ITD mutation in FLT3 tyrosine kinase promotes Warburg effect and renders therapeutic sensitivity to glycolytic inhibition

H-Q Ju1,6, G Zhan1,6, A Huang1, Y Sun1, S Wen1, J Yang1, W-h Lu1, R-h Xu1, J Li2, Y Li3, G Garcia-Manero4, P Huang1,5 and Y Hu1

Internal tandem duplication (ITD) mutation in Fms-like tyrosine kinase 3 gene (FLT3/ITD) represents an unfavorable genetic change in acute myeloid leukemia (AML) and is associated with poor prognosis. Metabolic alterations have been involved in tumor progression and attracted interest as a target for therapeutic intervention. However, few studies analyzed the adaptations of cellular metabolism in the context of FLT3/ITD mutation. Here, we report that FLT3/ITD causes a significant increase in aerobic glycolysis through AKT-mediated upregulation of mitochondrial hexokinase (HK2), and renders the leukemia cells highly dependent on glycolysis and sensitive to pharmacological inhibition of glycolytic activity. Inhibition of glycolysis preferentially causes severe ATP depletion and massive cell death in FLT3/ITD leukemia cells. Glycolytic inhibitors significantly enhances the cytotoxicity induced by FLT3 tyrosine kinase inhibitor sorafenib. Importantly, such combination provides substantial therapeutic benefit in a murine model bearing FLT3/ITD leukemia. Our study suggests that FLT3/ITD mutation promotes Warburg effect, and such metabolic alteration can be exploited to develop effective therapeutic strategy for treatment of AML with FLT3/ITD mutation via metabolic intervention.

INTRODUCTION

The internal tandem duplication (ITD) mutation in FLT3 tyrosine kinase is detected in ~30% of acute myeloid leukemia (AML) patients and predict poor prognosis.1–3 A recent study suggests that the FLT3/ITD mutation plays an important role in conferring drug resistance in AML.4 It has been known that ITD mutation leads to auto-phosphorylations and constitutive activation of FLT3 receptors, which promote cell proliferation through multiple signaling pathways including MAPK, STAT and PI3K/AKT.5–7 Small molecule tyrosine kinase inhibitors that target the constitutively activated FLT3 have been developed and investigated in clinical trials for AML patients.8 However, these inhibitors of FLT3 tested clinically thus far have only induced partial or transient response as single agents due to drug resistance. Indeed, acquired point mutations or overexpression of the target molecule associated with drug resistance have imposed challenge in treatment of AML patients using inhibitors of FLT3.8,9 This situation has prompted us to investigate an alternative approach that exploits the biochemical alterations that may be regulated by the oncogenic FLT3/ITD.

Increasing numbers of studies have suggested that metabolic alterations induced by oncogenes10–12 in solid tumors is associated with the aggressive phenotypes and drug sensitivity.13,14 However, the mechanistic link between the oncogenic FLT3/ITD and cellular metabolism in leukemia cells still remains largely unknown. The aim of this study was to identify the metabolic profile under the influence of FLT3/ITD and investigate the therapeutic implications of the metabolic alterations in FLT3/ITD leukemia.

In the current study, we found that compared with the parental murine hematopoietic BaF3 cells, BaF3/ITD overexpressing FLT3/ITD exhibited significant decrease of mitochondrial respiration, increase of glycolytic activity and phosphorylated form of mitochondrial hexokinase 2 (HK2). Glycolytic inhibitors including 3-Bromopyruvate propyl ester (3-BrOP) and 2-deoxyglucose (2-DG), exhibited a selective glycolytic inhibition and cytotoxicity in murine and human leukemia cells carrying FLT3/ITD mutation. Importantly, glycolytic inhibition substantially potentiated the anti-leukemia effect of the FLT3 inhibitor both in vitro and in vivo. Our study illustrates the metabolic alterations induced by FLT3/ITD and the biochemical basis to effectively kill FLT3/ITD leukemia cells using glycolytic inhibitors or in combination with FLT3 inhibitors.

