

Targeting of the Alox12-12-HETE in Blast Crisis Chronic Myeloid Leukemia Inhibits Leukemia Stem/Progenitor Cell Function

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Introduction: Chronic myeloid leukemia (CML) is a myeloid malignancy characterized by the oncogene BCR-ABL. CML responds well to therapy targeting BCR-ABL in the chronic phase but is resistant to treatment when it progresses to the blast phase (BP). This study attempted to address whether arachidonate 12-lipoxygenase (Alox12) confers to CML drug resistance.

Materials and Methods: We analyzed the expression of Alox12 using Western blotting, ELISA, and RT-PCR methods. Loss of functional analysis was performed using cellular activity assays on CML and normal hematopoietic stem/progenitor cells (HSPCs).

Results: Alox12 and 12-Hydroxyeicosatetraenoic acid (12-HETE) are overexpressed in BP-CML but not HSPCs, and that Alox12-12-HETE axis is regulated by BCR-ABL. The Alox12-12-HETE axis is required for CML. Specific Alox12 inhibitor inhibits colony formation, survival, and self-renewal capacity in BP-CML HSPCs, and to a significantly greater extent than in normal HSPCs. Of note, the Alox12 inhibitor significantly augments dasatinib's efficacy in BP-CML HSPCs. Mechanism studies show that Alox12 inhibition does not affect activities of essential signaling pathways involved in maintaining stem cell function, such as Wnt, p53, and bone morphogenetic protein (BMP). In contrast, we show that Alox12 inhibition disrupts nicotinamide adenine dinucleotide phosphate (NADPH) homeostasis and induces oxidative stress and damage in CML HSPCs and committed cells.

Conclusion: Alox12-12-HETE axis is a specific and critical regulator of BP-CML HSPCs functions. Pharmacological inhibition of Alox12 may be useful in BP-CML.

Keywords: chronic myeloid leukemia, Alox12, Bcr-Abl, resistance, stem cell

Introduction

Chronic myeloid leukemia (CML) is a lethal myeloproliferative neoplasm resulting from transformation of a hematopoietic stem/progenitor cell (HSPC) by the oncogene BCR-ABL.¹ Transformed HSPCs with deregulated tyrosine kinase activity of the BCR-ABL protein have aberrant proliferation and survival advantages over normal hematopoietic cells.² The remarkable clinical response has been achieved with BCR-ABL tyrosine kinase inhibitors (TKIs), such as imatinib and dasatinib, in CML patients at chronic phase.³ However, these TKIs are not effective when CML progresses to blast phase (BP), suggesting that additional transforming events contribute to the BP phenotype.⁴ The mechanisms underlying resistance to TKIs in the BP-CML HSPCs involve BCR-ABL-independent mechanisms.⁵ In this study, we set out to identify factors responsible for proper functions in BP-CML HSPC population that might be druggable.

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Lipoxygenases (LOXs) are dioxygenases that catalyze the formation of corresponding hydroperoxides from polyunsaturated fatty acids and have significant mitogenic and chemotactic effects, and stimulate the expression of oncogenes.^{6,7} LOXs family members, such as Alox15, Alox5 and Alox12, are expressed in hematopoietic cells.⁸ Particularly, Alox12 is predominantly distributed in blood platelets.⁹ Alox12 metabolizes arachidonic acid to 12S-hydroxyeicosatetraenoic acid (12S-HETE) by glutathione peroxidase.¹⁰ Alox12 metabolizes arachidonic acid to 12-hydroxyeicosatetraenoic acid (12-HETE). Alox12-12-HETE has recently garnered attention due to its ability in regulating growth, metastasis, survival, and chemoresistance in many cancers.¹¹⁻¹⁴ Studies have shown that Alox15 and Alox5 are required for myeloid leukemia stem cell to survive and function properly.^{15,16}

We investigated the expression and functions of the Alox12-12-HETE axis in normal and BP-CML HSPCs and its relationship with BCR-ABL activation. Our results show that BCR-ABL regulates the Alox12-12-HETE axis to promote leukemogenesis. In addition, the Alox12-12-HETE axis is required for CML proliferation and survival. Consistent with these findings, we show that the pharmacological inhibitor of Alox12 suppresses HSPC functions and augments BCR-ABL TKI's efficacy. Mechanism studies show that loss of NADPH homeostasis and induction of oxidative damage are involved in the inhibition of the Alox12-12-HETE axis in CML HSPC and committed cells.

