6): e66915. doi:10.1371/journal.pone.0066915

Editor: Kevin D. Bunting, Emory University, United States of America

Received February 25, 2013; Accepted May 10, 2013; Published June 25, 2013

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Funding: This work was supported by grants from Zhejiang Provincial Program for the Cultivation of High-Level Innovative Heath Talents, Natural Science Foundation of Zhejiang Province (number Y12H310029), Research Foundation of Education Bureau of Zhejiang Province (Y201121007), and Science Foundation of Chinese University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction
Acute myeloid leukemia (AML), characterized by a differentiation blockage and accumulation of immature myeloid cells, has been recognized as a heterogeneous disorder in clinic [1,2]. The concept of differentiation therapy has been considered as a promising approach for the treatment of AML since 1970s [3]. In 1980s, the successful use of all-trans-retinoic acid (ATRA) in acute promyelocytic leukemia (APL) that elicited complete remission (CR) of more than 90% of APL patients has brought differentiation therapy from theoretical exploration to clinical application [4,5]. Although great breakthrough in clinical oncology has been achieved by differentiation therapy with ATRA, ATRA-based therapy showed poor results in non-APL AMLs; thus tremendous efforts have been conducted to explore novel molecular targets as well as identify efficient differentiating compounds in AML treatment.

The off-target effects of tyrosine kinase inhibitors (TKIs) on inducing AML differentiation have been a topic of considerable interest in the last few years. Imatinib, the first BCR/ABL inhibitor, has been revealed to exert an unexpected effect on potentiating ATRA-induced AML differentiation [6]. The EGFR inhibitor gefitinib has also been demonstrated to enhance ATRA-induced differentiation of leukemic cells [7] and to trigger the differentiation of AML cell lines (HL60, kasumi-1 and U937) that lack the expression of EGFR [8] when used alone. Dasatinib, which targets a variety of tyrosine kinases, most notably the BCR/ABL fusion protein and the Src Family Kinases (SFKs), is used for the treatment of BCR/ABL+ CML and acute lymphocytic leukemia with imatinib resistance or intolerance to previous therapy [9,10,11,12]. In vitro studies have shown that dasatinib significantly inhibited the growth of a variety of AML cell lines and primary blasts when treated alone or in combination with cytotoxic or molecular-targeted agents [13,14]. Of note, dasatinib was also reported to promote ATRA-induced differentiation of AML cell lines and restore differentiating response of non-APL primary AML cells to ATRA, while dasatinib alone could not promote leukemic cell differentiation [15,16]. In contrast, Lainey
et al.’s study demonstrated that dasatinib by itself could overcome the AML-typical differentiation blockage as evidenced by dasatinib-induced differentiation of MOLM13 and HL60 cells [17]. This effect was further confirmed by a clinical study which provided proof of prolonged differentiation of myeloid blasts bearing the t(8;21) to mature after dasatinib treatment [18]. Inhibition of c-Kit and induction of CAAT-enhancer binding protein-α (C/EBPα) was demonstrated to contribute to this remarkable response-induced by dasatinib [10]. However, the mechanisms by which dasatinib trigger AML differentiation remains largely unknown.

In the present study, we first reported that dasatinib-induced differentiation of AML cells was accompanied with the activation of the signal transducer and activator of transcription 1 (STAT1), which was evidenced by the augmented phosphorylation of STAT1, the redistribution of STAT1 from cytoplasm to nucleus and the enhanced transcription of STAT1 direct target genes. We then showed that STAT1 knockdown significantly decreased the differentiating effect of dasatinib, indicating a potential role of STAT1 in dasatinib’s off-target effects. We further found that dasatinib-induced STAT1 activation was MEK/ERK cascade dependent since MEK inhibitors not only inhibited dasatinib-triggered phosphorylation of STAT1, but also abrogated dasatinib-induced differentiation of AML cells. In summary, our results suggested a novel mechanism associated with the differentiation-induction effect of dasatinib on leukemic cells, which may provide theoretical support for the anticaner approach of dasatinib in AML.

**Materials and Methods**

**Cell Culture and Chemical**

Human acute myeloid leukemia HL60 and NB4 cells were cultured in RPMI 1640 medium plus 10% heat-inactivated fetal bovine serum (FBS). Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle medium plus 10% FBS. All cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and grown in the medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO2. Dasatinib (purity: 99.1%) was a generous gift from Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). Nitroblue tetrazolium (NBT), PD98059, U0126, and AG490 were purchased from Calbiochem (San Diego, CA). All compounds were dissolved from Calbiochem (San Diego, CA) as well as p21, p27, p-c-Myc, ERK, JNK, MEK, p38, and GAPDH obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) overnight at 4°C, followed by Alexa Fluor 488-conjugated second antibody (Life Technologies Corp, Eugene, OR) and 4′, 6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Each incubation step was followed by three washes with PBS. Images of stained cells were obtained with a Leica fluorescence microscope and a Leica DFC300 FX charge-coupled device camera.