MATERIALS AND METHODS

Cell lines and cell culture

The BaF3/ITD cell line generated previously15 was a generous gift from Dr Donald Small from Department of Oncology, Johns Hopkins University School of Medicine. The human leukemia cell lines MOLM-13 and MV4-11 (FLT3/ITD) were obtained from DSMZ (Braunschweig, Germany). ML-1 and HL-60 (FLT3/wild type) were obtained from ATCC (Manassas, VA, USA). All cell lines were authenticated by short tandem repeats profiling and tested for mycoplasma contamination in house before use. Peripheral blood samples were obtained from AML patients (> 80% blasts;
Reagents and antibodies

The details of compounds used for cytotoxicity assay and antibodies used for co-immunoprecipitations and immunoblots are given in Supplementary Methods.

Determination of glycolytic activity and oxygen consumption

Cellular glycolytic activity was measured by glucose uptake and lactate production as described previously. We observed in addition, oxygen consumption and extracellular acidification rate were measured by Seahorse Bioscience XF-24 Extracellular Flux Analyzer as described before.

Animal study

Animal experiments were performed under the guidelines and approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University Cancer Center. Balb/c mice (4–6 weeks old, female, purchased from Guangdong Animal Experimental Center, China) were injected intravenously with 2 × 10⁶ BaF3/ITD cells. The mice were divided into four groups (15 mice each), and randomization of animal allocation to the groups was done by random numbers generated by a computer. Five days after cell injection, the treatment group received 2-DG (i.p., 1.5 mg/g daily), sorafenib (oral gavage, 3 mg/kg daily) or 2-DG plus sorafenib. The control group received an equal volume of solvent control.

Cell Signaling Technology, Beverly, MA, USA).

Statistical analysis

The statistical significance of the difference in cytotoxicity between BaF3 and BaF3/ITD cells induced by 3-BrOP and 2-DG was evaluated using two-way ANOVA. All other statistical significant difference analyses were evaluated using two-sided Student’s t-test. Median dose-effect analysis by the Calcsyn software (Biosoft, Ferguson, MO, USA) was used to characterize synergistic or antagonistic interactions between agents. The effects of sorafenib and 2-DG on mouse survival were estimated by the Kaplan–Meier method and the statistical difference in animal survival curves between different groups was analyzed using the log-rank test. All data analyses were performed using Prism software (GraphPad, San Diego, CA, USA). Sample size was chosen based on the need to have sufficient statistical power. Error bars indicate means ± s.d. or means ± s.e.m. of three independent experiments. The variance between the groups that are being statistically compared is similar. A P-value of < 0.05 was considered statistically significant.

RESULTS

Oncogenic transformation by FLT3/ITD leads to upregulation of glycolytic activity associated with increase of mitochondrial hexokinase 2

We first tested the effect of FLT3/ITD on glycolysis using the isogenic cell lines BaF3 and BaF3/ITD. Immunoblotting analysis demonstrated the high expression of FLT3 and increase of phosphorylation of FLT3 and AKT in BaF3/ITD cells, indicating activation of the downstream effector by FLT3. Hexokinase 2 (HK2), a key glycolytic enzyme, was upregulated in BaF3/ITD cells. No significant change of glyceraldehyde-3-phosphate dehydrogenase, another key enzyme in the glycolytic pathway, was detected in BaF3/ITD (Figure 1a). Biochemical analysis showed that BaF3/ITD cells exhibited a more active glucose uptake and lactate production compared with the parental cells (Figure 1b). The Seahorse XF24 extracellular flux analyzer demonstrated a significant decrease of basal oxygen consumption rate and maximal respiration, and increase of extracellular acidification rate (ECAR, a measure of lactate production from glycolysis) in BaF3/ITD (Supplementary Figure 1). These results indicated the attenuated mitochondrial respiration in BaF3/ITD cells and a metabolic alteration from oxidative phosphorylation to glycolysis induced by FLT3/ITD.