Materials and Methods

Patient Samples, Primary Cell Isolation, Cell Culture, Drugs and Antibodies

BP-CML CD34⁺ cells (5x10⁶ to 10⁸ per sample) were purified from bone marrow or peripheral blood of CML patients at blast phase seen at Wuhan Forth Hospital with informed consents approved by the Tongji Medical College Centralised Institutional Review Board (No. 2,017,016). Mononuclear cells were obtained by using Ficoll separation, followed by CD34⁺ cell selection using CD34 MicroBead kit (Miltenyi Biotec). BP-CML CD34⁺ and normal bone marrow (NBM) CD34⁺ cells (StemCell Technologies) were cultured using the same growth factors and cytokines supplemented serum-free medium as described in our previous study.¹⁷ Human CML cell lines were cultured in RPMI1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone) and

2mM glutamine (Invitrogen). Imatinib (LC laboratories) was reconstituted in PBS. Dasatinib (LC laboratories) and ML355 (Cayman chemical) were dissolved in dimethyl sulfoxide (DMSO). Antibodies against Bcr-Abl, p-Crk1, and ALOX12 and corresponding secondary HRP-conjugated antibodies were purchased from Santa Cruz and standard deviation z Biotechnology.

Cell Transduction and Transfection

NBM CD34⁺ cells overexpressing Bcr-Abl were generated by retroviral transduction by using NGFR and NGFR P210 (Addgene) vectors as previously described.¹⁸ Specific ALOX12 and BCL-ABL knockdown were conducted in CML cells by using nucleofection (Lonza) followed by electroporation. 100 nM scramble siRNA or human ALOX12 or BCR-ABL-specific siRNAs were used for transfection. The target siRNA sequence of BCR-ABL¹⁹ and ALOX12²⁰ is the same as previously reported.

Measurement of Proliferation and Apoptosis

Proliferation and apoptosis were measured after 3 days drug treatment or in transfected cells at 72-hour post-transfection. Cell proliferative and apoptotic activities were determined using the same methods as described in our previous study.¹⁷

Colony-Forming and Serial Replating Assays

CD34⁺ cells at 1000/well together with drugs were mixed with HSC-CFU methylcellulose medium (Miltenyi Biotec) and plated onto 6-well plate. After 2 weeks incubation, colonies were formed and counted. Individual colonies formed in colony-forming assay were picked, mixed well with HSC-CFU complete methylcellulose and plated onto 96-well plate. After 2 weeks incubation, wells with colonies were considered as positive. Individual colonies formed in 96-well plate were picked, mixed well with HSC-CFU complete methylcellulose and plated onto 96-well plate again. Serial replating capacity is determined by the percentage of number of positive wells among total number of colonies plated in each serial replating. Total three rounds of serial replating were performed.

Luciferase Reporter Assays

Reporter assays were carried out in CML cells by using nucleofection (Lonza) and electroporation. p53, Wnt or

BMP transcriptional activities were assessed by transfecting cells with PG13-luc (wt p53 binding sites), M50 Super 8x TOPFlash or pGL3 BRE plasmid. Cells were treated with ML355 for 24 hours followed by luciferase assays (Promega) according to manufacturer's instructions to assess β -catenin, p53 and BMP transcriptional activities.

Measurement of SIRT1 Enzyme Activity, NADPH, NADH, 12-Hydroxyeicosatetraenoic Acid (12-HETE) and ALOX12 Levels

After 24 hours drug treatment, cells were lysed using standard protocol. Changes in SIRT1 enzyme activity, NADPH, NADH, 12-HETE and ALOX12 levels were assessed using total cell lysates and were measured using a fluorometric SIRT-1 Activity Assay kit (Abcam), NADPH and NADH colorimetric quantification kits (Abcam, US), 12-HETE ELISA kit (Abcam) and ALOX12 ELISA Kit (MyBioSource).