**Real-time Quantitative PCR Assay**

Total RNA was isolated from HL60 cells treated with dasatinib or vehicle, using the Trizol reagent (Bio Basic Inc., Markham, Ontario, Canada). RNA was transcribed into cDNA using random hexamer primers and RevertAidTM M-MuLV Reverse Transcriptase (Fermentas International Inc., Burlington, Ontario, Canada). Equilibrated amounts of cDNA were taken for transcript PCR amplification, which was performed using SYBR Premix Ex Taq (Takara Biotechnology, Dalian, China). The housekeeping gene GAPDH was used as an internal standard. Primer sequences used for the PCR were as follows: RIG-G: Forward 5’-AAGCCTTGGCTTCTATCATGTC-3’, Reverse 5’-ACACCTTGCCCTTTGATTTC-3’; CXCL-10: Forward 5’-GAATCGAAGGCCATCAAGAA-3’, Reverse 5’-GCTCCCTCTGTGTTTAAAGG-3’; 5’-GACTCATGCCAATCTTTTG-3’, Reverse 5’-GAGCTTGAGGATTGTTCT-3’ [21]; CXCL-10: Forward 5’-GAATCGAAGGCCATCAAGAA-3’, Reverse 5’-GCTCCCTCTGTGTTTAAAGG-3’ [22]; GAPDH: Forward 5’-GTCATCCATGACAACATATTGG-3’, Reverse 5’-GAGGTTGAAGATGGTGTCT-3’ [23].

**Assessment of Cell Differentiation**

Fluorochrome conjugate of monoclonal antibody CD11b-PE was used to detect CD11b expression following the protocol from the manufacturer (BD Biosciences). The stained cells were run on a FACS Calibur flow cytometer and quantitated using CellQuest Pro software. At least 10 000 cells were analyzed for each data point. Cells stained with mouse IgG-PE served as negative controls. For NBT reduction and morphology analysis, cytospin preparations were made using a Shandon cytosgn 4 cytoreference. NBT reduction was performed to examine for cells containing the precipitated formazan particles as described [19]. At least 200 cells were assessed for each experiment. Cell morphology was evaluated by Wright-Giemsa staining and examined under a Leica microscope and captured with a Leica DFC300 FX charge-coupled device camera.

**Immunofluorescence**

HL60 cells treated with dasatinib or vehicle were collected and cytopsioned onto slides, fixed in 4% paraformaldehyde at room temperature. Cells were then permeabilized with 0.1% triton X-100 for 10 min, washed with PBS, and blocked with 10% FBS for 10 min. After that, cells were stained with the indicated primary antibodies-STAT1 (Santa Cruz, CA) overnight at 4°C, followed by Alexa Fluor 488-conjugated secondary antibody (Life Technologies Corp, Eugene, OR) and 4′, 6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Each incubation step was followed by three washes with PBS. Images of stained cells were obtained with a Leica fluorescence microscope and a Leica DFC300 FX charge-coupled device camera [20].

**Western Blotting**

Protein extracts were separated on SDS-PAGE and electroblotted onto PVDF membranes according to standard procedures. The membranes were blocked with 5% nonfat dry milk and incubated with the respective primary antibodies: AKT, p-AKT, c-Myc, ERK, JNK, MEK, p38, and GAPDH obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) as well as p21, p27, p-ERK, p-JNK, p-MEK, p-p38, p-STAT1(Y701), p-STAT1(S727), STAT1, p-STAT3(Y705), p-STAT3(S727), STAT3,
Figure 1. Dasatinib exhibits dose- and time-dependent effect on inducing differentiation of HL60 cells. HL60 cells were treated with dasatinib for the indicated times. Aliquots of the cultures were withdrawn at each time point and subjected to manual counting, following trypan blue staining for determination of (A) the total number of viable cells and (B) the percentage of viable cells. Data presented are the mean ± SD of cell numbers from triplicate wells. The experiments were repeated twice. *** P < .001 versus vehicle treated cells. (C) and (E) The percentages of CD11b

F

G

Das (10 μM)

0  1  3  6  12  24  72 h

c-Myc

p21

p27

GAPDH

Figure 1. Dasatinib exhibits dose- and time-dependent effect on inducing differentiation of HL60 cells. HL60 cells were treated with dasatinib for the indicated times. Aliquots of the cultures were withdrawn at each time point and subjected to manual counting, following trypan blue staining for determination of (A) the total number of viable cells and (B) the percentage of viable cells. Data presented are the mean ± SD of cell numbers from triplicate wells. The experiments were repeated twice. *** P < .001 versus vehicle treated cells. (C) and (E) The percentages of CD11b
expression cells were analyzed as described. Data presented are the mean ± SD of three independent experiments. *** P < .001 and * P < .05 versus vehicle treated cells. (D) and (F) Cell cycle proportion was analyzed by flow cytometry, following propidium iodide staining. Data presented are the mean ± SD of three independent experiments. *** P < .001, ***/## P < .01, */# P < .05 versus vehicle treated cells. (G) Cell extracts were subjected to Western blotting analysis with specific antibodies. The results are representative of three independent experiments.

doi:10.1371/journal.pone.0066915.g001

STAT5(Y694) and STAT3 purchased from Cell Signaling Technology (Danvers, MA). Western blotting were visualized with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by enhanced chemiluminescence detection (Biological Industries, Beit Haemek, Israel).