It has been reported that the highly glycolytic phenotype in solid tumors is supported by hexokinase bound to the outer membrane of mitochondria with voltage-dependent anion channel (VDAC). When phosphorylated by AKT, mitochondrial HK2 not only promotes glycolysis but also prevents apoptosis by stabilizing the mitochondrial permeability transition pore complex, which controls the mitochondrial membrane permeability and release of cytochrome c. As PI3K/AKT is known to be a major downstream survival pathway of FLT3, we reasoned that activation of FLT3/ITD may promote glycolytic activity through PI3K/AKT-activated mitochondrial HK2, which contribute to the survival advantage of AML harboring FLT3/ITD. Indeed, we observed a substantial increase of total HK2 and phosphorylated-HK2 activated by AKT in the mitochondrial fraction of BaF3/ITD cells, and the phosphorylation was completely inhibited by a PI3K inhibitor LY294002 (Figure 1c). Confocal microscopic analysis was used to further test the localization of HK2 in BaF3 and BaF3/ITD cells. Compared with BaF3, BaF3/ITD cells exhibited intense green fluorescent signal of HK2 and orange signal due to overlap with the red fluorescence of mitochondria. The fluorescent signal of mitochondrial HK2 was markedly reduced by the FLT3 inhibitor (sorafenib, 5 μM for 18 h) (Figure 1d, left panel). Metabolically, sorafenib significantly inhibited glycolytic activity in BaF3/ITD cells in a dose-dependent manner, yet apparently did not affect the parental BaF3 cells (Supplementary Figure 2a). The co-immunoprecipitation analysis of mitochondrial proteins showed increased binding of HK2 to VDAC in BaF3/ITD cells, accompanied by increased HK2 not only promotes glycolysis but also prevents apoptosis by stabilizing the mitochondrial permeability transition pore complex, which controls the mitochondrial membrane permeability and release of cytochrome c. As PI3K/AKT is known to be a major downstream survival pathway of FLT3.

Taken together, our results indicate that FLT3/ITD signaling may upregulate glycolytic activity through PI3K/AKT–mitochondrial HK2 pathway and in part explain the poor prognosis of FLT3/ITD AML.

Glycolytic inhibition sensitizes FLT3/ITD cells to FLT3 inhibitor sorafenib

Based on the significant difference between cells with and without FLT3/ITD mutations in their glycolytic activity, we reasoned that leukemia cells with FLT3/ITD mutations might be more dependent on this metabolic pathway for survival and proliferation, and thus highly sensitive to glycolytic inhibition.

We previously showed that 3-bromopyruvate is able to inhibit the activity of HK2 and cause dissociation of HK2 from mitochondria and subsequent apoptotic cell death. As shown in Figure 2a, 20 μM 3-bromopyruvate propyl ester (3-BrOP) for 3 h caused substantial reduction of glucose uptake and ATP depletion in BaF3/ITD cells, and only moderate decrease in the parental BaF3 cells. Furthermore, 20 μM 3-BrOP caused a time-dependent loss of
mitochondrial transmembrane potential in BaF3/ITD cells as detected by a fluorescent dye rhodamine-123 (Rho-123). In contrast, only a small portion of BaF3 cells exhibited loss of Rho-123 signal after same treatment (Supplementary Figure 4a). The temporal relationship between early inhibition of glycolytic activity and the relatively late decrease of mitochondrial integrity further suggests the metabolic alteration as a therapeutic target of FLT3/ITD cells. Consistently, treatment of 3-BROP for 18 and 24 h effectively induced cell death in BaF3/ITD cells in a dose-dependent manner, while BaF3 cells were significantly less sensitive to the same treatment (Supplementary Figure 4b and Figure 2b).