Measurement of Intracellular Reactive Oxygen Species (ROS) and Oxidative DNA Damage

ROS and oxidative DNA damage were measured after 24 hours drug treatment. Cells were incubated with 10 μ M CM-H₂DCFDA (Life Technologies, US) at 37°C for 1 h, followed by measuring absorbance at ex/em of 495/525 nm were on Spectramax M5 microplate reader. DNA was extracted using the DNEasy Mini Kit (Qiagen). 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were quantified using the OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs).

Statistical Analyses

All data are obtained from at least three independent experiments and expressed as mean and standard deviation. Student's *t*-test was performed to determine statistical significance. Values were considered statistically significant at $P < 0.05$.

Results

BCR-ABL Regulates Alox12-12-HETE Axis in Normal and Malignant Hematopoietic Cells

We firstly investigated the expression level of Alox12 and 12-HETE in hematopoietic stem cells isolated from BP-

CML patients and compared with normal bone marrow (NBM) counterparts. Among hematopoietic stem/progenitor cell (HSPC) makers, CD34 is well known for its unique expression on HSPCs.²¹ ELISA analysis showed that the average level of Alox12 protein and 12-HETE was significantly higher in BP-CML (n=8) than NBM (n=5) CD34⁺ cells (Figure 1A). Western blot analysis showed Alox12 protein level in individual BP-CML (n=6) and NBM (n=3) samples and the quantification of band density confirmed the finding obtained from ELISA assay (Figure 1B and C). In addition, NBM#1 and #2 displayed less Alox12 level compared to all tested BP-CML samples whereas NBM#3 displayed a similar level to BP-CML#1 and #4, suggesting that Alox12 upregulation occurs in some but not all BP-CML patients.

We next investigated whether BCR-ABL expression regulates Alox12 in CML. We transduced NBM CD34⁺ cells using BCR-ABL retroviral vector NGFR P210 and confirmed the overexpression of BCR-ABL after retroviral transduction (Figure 1D). Of note, Alox12 protein level and 12-HETE level were significantly increased after BCR-ABL overexpression (Figure 1D and E), demonstrating the change of Alox12 expression during BCR-ABL transformation. In contrast, BCR-ABL depletion decreased the Alox12 protein level in CML cells (Figure 1F and G). Consistent with the previous report,²² imatinib decreased p-Crk1 (a marker of BCR-ABL function in response to BCR-ABL inhibitors) in CML cells, demonstrating the inhibition of BCR-ABL kinase activity by imatinib (Figure 1H and I). We observed that both Alox12 and 12-HETE levels were decreased in imatinib-treated CML cells (Figure 1H to J). These demonstrate that BCR-ABL regulates Alox12-12-HETE axis in normal and malignant hematopoietic cells.

Alox12-12-HETE Axis is Required for CML Cell Growth and Survival

To understand the biological function of Alox12-12-HETE axis in CML cells, we depleted Alox12 in CML cells and examined the growth and survival in the absence and presence of 12-HETE. We observed that specific ALOX12 siRNA resulted in minimal Alox12 protein level and remarkable reduction of 12-HETE in K562 cells (Figure 2A). We further found that Alox12 depletion significantly decreased growth and increased apoptosis in K562 cells, and furthermore that these inhibitory effects were reversed by the addition of 12-HETE (Figure 2B and