Virus Production and Transduction of Cells

A human pLKO.1 lentiviral STAT1 shRNA set (RHS4533, including 5 clones) and pLKO.1 empty lentiviral vector (RHS4080) were purchased from Open Biosystems. The pLKO.1 Non-target shRNA control plasmid was purchased from Sigma. Infectious Lentivirus was produced by cotransfecting 293T cells with shRNA-expressing lentiviral plasmids together with the packaging plasmid pR8.9 and envelope plasmid pMD.G. Supernatants containing infectious virus particles were collected 48 h after transfection and then used to transduce HL60 cells by spinoculation in the presence of polybrene (6 μg/mL). Positive transductants were selected with puromycin, and then the expression of STAT1 was analyzed by Western blotting.

Statistical Analysis

The statistical significance of differences between groups was evaluated by the unpaired Student’s t test and indicated with *** P < .001, ***/## P < .01, */# P < .05. All statistical tests were two-sided.

Results

Dasatinib Induces Differentiation of AML Cells

Exponentially growing HL60 cells were treated with different concentrations of dasatinib (1.25–20 μM) with indicated exposure times. Compared with control group, dasatinib significantly inhibited the proliferation of HL60 cells in a dose- and time-dependent manner (Fig. 1A). Interestingly, we found that dasatinib exhibited little effect on viability of HL60 cells except high concentration (20 μM), at which the apoptosis of HL60 cells was observed (Fig. 1B and data not shown). To explore the antineoplastic effect of dasatinib within the concentration of 1.25–10 μM, we studied its effect on triggering cell differentiation and cell cycle arrest after 120 h treatment (Fig. 1C and D). As shown in Fig. 1C, dasatinib induced the expression of CD11b, a myeloid differentiation marker, in a dose-dependent manner. In consis-

Figure 2. Dasatinib induces differentiation of NB4 cells. NB4 cells were treated with different concentrations of dasatinib for the indicated times. (A) The percentages of CD11b expression cells were determined as described. Data presented are the mean ± SD of three independent experiments. *** P < .001, ** P < .01 versus vehicle treated cells. (B) Cell cycle proportion was analyzed as described. Data presented are the mean ± SD of three independent experiments. *** P < .001, ** P < .01, */# P < .05 versus vehicle treated cells. (C) HL60 cells were treated with dasatinib (10 μM) for 72 h and NB4 cells were treated with dasatinib (5 μM) for 48 h. Cells were collected and cytospined onto slides, then stained with Wright-Giemsa stain. Stained cells were observed under microscope (×40). The data are representative of three independent experiments.

doi:10.1371/journal.pone.0066915.g002
tence with previous study [17], dasatinib-induced differentiation was paralleled by G0/G1 phase arrest (Fig. 1D).

To study the progression of dasatinib-induced differentiation, HL60 cells were treated with dasatinib (10 mM) for 0–120 h. Of note, dasatinib-induced CD11b expression was already detectable as early as 24 h, albeit slightly, with approximately 10% positive cells. After that the population increased to 48% and 68% at 72 h and 120 h, respectively (Fig. 1E), and even reached >90% at day 11 (data not shown). Moreover, dasatinib-induced G0/G1 phase arrest was also in line with the differentiation progression, which was detectable at 24 h of treatment (Fig. 1F). Consistently, dasatinib significantly decreased the expression of c-Myc which is involved in leukemic cell proliferation and increased the expression of p21 and p27 which play important roles in preventing cell cycle progression (Fig. 1G).

The differentiation-inducing capacity of dasatinib was further determined on another AML cell line NB4 cells. Dasatinib-induced expression of CD11b was in a dose-dependent manner at low concentrations (1.25 and 2.5 mM). However, high concentrations (5 and 10 mM) of dasatinib did not induce an enhanced expression of CD11b (Fig. 2A). This could be attributed to dasatinib-induced increase of hypodiploid cells at 72 h. Upon high concentrations (5 and 10 mM) of dasatinib treatment, approximately more than 30% of NB4 cells were accumulated in Sub G0 phase. Actually, dasatinib-induced cell-cycle blockade was manifested before the accumulation of Sub G0 phase cells. An obvious G0/G1 phase arrest was already detectable at 24 h after dasatinib treatment (2.5–10 mM), when no hypodiploid cells were observed (Fig. 2B). Furthermore, the ability of dasatinib to induce AML differentiation was also confirmed by morphologic changes of HL60 and NB4 cells, namely a reduction of nucleus to cytoplasm ratio and an obvious nuclear segmentation compared with control group (Fig. 2C). Taken together, these results indicated that dasatinib exerted a differentiation effect on AML cells.