Sorafenib is a tyrosine kinase inhibitor that selectively reduces leukemia blasts in AML patients with FLT3/ITD. However, FLT3 inhibitors tested clinically thus far have only induced partial or transient response as single agents. Owing to the selective effect of both sorafenib and 3-BROP in FLT3/ITD leukemia cells, we reasoned that glycolytic inhibition may significantly enhance the
anti-leukemia activity of sorafenib and cause a better therapeutic outcome. As shown in Figure 2c, sorafenib treatment alone for 18 h caused a dose-dependent decrease in glycolytic activity in BaF3/ITD cells and such inhibitory effect was significantly enhanced by addition of 7.5 μM 3-BrOP. Combination of 5 nM sorafenib for 12 h followed by 7.5 μM 3-BrOP for 6 h caused an almost complete depletion of ATP. 3-BrOP consistently enhanced the cytotoxicity induced by sorafenib at various concentrations (Supplementary Figure 6a and Figure 2d left panel). The combination index CI indicated a synergistic interaction between these two compounds (Figure 2d, right panel). In contrast, such combination did not cause any detectable change
Combination of sorafenib and glycolytic inhibition is effective in killing primary AML cells with FLT3/ITD and exhibit significant therapeutic activity in vivo

Finally, the combination effects of sorafenib and glycolytic inhibition against FLT3/ITD leukemia were tested ex vivo and in vivo. Mononuclear cells were isolated from peripheral blood draws from AML patients with FLT3/ITD, FLT3/wild-type and healthy donors. Treatment of various concentrations of 3-BrOP for 24 h caused significantly greater cell death in primary FLT3/ITD AML cells compared with FLT3/wild type (Figure 3a, left panel). While 3-BrOP induced apoptotic cell death in a dose-dependent manner against FLT3/ITD cells, no significant cytotoxicity was detected in the healthy donor samples (Figure 3a, right panel). As the AML cells with FLT3/ITD showed a 9% 'spontaneous' cell death, incubation with sorafenib (1 μM) or 3-BrOP (10 μM) induced additional 16% and 44% cell death, respectively (Figure 3b). Pretreatment with sorafenib for 18 h followed by 3-BrOP for 12 h resulted in a significant increase in cell killing compared with sorafenib (P = 0.0001) or 3-BrOP (P = 0.0026) alone (Figure 3c, left panel). In contrast, same treatment of sorafenib or 3-BrOP caused only 3 and 13% additional cell death in FLT3/wild-type cells and their combination did not increase significant cell death (Figure 3c, right panel).

Although various studies have shown the potent anti-cancer activity of 3-bromopyruvate as a glycolytic inhibitor in of either glycolytic activity or cytotoxicity in BaF3 cells (Figure 2c, Supplementary Figure 5).

Finally, the preferential inhibition of glycolysis and cytotoxicity induced by 3-BrOP and combination with sorafenib against FLT3/ITD was further validated in human leukemia cells with FLT3/ITD (MV4-11, MOLM13), in comparison with leukemia cells with FLT3/wild type (HL-60, ML-1; Supplementary Figures 6 and 7). Combination of sorafenib and glycolytic inhibition is effective in killing primary AML cells with FLT3/ITD and exhibit significant therapeutic activity in vivo.
experimental models, its efficacy has been tested clinically in a limited number of patients.27–28 2-DG, a glucose analog that inhibits glycolytic activity, has been investigated in clinical trials as monotherapy or in combination with radiotherapy or chemotherapy.29–31 Here, we demonstrated that 2-DG preferentially inhibited cell proliferation and induced cell death in BaF3/ITD cells in a dose-dependent manner. BaF3 cells were less sensitive to 2-DG at all concentrations tested (Supplementary Figures 8a and b). Intriguingly, while 2-DG caused apoptotic cell death in a dose-dependent manner in human leukemia cells with FLT3/ITD (MV4-11) and potentiate the cytotoxic effect of sorafenib, the same treatment did not cause any detectable cell death in leukemia cells with FLT3/wild type (HL60 and ML-1; Supplementary Figure 9). Consistent with the findings with 3-BrOP, 2-DG caused preferential killing of primary AML cells from FLT3/ITD patients (Supplementary Figure 10a). In addition, combination of sorafenib and 2-DG caused significant increase of net killing of primary leukemia cells with FLT3/ITD compared with sorafenib or 2-DG alone. Such combination effect was not observed in cells with FLT3/wild type (Supplementary Figure 10b).