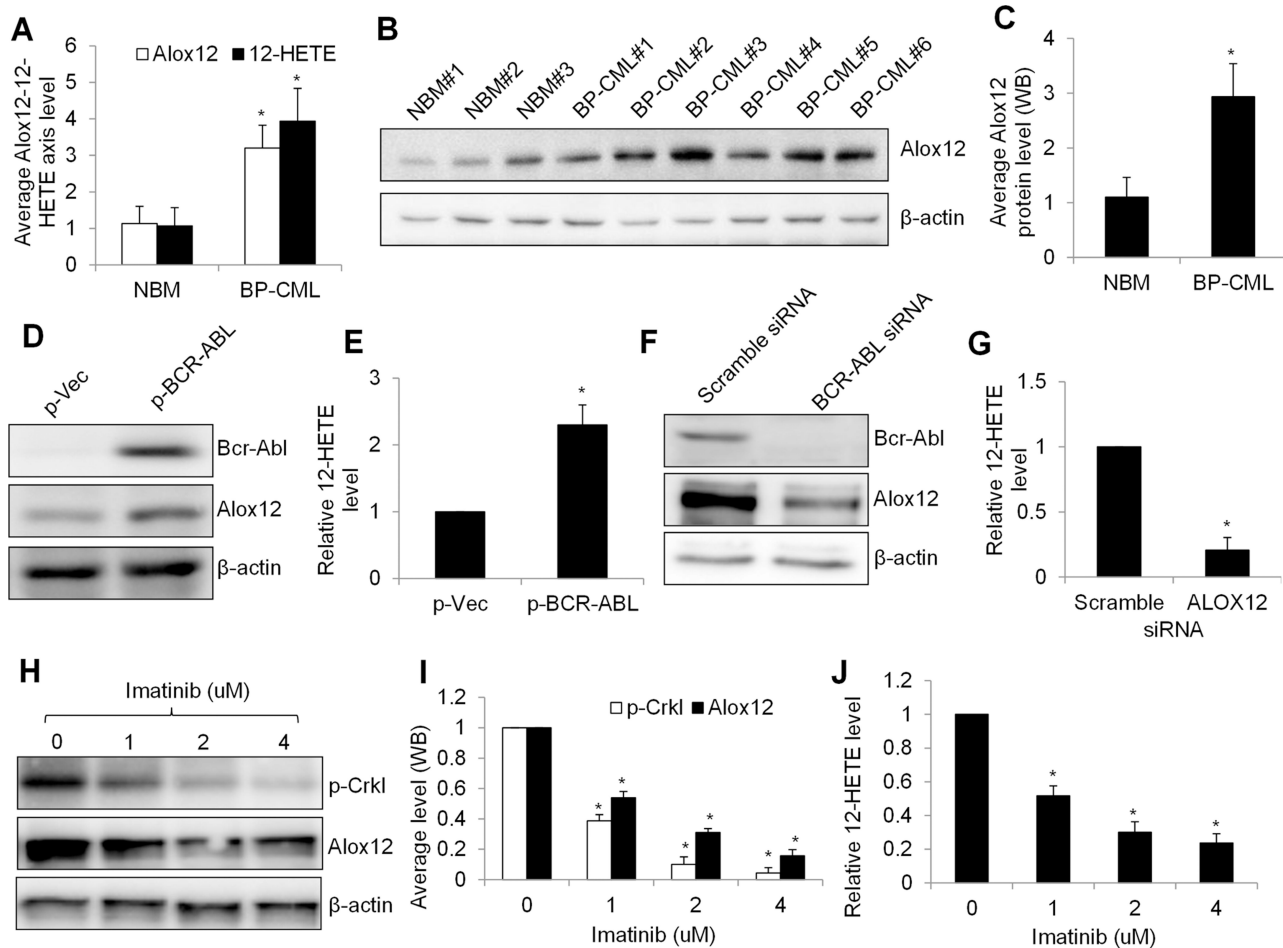


Figure 1 BCR-ABL regulates Alox12-12-HETE axis in human normal and malignant hematopoietic progenitor cells. (A) Alox12 and 12-HETE levels are higher in BP-CML (n=8) than NBM (n=5) CD34⁺ cells. (B) WB image shows Alox12 protein level in individual NBM and BP-CML CD34⁺ cells. (C) Quantification of WB shows average of Alox12 in NBM (n=2) and BP-CML (n=7) CD34⁺ cells. Overexpression of BCR-ABL in NBM CD34⁺ cells increased Alox12 protein level (D) and 12-HETE (E). BCR-ABL was transfected to NBM CD34⁺ cells. BCR-ABL knockdown by siRNA decreases Alox12 expression (F) and 12-HETE (G) in K562 cells. Cells are electroporated with 100 nM scramble or ABL siRNA. Image (H) and quantification (I) of WB shows decreased Alox12 in K562 cells after imatinib treatment. (J) Imatinib significantly decreases 12-HETE in K562 cells. Cells were harvested for WB and ELISA analysis after 24 hours treatment or 72 hours transfection. Densitometry was performed using Image J. *p<0.05, compared to NBM, p-Vec or 0 uM imatinib.