Dasatinib Induces the Activation of STAT1

Numerous reports have demonstrated the essential role of STAT proteins in myeloid differentiation; here we intended to...
determine whether dasatinib-induced AML differentiation was mediated by STAT signal pathway. HL60 cells were incubated with dasatinib (10 μM) for indicated times and then analyzed for the phosphorylation of STAT proteins. As shown in Fig. 3A, dasatinib treatment led to an increased phosphorylation of STAT1 (Y701 and S727) within 1 h, which was then augmented with time, whereas dasatinib significantly decreased the phosphorylation of STAT3 (Y705), which was almost undetectable after 3 h.

**Figure 4. Silencing of STAT1 expression by shRNA inhibits dasatinib-induced differentiation of HL60 cells.** HL60 cells transduced with empty vector, Non-target shRNA or STAT1 shRNA (shSTAT1-1 and shSTAT1-2) were treated with dasatinib (10 μM) for 72 h. (A) STAT1 expression was detected by Western blotting. Band intensities were quantified using Quantity One 4.4.0 software (Bio-Rad) and then normalized to the amount of GAPDH in each sample. All samples were compared with the signal detected in Non-target shRNA transduced cells. The data are representative of three independent experiments. (B) NBT reduction assay was performed as described. The percentage of NBT-positive cells was calculated by counting at least 200 cells under a light microscope. Data presented are the mean ± SD of three independent experiments. *** P<.001 (C) CD11b expression was measured by flow cytometer. Data presented are the mean ± SD of three independent experiments. ** P<.01, * P<.05 (D) Cells were collected onto slides by cytospin, stained by Wright–Giemsa stain and observed under microscope. Original magnification, ×40. The data are representative of three independent experiments.

doi:10.1371/journal.pone.0066915.g004

**Figure 5. Dasatinib-induced HL60 cells differentiation could not be abrogated by JAK inhibitor AG490.** HL60 cells were treated with dasatinib (10 μM) in the presence of U0126 (5 μM) or AG490 (10 μM) for 72 h. (A) The percentages of CD11b expression cells were determined as described. Data presented are the mean ± SD of three independent experiments. *** P<.001 (B) The expression of p-MEK, p-ERK, p-STAT1(Y701) and p-STAT1(S727) as well as their total proteins were analyzed by Western blotting. The experiments were repeated three times and the data show the representative results.

doi:10.1371/journal.pone.0066915.g005
Differentiation

STAT1 knockdown Inhibits Dasatinib-induced AML cells. Returned to baseline by 12 h (Fig. 3D). These results indicated that returned to baseline by 48 h, while the level of p-STAT1 (S727) was virtually detectable at 0.5 h after dasatinib treatment, but the level of p-STAT1 (Y701) and p-STAT1 (S727) were virtually detectable at 1 h and then maintained at a high level for at least 72 h. We then determined the effect of dasatinib on the phosphorylation of other MAPK family members, p38 and JNK. Interestingly, the phosphorylation of STAT1 on both sites (Y701 and S727) was strikingly inhibited by PD98059 in HL60 and NB4 cells, while dasatinib-induced phosphorylation of STAT1 was not affected by PD98059.

Figure 6. Dasatinib enhances the phosphorylation of MEK/ERK cascades. HL60 cells were treated with dasatinib (10 μM) for the indicated times. The expressions of phosphorylated MEK, ERK, p38, JNK and AKT as well as their total proteins were analyzed by Western blotting. All the experiments were repeated three times and the data show the representative results. doi:10.1371/journal.pone.0066915.g006

treatment. In addition, dasatinib exhibited little effects on the phosphorylation of STAT3 (S727) and STAT5 (Y694) (Fig. 3A and data not shown). Given that phosphorylated STAT1 will translocate into the nucleus to exert its gene regulation effects, we then determined the localization of STAT1 by immunofluorescence assay. In the absence of dasatinib, STAT1 was predominantly cytoplasmic, while dasatinib treatment induced the redistribution of STAT1 from the cytoplasm to the nucleus (Fig. 3B). To further confirm the ability of phosphorylated STAT1 on gene transcription regulation, the expressions of two direct STAT1 target genes (RIG-G and CXCL-10) were tested by real time PCR. As shown in Fig. 3C, the transcription of both STAT1 target genes was enhanced by dasatinib in a time-dependent manner. In consistence with these results, dasatinib-induced activation of STAT1 was also observed in NB4 cells. A modest increase of p-STAT1 (Y701) and p-STAT1 (S727) were virtually detectable at 0.5 h after dasatinib treatment, but the level of p-STAT1 (Y701) returned to baseline by 48 h, while the level of p-STAT1 (S727) returned to baseline by 12 h (Fig. 3D). These results indicated that STAT1 might play a role in dasatinib-induced differentiation of AML cells.