The selectivity of 2-DG against FLT3/ITD transformed cells in vitro prompted us to evaluate whether glycolytic inhibition could potentiate the anti-leukemia activity of sorafenib in vivo. We conducted the animal experiment using a BaF3/ITD leukemia model reported before.15,32 As shown in Figure 8a, the leukemia-bearing mice started to die about 13 days after cell injection. Interestingly, although 2-DG alone did not appear to significantly prolong median (16 days) survival compared with the untreated control (15 days), 2-DG plus sorafenib provided a significant therapeutic benefit over sorafenib alone (2-DG plus sorafenib, 41 days; sorafenib, 32 days; P < 0.0001). The combination substantially prolonged the median survival by 173% from 15 days in the control group to 41 days (P < 0.0001). No severe toxicity was observed, although some mice exhibited temporary low physical activity after injection of 2-DG. Wright–Giemsa staining of the peripheral blood smears from the mice demonstrated that the control group without treatment contained ~65% leukemia cells per microscopic field. The 2-DG and sorafenib-treated group contained 54 and 26% leukemia cells, respectively. Importantly, the combination of 2-DG and sorafenib almost completely depleted the leukemia cells in the peripheral blood (Figure 4b). Representative Wright–Giemsa staining of peripheral blood smears showed that numerous immature forms/blasts, including cells with deep nuclear indentations were observed in the untreated group and single agent-treated group (2-DG or sorafenib). In contrast, immature forms/blasts were not seen in the group treated with 2-DG plus sorafenib. In addition, the FLT3/ITD leukemia mice exhibited an aggressive and disseminated disease with infiltration of other tissues including spleens, livers and lungs (Figure 4c). Such infiltration was substantially inhibited by combination of 2-DG and sorafenib compared with 2-DG or sorafenib alone.

**DISCUSSION**

Oncogenes including K-ras, c-Myc and Bcr-Abl have been shown to promote glycolysis,33–35 and such metabolic alteration have emerged as a potential target for treatment of cancers. ITD mutation of the FLT3 tyrosine kinase has been detected in high proportion of AML patients and associated with poor outcomes.2,3,36 However, the association between FLT3/ITD oncogenic signaling and glycolytic activity in leukemia and its therapeutic implications remain to be investigated. Using isogenic cell lines, we showed that FLT3/ITD transformation leads to decrease of mitochondrial respiration and elevation of glycolytic activity. Pharmacological inhibition of FLT3 and its downstream effector PI3K/AKT selectively inhibited glycolytic activity in BaF3/ITD cells compared with BaF3 cells. Therefore it is possible to exploit the metabolic alteration as a basis to effectively kill FLT3/ITD leukemia cells by glycolytic inhibition.

The observation that constitutive activation of FLT3 by ITD mutation caused upregulation of mitochondrial HK2 is a novel finding in this study. Hexokinase is the first and rate-limiting enzyme that catalyzes the ATP-dependent phosphorylation of glucose to form glucose-6-phosphate (G-6-P) in the glycolytic pathway. The mitochondrial localization of HK2 may allow them the advantage of direct access to ATP production from oxidative phosphorylation.37 As such, FLT3/ITD may promote glycolytic activity through mitochondrial hexokinase. We demonstrated increase of HK2 bound to the VDAC protein, which is the major component of the mitochondrial permeability transition pore complex. It is known that the Bcl-2 family proteins regulate the apoptosis process by their interactions with the mitochondrial permeability transition pore complex and control the mitochondrial membrane permeability for release of cytochrome c.38–40 Here, we showed upregulation of anti-apoptotic protein Bcl-XL and decrease of pro-apoptotic protein Bax bound to the HK2–VDAC complex in BaF3/ITD cells. Taken together, our studies demonstrated metabolic signaling events linked to the resistance to apoptotic pathway induced by constitutive activation of FLT3/ITD, which may contribute to a substantial growth advantage to leukemic stem and progenitor cells. As implied above, direct inhibition of HK activity or interfering with the mitochondrial-bound HK complex may release the inhibition on mitochondrial permeability transition pore and induce the apoptotic events. Indeed, we showed that inhibition of FLT3/PI3K/Akt pathway suppressed the phosphorylation of mitochondrial HK2, leading to mobilization of the Bcl-2 family proteins and selective apoptotic cell death in BaF3/ITD cells. Compared with FLT3/wild type, FLT3/ITD mutated leukemia cells were highly sensitive to glycolytic inhibitors including 3-BrOP and 2-DG that target hexokinase.