C). Consistent with siRNA knockdown, pharmacological inhibition of Alox12 using ML355, a potent and selective Alox12 inhibitor,²³ led to a significant reduction of growth and survival (Figure 2D–F). In addition, the decreased proliferation and increased apoptosis induced by Alox12 inhibition is not limited to K562 cells; LAMA82 cells responded in a similar manner (Figure S1), suggesting a general regulatory mechanism by Alox12-12-HETE in CML cell growth and survival.

Alox12 Inhibition Selectively Targets BP-CML HSPC Cells, and Acts Synergistically with Dasatinib

We further investigated the effects of Alox12 inhibition in NBM and BP-CML HSPCs. Apart from apoptosis assay, we

performed colony formation and serial replating assays using HSPCs as the hallmark characteristics of HSPCs are to proliferate, differentiate, and self-renew.²⁴ As shown in Figure 3A, ML355 induced apoptosis in BP-CML CD34⁺ cells in a dose-dependent manner, and to a significantly greater extent than in NBM counterparts (Figure 3A). ML355 also significantly decreased colony formation of BP-CML CD34⁺ cells and was less effective in NBM counterparts (Figure 3B), suggesting that ML355 preferentially inhibits BP-CML HSPCs growth and differentiation. It is worth noting that the combination of ML355 with dasatinib further enhanced the apoptosis induction and colony formation inhibition in BP-CML but not NBM HSPCs than dasatinib alone (Figure 3A and B).

In serial replating assay, we observed that BP-CML and NBM HSPC control cells were capable of forming colonies up

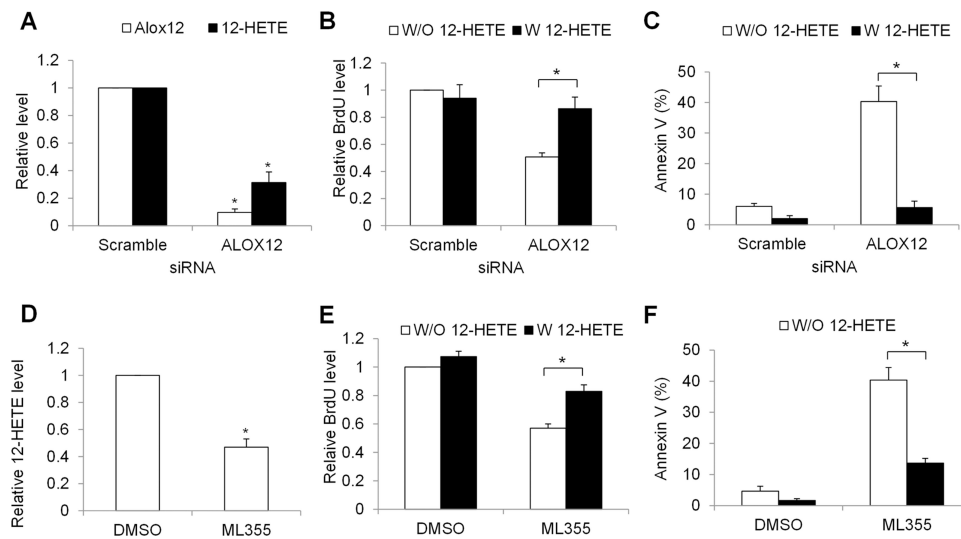


Figure 2 Alox12-12-HETE axis is required for CML cell growth and survival. **(A)** ALOX12 knockdown decreases Alox12 and 12-HETE level in K562 cells. **(B)** Proliferation is decreased and **(C)** apoptosis is increased in ALOX12- depleted K562 cells, and the effects are reversed by the addition of 12-HETE (1 μ M). Cells are electroporated with 100 nM scramble or ALOX12 siRNA and cultured for 24 hours prior to assays. ML355 (10 μ M) significantly decreases 12-HETE level **(D)**, inhibits proliferation **(E)** and induces apoptosis **(F)** of K562 cells. Proliferation and apoptosis were determined after 72 hours drug treatment. * $p < 0.05$, compared to scramble siRNA or DMSO.