STAT1 knockdown Inhibits Dasatinib-induced Differentiation

The finding that STAT1 was significantly activated in AML cells after dasatinib treatment prompted us to investigate whether STAT1 contributed to dasatinib-induced differentiation. The endogenous expression of STAT1 was knocked down by shRNA. Of the 3 shRNA clones, two shRNA sequences that exhibited the strongest knockdown efficiency were selected to silence STAT1 expression in HL60 cells. As shown in Fig. 4A, expression of shSTAT1-1 and shSTAT1-2 significantly reduced STAT1 expression in HL60 cells compared with Vector or Non-target shRNA. As expected, silencing of STAT1 expression significantly inhibited dasatinib-induced AML differentiation. In detail, STAT1-knockdown HL60 cells showed markedly reduced NBT positive cells after dasatinib treatment compared with Vector and Non-target shRNA-expressing cells (78.5±1.4, 83.1±2.1, 48.2±2.3, and 65.0±3.4 for Vector, Non-target shRNA, shSTAT1-1, and shSTAT1-2, respectively; Fig. 4B). STAT1-knockdown HL60 cells also demonstrated significantly reduced CD11b expression after dasatinib treatment compared with Vector and Non-target shRNA-expressing cells (56.2±6.3, 54.5±6.1, 24.7±8.5, and 36.5±4.7 for Vector, Non-target shRNA, shSTAT1-1, and shSTAT1-2, respectively; Fig. 4C). Moreover, STAT1 shRNA-transduced cells exhibited less mature granulocyte morphology compared with Vector and Non-target shRNA-transduced cells, as evidenced by a decreased ratio of cytoplasm to nucleus (Fig. 4D). These results indicated that knockdown of STAT1 in HL60 cells resulted in a significantly decreased differentiation response to dasatinib stimulation.

Dasatinib Induces Activation of MEK/ERK Cascade

Given that STAT1 is phosphorylated at tyrosine 701 by the Janus kinase (JAK) family members and at serine 727 by MAPK cascades including MEK, ERKs, p38 and JNKs as well as PI3K-AKT pathway, we first evaluated the effects of dasatinib on JAK1, JAK2 and Tyk2 kinases. Unexpectedly, dasatinib had no effect on the phosphorylation of JAK1 or JAK2 (data not shown). In addition, neither dasatinib-induced CD11b expression nor dasatinib-enhanced phosphorylation of STAT1 was affected by the JAK inhibitor AG490 (Fig. 5). In contrast, the phosphorylation of MEK was augmented within 1 h and increased to a high level at 72 h of dasatinib treatment (Fig. 6). In consistence, the enhanced phosphorylation of ERK was also detectable as early as 1 h and then maintained at a high level for at least 72 h. Then we determined the effect of dasatinib on the phosphorylation of other MAPK family members, p38 and JNK. As shown in Fig. 6, phosphorylation of p38 was transiently abolished within 1 h, and then returned to baseline at 72 h of dasatinib treatment, while dasatinib exhibited little effects on the phosphorylation of JNK and AKT.

Activation of STAT1 is Dependent on MEK/ERK Activation

To determine whether the phosphorylation of MEK/ERK cascade was indispensable for dasatinib-induced AML differentiation, HL60 and NB4 cells were treated with dasatinib in the presence of MEK inhibitor PD98059 and then analyzed for CD11b expression. The concentration of PD98059 to treat HL60 and NB4 were 20 and 10 μM, respectively, at which no cytotoxicity was observed in both cell lines when treated with PD98059 alone or in combination with dasatinib (data not shown). As shown in Fig. 7A, PD98059 significantly inhibited dasatinib-induced CD11b expression in both cells. In detail, the population of CD11b-positive cells decreased from 50% to 16% in HL60 cells and from 57% to 12% in NB4 cells in the presence of PD98059, respectively. In addition, PD98059 also significantly inhibited dasatinib-induced G0/G1 phase arrest, especially in NB4 cells of which the percentage of G0/G1 phase cells decreased from 80% to 40% (Fig. 7B). As expected, the activation of MEK and ERK was successfully inhibited by PD98059 (Fig. 7C and D). Interestingly, the phosphorylation of STAT1 on both sites (Y701 and S727) was strikingly inhibited by PD98059 in HL60 and NB4 cells. These results were further corroborated by another MEK inhibitor U0126. Dasatinib-induced CD11b expression decreased from 50% to 11% in the presence of U0126 (Fig. 5A). Western blotting analysis also confirmed that U0126 not only inhibited dasatinib-induced serine phosphorylation of STAT1 (S727), but also abrogated the tyrosine phosphorylation of STAT1 (Y701).
Taken together, dasatinib-induced activation of STAT1 was dependent on MEK/ERK activation, thus leading to the differentiation of leukemic cells.

Discussion

The present study demonstrated the capacity of dasatinib to induce leukemic cell differentiation, as evidenced by the enhanced expression of CD11b, G0/G1 phase arrest and the decreased ratio of nuclear to cytoplasm in dasatinib-treated AML cells. Notably, we found that the biological function of dasatinib on inducing AML differentiation was mediated by MEK/ERK dependent activation of STAT1.