Owing to the selective cytotoxicity of both sorafenib and glycolytic inhibitors against FLT3/ITD mutated cells, it seems logical to use such combination to target both the regulatory and glycolytic proteins involved in nutrient metabolism and the apoptotic pathway. We indeed demonstrated that such combination caused synergistic cytotoxicity in the established murine and human leukemia cell lines with FLT3/ITD mutation and did not show any combination effect in those with FLT3/wild type.

This mechanism-based drug combination was equally effective in primary leukemia cells isolated from patients and in a murine model with FLT3/ITD leukemia. It is worth noting that the effective concentration of sorafenib (1 μM) in combination with glycolytic inhibitor may be achievable in human, as a pharmacokinetics study showed that 400 mg sorafenib twice daily resulted in steady-state plasma concentration of more than 1 μM.41 We used a commonly used glycolytic inhibitor 2-DG that has been tested in clinical trials39–41 for *in vivo* study. Raez *et al.*31 conducted the first clinical trial that combines 2-DG with a conventional chemotherapeutic agent. The authors suggested that the overall anti-tumor effect of 2-DG depends on the potency of the combined agent. Indeed, we found that 2-DG alone had no significant effect on the survival of animals with FLT3/ITD leukemia. However, this compound substantially prolonged the survival and inhibited the leukemia burden of animals treated with sorafenib alone. Interestingly, we recently established a sorafenib-resistant leukemia model and found that compared with the non-resistant counterpart, the resistant cells are highly sensitive to glycolytic inhibitors including 3-BrOP and 2-DG.17 Therefore, our findings indicated the advantage of combining glycolytic inhibitors with sorafenib compared with mono therapies.

Although the efficacy of 2-DG observed in clinical trials may limit further use of this compound as monotherapy for cancer treatment, our observations that FLT3/ITD promotes Warburg effect, pharmacological inhibition of glycolysis sensitized FLT3/ITD leukemia cells to sorafenib, and that 2-DG provided significant
Figure 4. 2-DG enhances the therapeutic effect of sorafenib in mice bearing FLT3/ITD leukemia. (a) The survival rate of the combination group was significantly different from that of the control \((P < 0.0001)\), 2-DG alone \((P < 0.0001)\) and sorafenib alone \((P < 0.0001)\). (b) Leukemia cell counts in the peripheral blood smears from leukemic mice with different treatments. Values represent mean percentage of leukemia cells per microscopic field ± s.e.m. \((n = 5)\). (c) Representative peripheral blood smear and hematoxylin and eosin stain of the spleens, lungs and livers from FLT3/ITD mice. Arrows indicate leukemia cells (untreated, 2-DG and sorafenib) and red blood cells (combination) shown in the insets. Original magnification \(\times 400\), insets, \(\times 1000\); leukemic spleens have red pulp effaced with leukemic cells indicated by arrows. Original magnification \(\times 200\); leukemic cells infiltrate the lungs focally in the untreated, 2-DG or sorafenib-treated groups. Original magnification \(\times 200\); leukemic cells are present in perportal areas and infiltrate the sinusoids of the liver in the untreated, 2-DG or sorafenib-treated groups. Original magnification \(\times 400\), insets, \(\times 1000\). Infiltration of the spleen, lung and liver were substantially inhibited by treatment of 2-DG plus sorafenib in the combination group.
therapeutic benefit in a murine model with FLT3/ITD leukemia in combination with sorafenib, support the notion that modulation of glycolytic metabolism may represent a novel strategy for the treatment of FLT3/ITD leukemia. In addition, we are currently testing other novel compounds that target glycolysis in preclinical models and may prove more effective in the future. Combination of glycolytic inhibitors with FLT3 tyrosine kinase inhibitors including sorafenib warrants further investigation.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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