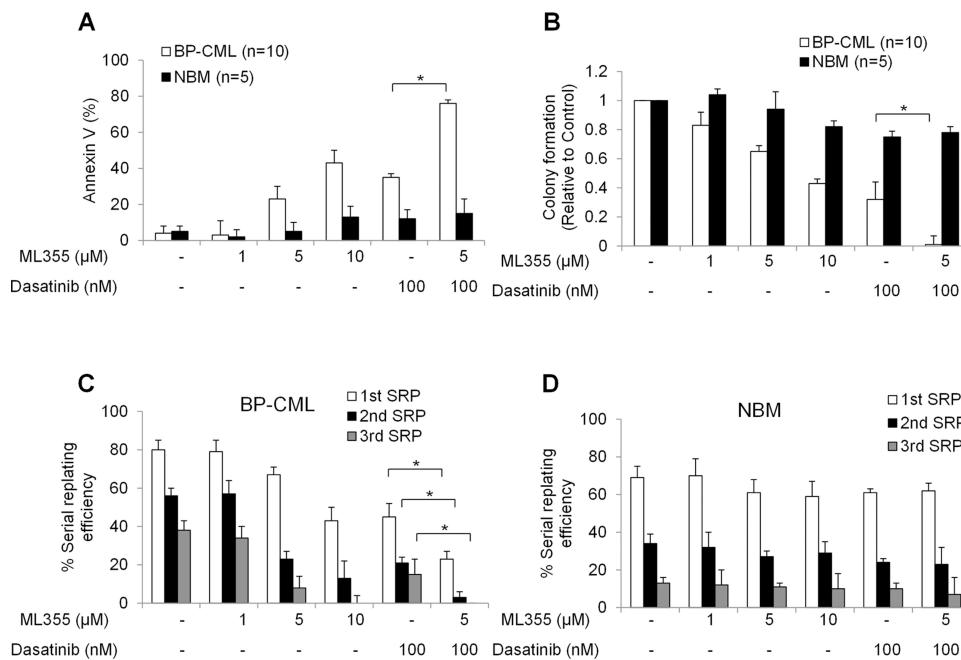


Figure 3 Alox12 inhibition selectively targets BP-CML CD34 stem/progenitor cells, and acts synergistically with dasatinib. Combination of ML355 and dasatinib selectively induces apoptosis **(A)**, inhibits colony formation **(B)** and self-renewal capacity **(C and D)** in CML CD34 cells (n=10) without affecting NBM CD34 cells (n=5). Colonies were enumerated and individually picked for serial replating. Graphs presented are mean of the results obtained from ten BP-CML patients and five NBM. * $p < 0.5$, compared to control or dasatinib alone.

to three rounds of serial replating (equivalent to >8 weeks in vitro) and that the efficiency was reduced with each replating (Figure 2D and E). However, exposure to ML355 significantly impaired the ability of BP-CML but not NBM HSPCs

to serially replate (Figure 3C and D). The combination of ML355 and dasatinib completely abolished the self-renewal ability of BP-CML HSPCs during the third replating without affecting NBM HSPCs (Figure 3D).