STAT family proteins, activated by cytokines, hormones, and growth factors function as transcription factors that mediate a variety of biologic processes, such as cell proliferation and apoptosis [31]. Among at least seven members, STAT1 has been identified to play essential roles in myeloid differentiation. STAT1 activation was first identified in ATRA-induced myeloid differentiation of various leukemia cell lines [32]. In addition, STAT1 was also reported to be activated by Bryostatin 1 to mediate the differentiation of chronic lymphocytic leukemia cells (CLL). Here we observed a significant increase of tyrosine and serine phosphorylation of STAT1 after dasatinib treatment, accompanied by the translocation of STAT1 from the cytoplasm to the nucleus, thus leading to the enhanced transcription of STAT1 target genes. Silencing of STAT1 expression by shRNA significantly lessened dasatinib-induced differentiation, suggesting an essential role of STAT1 in dasatinib's differentiation effect. In contrast to the tumor suppressor function of activated STAT1, STAT3 is mainly identified as an oncogene [33,34]. Constitutive tyrosine and serine phosphorylation of STAT3 has been demonstrated in many different malignancies, including AML [35,36]. Inhibition of STAT3 activation induces apoptosis in AML cell lines and primary samples [36]. However, the important roles for STAT3 in granulocytic differentiation have also been reported in vitro and in vivo studies [37,38]. Thus, the exact role of STAT3 in leukemogenesis remains controversial. In our study, the tyrosine phosphorylation of STAT3 was significantly down-regulated by dasatinib, while the serine phosphorylation of STAT3 was unaffected. A previous study has demonstrated that constitutive phosphorylation of STAT3 could lead to the survival of AML cells [39].

Figure 7. Dasatinib-induced AML differentiation requires MEK/ERK-dependent activation of STAT1. HL60 cells were treated with dasatinib (10 μM) in the presence of PD98059 (20 μM) for 72 h, and NB4 cells were treated with dasatinib (5 μM) in the presence of PD98059 (10 μM) for 48 h. HL60 and NB4 cells were pretreated with PD98059 for 1 h, and then incubated with dasatinib. (A) The percentages of CD11b expression cells were determined as described. Data presented are the mean ± SD of three independent experiments. (B) Cell cycle proportion was analyzed as described. Data presented are the mean ± SD of three independent experiments. *** P < .001, * P < .05. (C) and (D) The expression of p-MEK, p-ERK, p-STAT1(Y701) and p-STAT1(S727) as well as their total proteins in HL60 and NB4 cells were analyzed by Western blotting. The experiments were repeated three times and the data show the representative results.

doi:10.1371/journal.pone.0066915.g007
phosphorylation of STAT3 on serine 727, not tyrosine 705, was required for DNA-binding and transcription activation of target genes for survival [39]. Thus the serine phosphorylation of STAT3 here might disrupt apoptotic signal pathway caused by the decrease of tyrosine phosphorylation, thereby conferring a survival advantage that was necessary for differentiation of HL60 cells.

JAK1, JAK2, and Tyk2, which are reported to directly phosphorylate STAT1 protein at tyrosine site (Y701) following activation of the IFN receptors by their respective ligands [31,40], did not seem to be potential targets for dasatinib. In detail, dasatinib did not cause any changes in the levels of JAK1, JAK2 or Tyk2 phosphorylation and neither dasatinib-induced CD11b expression nor dasatinib-enhanced phosphorylation of STAT1 (Y701 or S727) was affected by JAK1 inhibitor AG490. Previous studies have shown that STAT1 was phosphorylated at serine site by several kinases, including MEK, ERK, JNK, p38 kinases and PI3K/AKT pathway. Here we found that MEK and ERK were significantly activated in dasatinib treated AML cells. However, the phosphorylation of p38 was totally abrogated by dasatinib except a recovery at 72 h and the phosphorylation of JNK and AKT were hardly changed after dasatinib treatment. We extended those observations to show that activation of MEK/ERK cascade was indispensable for dasatinib’s differentiating effect. Critically, we showed that dasatinib-induced expression of CD11b and G0/G1 arrest were significantly inhibited by MEK inhibitors PD98059 and U0126. In line with these facts, dasatinib-induced phosphorylation of STAT1 (both tyrosine 701 and serine 727 sites) were inhibited by MEK inhibitors, indicating MEK/ERK-dependent STAT1 activation might be indispensable for the differentiation effect of dasatinib.

SFKs have been reported to activate the Ras/Raf/MEK/ERK pathway via recruitment of Shc and Shp2 proteins [41]. Here we observed that dasatinib, as an efficient SFKs inhibitor, did not inhibit the MEK/ERK cascades in AML cells. On the contrary, dasatinib significantly induced the phosphorylation of MEK and ERK, which was approved to be indispensable for dasatinib’s differentiating effect. This phenomenon could be partially explained by the fact that dasatinib is also a weak RAF inhibitor. Packer et al. [42] reported that the binding of weak RAF inhibitors (imatinib, nilotinib and dasatinib) to BRAF and CRAF drives BRAF:CRAF heterodimer as well as BRCF and CRAF homodimer formation in the presence of oncoenetic RAS, thus leading to paradoxical activation of both BRAF and CRAF, followed by the activation of MEK and ERK. Considering the complex mechanisms involved in activation of Ras-Raf-MEK-ERK pathway, further studies will be performed to better understand the effect of dasatinib-induced activation of MEK/ERK kinases in our future work.

In summary, the present results demonstrated for the first time that the differentiation-induction effect of dasatinib required the activation of STAT1, which depended on MEK/ERK cascade activation. Our results suggest that attempts to treat AML with a combination of dasatinib and MEK inhibitors should be made with great caution. The identification of potential molecular mechanisms for dasatinib’s biological effect on AML differentiation may provide some molecular theoretical basis for clinical application when dasatinib is combined with other target based drugs in leukemia therapy.

**Author Contributions**

Conceived and designed the experiments: YFF QJH. Performed the experiments: LKZ MHL XLZ HJ. Analyzed the data: YFF MDP PHL BY QJH. Wrote the paper: YFF QJH.

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**References**

1. Wouters BJ, Lowenberg B, Delwel R (2009) A decade of genome-wide gene expression profiling in acute myeloid leukemia: flashback and prospects. Blood 113: 291–298.

2. Burnett A, Wetzler M, Lowenberg B (2011) Therapeutic advances in acute myeloid leukemia. J Clin Oncol 29: 487–494.

3. Nowak D, Stewart D, Koeffler HP (2009) Differentiation therapy of leukemia: 3 decades of development. Blood 113: 3655–3665.

4. Petrie K, Zelen A, Waxman S (2009) Differentiation therapy of acute myeloid leukemia: past, present and future. Curr Opin Hematol 16: 84–91.

5. Wang ZY, Chen Z (2008) Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 111: 2505–2515.

6. Gianni M, Kalac Y, Ponzanelli A, Rambaldi A, Terao M, et al. (2001) Tyrosine kinase inhibitor STI571 potentiates the pharmacologic activity of retinoic acid in acute promyelocytic leukemia cells: effects on the degradation of RARalpha and PML-RARalpha. Blood 97: 3234–3243.

7. Miranda MB, Duan R, Thomas SM, Grandis JR, Redner RL, et al. (2008) Gefitinib potentiates myeloid cell differentiation by ATRA. Leukemia 22: 1624–1627.

8. Stengaurer K, Corsello SM, Ross KN, Wong JS, Deangelo DJ, et al. (2005) Gefitinib induces myeloid cell differentiation by ATRA. Leukemia 22: 1624–1627.

9. Sullivan C, Peng C, Chen Y, Li D, Li S (2010) Targeted therapy of chronic myeloid leukemia. J Clin Oncol 28: 497–508.

10. Rosenzweig SA (2012) Acquired resistance to drugs targeting receptor tyrosine kinase inhibitors as first-line therapy. Leukemia 26: 214–224.

11. Stegmaier K, Deininger M (2012) Evolving treatment strategies for patients newly diagnosed with chronic myeloid leukemia: the role of second-generation BCR-ABL inhibitors as first-line therapy. Leukemia 26: 214–224.

12. Darbrioli A, Caldarelli I, Basile MA, Bencivenga D, Tramontano A, et al. (2011) The tyrosine kinase inhibitor dasatinib induces a marked adipogenic differentiation of human monopotent mesenchymal stromal cells. PLoS One 6: e28555.

13. Guerrouahen BS, Futami M, Vaklavas C, Kanerva J, Whichard ZL, et al. (2010) Initial validation that was necessary for differentiation of HL60 cells.

14. Kolb EA, Gotrnick R, Houghton PJ, Morton CL, Lock RB, et al. (2008) Initial validation that was necessary for differentiation of HL60 cells.

15. Gropp PL, Wang L, Zang Y, Redner RL, Johnson DE (2010) Dasatinib promotes ATRA-induced differentiation of AML cells. Leukemia 24: 663–665.

16. Congleton J, Macdonald R, Yen A (2012) Src inhibitors, PP2 and dasatinib, increase retinoic acid-induced association of Lyn and c-Src (5259) and enhance MAPK-dependent differentiation of myeloid leukemia cells. Leukemia 26: 1180–1188.

17. Lainey E, Thepot S, Bouloucoup C, Sebert M, Ales L, et al. (2011) Tyrosine kinase inhibitors for the treatment of acute myeloid leukemia: delineation of anti-leukemic mechanisms of action. Biochem Pharmacol 82: 1457–1466.

18. Chevalier N, Solari ML, Becker H, Paniec M, Gardner F, et al. (2010) Robust in vivo differentiation of t(8;21)-positive acute myeloid leukemia blasts to neutrophilic granulocytes induced by treatment with dasatinib. Leukemia 24: 1729–1731.

19. Fang Y, Zhou X, Lin M, Ying M, Luo P, et al. (2011) Inhibition of all-trans-retinoic acid-induced proteasome activation potentiates the differentiating effect of retinoid in acute myeloid leukemia cells. Mol Carcinog 50: 24–35.