Alox12 Inhibition Disrupts NADH Homeostasis and Induces Oxidative Damage in CML

Wnt/ β -catenin, p53 and BMP pathways have been reported to play essential roles in maintaining BP-CML HSPC functions, including survival and self-renewal.^{25–27} Although Alox12 inhibition negatively affected BP-CML HSPCs, Alox12 inhibition by ML355 did not affect transcriptional activities of Wnt/ β -catenin, p53, and BMP as shown by luciferase reporter assays (Figure 4A). In contrast, we found that Alox12 inhibition by both ML355 and siRNA knockdown significantly decreased levels of nicotinamide adenine dinucleotide phosphate (NADPH) and its reduced form NADH in K562 and BP-CML HSPCs (Figure 4B and Figure S2A), suggesting that Alox12 inhibition disrupts NADH homeostasis. To further define the biochemical consequences of altered NADH homeostasis, we measured sirtuin1 enzyme activity which is dependent on oxidized NADH. As expected, Alox12 inhibition significantly suppressed sirtuin1 enzyme activity in K562 and BP-CML HSPCs (Figure 4C and Figure S2B). In addition, we found that Alox12 significantly increased ROS and 8-OHdG (an oxidized DNA byproduct). These results indicate that Alox12 inhibition disrupts NADH homeostasis and induces oxidative damage in CML cells.

Discussion

BCR-ABL TKIs are less effective as single agents in targeting leukemia stem cells which represent a reservoir of resistance and source of relapse. The resistance mechanisms are

not fully understood. A systematic analysis of potential drug resistance pathways is important to identify potential therapeutic targets. This aims to sensitize BCR-ABL TKI's efficacy to leukemia stem cells and induce a better patient response. Our prior work had implicated the role of Wnt/ β -catenin and ERK/MNK/eIF4E pathways in TKI resistance in CML.^{17,28} In the present study, we found that Alox12-12-HETE activation is a feature of BP-CML HSPCs and is preferentially required for leukemia HSPCs and committed cells to function properly. The ability of Alox-12-12-HETE inhibition to specifically target leukemia while sparing normal HSPCs is of interest and will contribute to improved control of BP-CML patients.

We demonstrate direct evidence obtained from patient samples that Alox12 and 12-HETE are particularly increased in BP-CML HSPCs compared to normal counterparts (Figure 1A–C), suggesting that BCR-ABL transformation activates the Alox12-12-HETE axis. This conclusion is further supported by our experimental evidence that BCR-ABL activation induces Alox12-12-HETE expression in normal HSPCs or vice versa in CML committed cells (Figure 1D–J). We further noted that Alox12 overexpression is not detected in all but some BP-CML samples (Figure 1B), suggesting that other factors rather than BCR-ABL also mediate Alox12 expression. Alox12 overexpression has been reported in lung, gastric and breast cancers.^{29–31} Our work supports and further extends the previous findings that apart from cancer committed cells, Alox12-12-HETE axis is also upregulated in primary

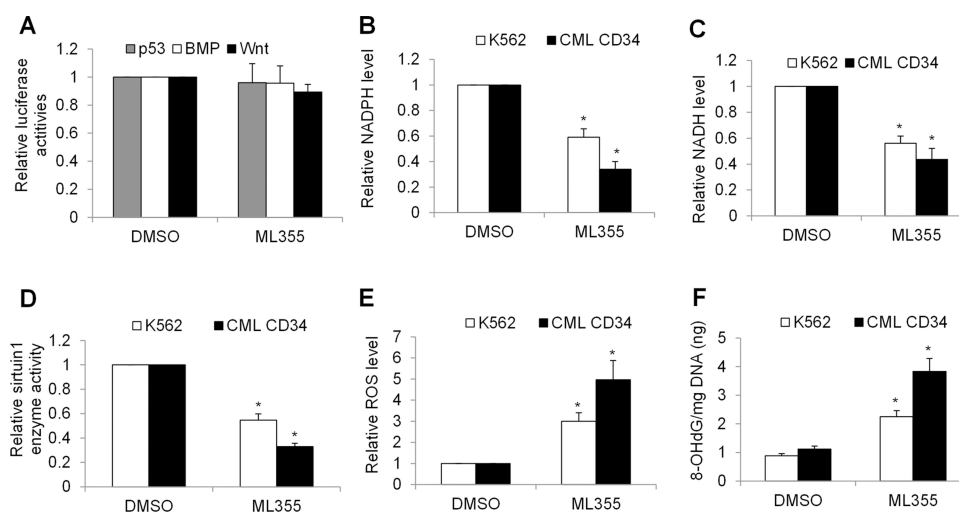


Figure 4 Alox12 inhibition decreases levels of NADPH, NADH and sirtuin1 activity, and induces oxidative stress and damage in CML cells. (A) ML353 (10 μ M) does not affect Wnt, p53 or BMP transcriptional activity in K562 cells. Cells were transfected with luciferase-based reporter constructs as described in methods. ML353 significantly decreases NADPH (B), NADH (C) and sirtuin1 enzyme activity (D) in K562 cells. ML353 significantly increases ROS (E) and 8-OHdG (F) level in K562 cells. * $p < 0.05$, compared to control.