20. Luo P, Lin M, Li L, Yang B, He Q (2011) The proteasome inhibitor bortezomib enhances ATRA-induced differentiation of neuroblastoma cells via the JNK mitogen-activated protein kinase pathway. PLoS One 6: e27298.

21. Gu ZM, Liu CX, Wu SF, Zhao M, Xu HZ, et al. (2011) PU.1 directly regulates RARalpha expression profiling in acute myeloid leukemia: flashback and prospects. Blood 70: 3618–3623.

22. Rivieccio MA, John GR, Song X, Suh HS, Zhao Y, et al. (2005) The cytokine proteasome pathway plays essential roles in ATRA-induced leukemia cells G0/G1 phase arrest and transition into granulocytic differentiation. Cancer Biol Ther 10: 1317–1326.

23. Fang Y, Zhou X, Lin M, Jing H, Zhong L, et al. (2010) The ubiquitin-proteasome pathway plays essential roles in ATRA-induced leukemia cells G0/G1 phase arrest and transition into granulocytic differentiation. Cancer Biol Ther 10: 1317–1326.

24. Fey A, Eicher S, Flavier S, Christen R, Hölle MG, et al. (2004) Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using Salmonella as a model organism. Appl Environ Microbiol 70: 3618–3623.

25. Xiao S, Li D, Zha HQ, Song MG, Pan XR, et al. (2006) RIG-G as a key mediator of the antiproliferative activity of interferon-related pathways through enhancing p21 and p27 proteins. PLoS Acad Sci U S A 103: 16449–16453.

26. Weber S, Maas F, Schuemann M, Krause E, Suske G, et al. (2009) PRMT1-mediated arginine methylation of PIAS1 regulates STAT1 signaling. Genes Dev 23: 118–132.
27. Dimberg A, Nilsson K, Oberg F (2000) Phosphorylation-deficient Stat1 inhibits retinoic acid-induced differentiation and cell cycle arrest in U-937 monoblasts. Blood 96: 2870–2878.
28. Zhang Y, Cho YY, Petersen BL, Zhu F, Dong Z (2004) Evidence of STAT1 phosphorylation modulated by MAPKs, MEK1 and MKS1. Carcinogenesis 25: 1165–1173.
29. Li SW, Lai CC, Ping JF, Tsai FJ, Wan L, et al. (2011) Severe acute respiratory syndrome coronavirus papain-like protease suppressed alpha interferon-induced responses through downregulation of extracellular signal-regulated kinase 1-mediated signalling pathways. J Gen Virol 92: 1127–1140.
30. Nguyen H, Ramana CV, Bayes J, Stark GR (2001) Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. J Biol Chem 276: 33361–33366.
31. Benecke M, Baumann H, Wetzler M (2009) Targeting signal transducer and activator of transcription signaling pathway in leukemias. J Clin Oncol 27: 4422–4432.
32. Gianni M, Terao M, Fortino I, LiCalzi M, Viggiano V, et al. (1997) Stat1 is induced and activated by all-trans retinoic acid in acute promyelocytic leukemia cells. Blood 89: 1001–1012.
33. Schindler C, Levy DE, Decker T (2007) JAK-STAT signaling: from interferons to cytokines. J Biol Chem 282: 20009–20063.
34. Qing Y, Stark GR (2004) Alternative activation of STAT1 and STAT3 in response to interferon-gamma. J Biol Chem 279: 41679–41685.
35. Schuringa JJ, Wierenga AT, Kruijer W, Vellenga E (2000) Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6. Blood 95: 3765–3770.
36. Redell MS, Ruiz MJ, Alonso TA, Grebing RB, Tweardy DJ (2011) Stat3 signaling in acute myeloid leukemia: ligand-dependent and -independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor. Blood 117: 5701–5709.
37. Panopoulos AD, Zhang L, Snow JW, Jones DM, Smith AM, et al. (2006) STAT3 governs distinct pathways in emergency granulopoiesis and mature neutrophils. Blood 108: 3682–3690.
38. Miranda MB, Johnson DE (2007) Signal transduction pathways that contribute to myeloid differentiation. Leukemia 21: 1363–1377.
39. Hazan-Halevy I, Harris D, Liu Z, Liu J, Li P, et al. (2010) STAT3 is constitutively phosphorylated on serine 727 residues, binds DNA, and activates transcription in CLL cells. Blood 115: 2832–2863.
40. Vera J, Rateitschak K, Lange F, Kossow C, Wolkenhauer O, et al. (2011) Systems biology of JAK-STAT signalling in human malignancies. Prog Biophys Mol Biol 106: 426–434.
41. Johnson DE (2008) Src family kinases and the MEK/ERK pathway in the regulation of myeloid differentiation and myeloid leukemogenesis. Adv Enzyme Regul 48: 98–112.
42. Packer LM, Rana S, Hayward R, O'Hare T, Eide CA, et al. (2011) Nilotinib and MEK inhibitors induce synthetic lethality through paradoxical activation of RAF in drug-resistant chronic myeloid leukemia. Cancer Cell 20: 713–727.