cancer stem cells, and furthermore that this axis is mediated by oncogenic stress (eg, the presence of BCR-ABL) in cancer. Alox12 elevation has been shown to be positively associated with an advanced stage and poor differentiation in prostate cancer.³² Whether Alox12 expression or mutations can serve as a prognostic marker in CML is worth of future investigation.

We demonstrate that Alox12 inhibition suppresses proliferation and induces apoptosis in CML cells, and its effects are reversed by the addition of 12-HETE (Figure 2 and Figure S1). This data adds to the recent evidence supporting the important role of Alox12-12-HETE in the growth and survival of cancer.^{29–31,33,34} Notably, we find that Alox12 inhibition impairs CML HSPCs functions (Figure 3). This finding is consistent with the previous reports on the other two ALOX family members that loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia in mice,³⁵ and Alox15 is required for chronic myeloid leukemia stem cell survival,¹⁵ suggesting the important roles of ALOX family in leukemia stem cells. Our work also highlights the selectivity of Alox12 inhibition in BP-CML while sparing normal HSPCs, suggesting the therapeutic window of pharmacological Alox12 inhibitor in CML. The synergistic effect of Alox12 inhibitor with dasatinib in targeting BP-CML stem cells suggests the value of targeting Alox12 in overcoming resistance to BCR-ABL TKIs in CML.

Our work also addresses the mechanisms by which targeting of the Alox12-12-HETE axis inhibits myeloid leukemia. In CML HSPCs and committed cells, we show that Alox12 inhibition does not affect Wnt/ β -catenin, BPM, and p53 activities which are well-known signaling pathways playing critical roles in leukemia stem cells (Figure 4A). In contrast, we show that Alox12 inhibition decreases levels of NADPH, NADH and sirtuin1 enzyme activity. In addition, Alox12 inhibition induces oxidative stress and damage (Figure 4B–F and FigureS2). Oxidative stress has emerged as a putative mechanism in the development of BCR-ABL1 (+) and (-) myeloproliferative neoplasms.^{36–40} Oxidative stress levels are higher in CML patients compared with healthy controls.⁴⁰ Oxidative stress levels are positively correlated with JAK2V617F mutational status and thrombotic complications in patients with essential thrombocythemia.³⁸ The biological roles of Alox12 in oxidative stress and NADPH homeostasis are complex and are not well understood.⁴¹ Our study demonstrates the correlation of Alox12 inhibition with oxidative stress induction and

disruption of NADPH homeostasis in CML stem and committed cells. However, the exact mechanisms on how Alox12 inhibition leads to oxidative stress and disrupted metabolic reprogramming in CML are not clear and are worthy of further investigation to identify what are the biologically active lipid mediators involved.

Although BCR-ABL is not the exclusive regulator of the Alox12-12-HETE axis, our findings demonstrate that this axis is preferentially activated in BP-CML stem cells and plays important role in CML growth, survival, differentiation, and self-renewal. We expand the previously known BCR-ABL-regulated molecular network in leukemogenesis. Inhibition of Alox12 is selective and effective in sensitizing BP-CML stem cells to dasatinib treatment. Our results suggest that pharmacological Alox12 inhibitors may have utility in treating BP-CML patients. Pharmacological inhibitors of Alox12 have been developed and shown to have anti-cancer activities in many cancers, such as breast cancer and lung cancer. Our pre-clinical evidence may accelerate the initiation of clinical trials testing Alox12 inhibitors in combination with BCR-ABL inhibitors for the treatment of BP-CML patients.

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Disclosure

All authors declare no conflicts of interest.

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