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Synergistic cell death in FLT3-ITD positive acute myeloid leukemia by combined treatment with metformin and 6-benzylthioinosine

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Abstract

Current therapy for acute myeloid leukemia (AML) primarily includes high-dose cytotoxic chemotherapy with or without allogeneic stem cell transplantation. Targeting unique cellular metabolism of cancer cells is a potentially less toxic approach. Monotherapy with mitochondrial inhibitors like metformin have met with limited success since escape mechanisms such as increased glycolytic ATP production, especially in hyperglycemia, can overcome the metabolic blockade. As an alternative strategy for metformin therapy, we hypothesized that the combination of 6-benzylthioinosine (6-BT), a broad-spectrum metabolic inhibitor, and metformin could block this drug resistance mechanism. Metformin treatment alone resulted in significant suppression of ROS and mitochondrial respiration with increased glycolysis accompanied by modest cytotoxicity (10–25%). In contrast, 6-BT monotherapy resulted in inhibition of glucose uptake, decreased glycolysis, and decreased ATP with minimal changes in ROS and mitochondrial respiration. The combination of 6-BT with metformin resulted in significant cytotoxicity (60–70%) in monocytic AML cell lines and was associated with inhibition of FLT3-ITD activated STAT5 and reduced c-Myc and GLUT-1 expression. Therefore, although the anti-tumor and metabolic effects of metformin have been limited by the metabolic reprogramming within cells, the novel combination of 6-BT and metformin targets this bypass mechanism resulting in reduced glycolysis, STAT5 inhibition, and increased cell death.

Graphical abstract

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Keywords

metformin; cancer metabolism; oxidative phosphorylation; glycolysis; leukemia

Introduction

All cells predominantly use two methods of energy production – oxidative phosphorylation within mitochondria and anerobic glycolysis within the cytoplasm [1]. Demonstration of metabolic differences between normal and cancer cells in early 1950's by Otto Warburg revolutionized the field of cellular metabolism [2]. Since then generation of adenosine triphosphate (ATP) by glycolysis even in the presence of oxygen has been found to be the hallmark of cancer cell metabolism within both solid and hematopoietic tumors [3]. Acute myeloid leukemia (AML) is the most common myeloid neoplasm in adults and overall cure rates are low at 30–40% with relapse being a major cause of mortality [4]. Metabolomic analysis has identified key differences in the glycolytic, gluconeogenic, and tri-carboxylic acid (TCA) pathways between normal and AML cells [5, 6]. AML cells use abnormally high amounts of glucose from their microenvironment and exhibit high levels of glycolysis. These metabolic differences can enable risk stratification into favorable and unfavorable prognostic groups and glycolysis suppression enhances sensitivity to chemotherapy with cytosine arabinoside (AraC) [7].

Signaling pathways upregulated in AML show strong correlation with cellular metabolism. FLT3-ITD⁺ poor prognosis AML is characterized by high levels of phosphorylated nuclear STAT5 and reactive oxygen species (ROS) [8]. The PI3K-Akt-mTOR signaling pathway and STAT5/PI3K-Akt cross-talk can promote survival in myeloid malignancies [9, 10]. Akt (Protein Kinase B), via its control on mTORC1 (mTOR complex 1), plays a key role in cellular metabolism by activating transcription factors c-Myc and HIF-1 α (hypoxia inducible factor - 1 α) [11, 12]. Both these factors up regulate GLUT-1 (glucose transporter 1) and enhance glucose entry into the cell making it freely available for glycolytic breakdown [13, 14].

Metabolic inhibitors are being studied to exploit the differences between normal and cancer cells. Metformin, used extensively in treatment of Type 2 diabetic mellitus has generated considerable interest as an anti-neoplastic agent and is in clinical trials for breast and prostate cancer [15, 16]. Both normoglycemic and hyperglycemic states in *in vivo* mouse models have been shown to be sensitive to metformin with increased c-Myc expression contributing to the lower response with hyperglycemia [17]. Although metformin has

multiple mechanisms of potential anti-cancer activity, it activates LKB1/AMPK (AMP activated protein kinase) by inhibiting complex 1 of the mitochondrial respiratory chain, resulting in mTOR inhibition via TSC1/2 [18]. Inhibition of complex 1 also prevents fatty acid oxidation while shifting the balance toward glycolysis as a means of ATP production. Increase in cellular AMP levels results in widespread metabolic effects including increased glucose uptake and glycolysis, and decreased gluconeogenesis [19, 20]. In prostate and liver cancer cells, AMPK activation and depletion of ATP (change in AMP/ATP ratio) induces apoptosis and cell death [21, 22]. Using the same mechanism, a potential role for metformin in AML therapy has been considered, however high levels of Akt in AML enhance glycolysis and can account for metformin resistance [23, 24].

In order to circumvent this resistance mechanism, we tested a broad spectrum metabolic inhibitor called 6-benzylthioinosine (6-BT). It is an inhibitor of human ENT1 (es) nucleoside transporter and was shown to have limited uptake into normal cells through an ENT independent route [25, 26]. We wanted to test whether 6-BT could be cytotoxic in AML in combination with the potent ATP inhibitor metformin. Here we report that 6-BT was able to effectively reverse the metabolic reprogramming resulting from metformin treatment, synergize with metformin to inhibit STAT5 phosphorylation, reduce c-Myc expression and induce cell death in FLT3-mutant leukemia cells.

Materials and Methods

Cell lines and umbilical cord blood cells

MV4-11, MOLM-14, OCI-AML3, Nomo-1, THP-1 and HL-60 cell lines were used for analysis. Umbilical cord blood cells were obtained from the Duke University Cord Blood Center upon Emory University IRB approval.

Compounds

6-Benzylthioinosine (6-BT) was obtained from the NCI chemical repository. Metformin and N-Benzylloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) were purchased from Sigma-Aldrich. AZD-8055 was purchased from Chemietek.

Cytotoxicity and apoptosis assays

Cell lines were treated with vehicle (DMSO) or drug and cell death was measured 48 hours later by trypan blue exclusion assay. Apoptosis and cell death were determined by flow cytometry using Annexin V and DAPI staining.

Measurement of ROS, ATP and Glucose Levels

Refer to Supplemental Materials.

Seahorse Metabolic Analysis

The real time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at 37°C using an XF24 extracellular flux analyzer. Refer to Supplemental Materials for details.

Statistical Analysis

All data was derived as a result of three independent experiments. Two tailed t-test was used to calculate p-values and values less than 0.05 were considered to be significant.

See Supplemental Materials for additional methods.

Results

Treatment of AML cell lines with metformin results in modest cytotoxicity which is enhanced by combination with 6-BT

AML cell lines were treated with 6-BT (10 μ M) and metformin (10 mM) for 48 hours and cell death was measured (Figure 1A). Millimolar concentrations of metformin are typically used in *in vitro* since glucose concentration in cell culture media is much higher than normal blood and mimics the hyperglycemic conditions seen in diabetic patients [24]. As expected with single drug, cytotoxicity was modest (ranging from 9.8–42.7%). Metformin inhibits the activity of mitochondrial respiratory chain complex 1 resulting in ATP depletion [27, 28] and 6-BT depletes ATP [26], so we hypothesized that the combination of these two agents might show improved cytotoxicity in the AML cell lines. We observed remarkable cytotoxicity (60–70%) in FLT3-ITD⁺ MV4-11 and MOLM-14 cells at 48 hours after treatment with both drugs. Non FLT3-ITD cell lines did not demonstrate this striking cell death with the combination. We also measured the effects of the drug combination on cell growth in all the cell lines (Figure 1B). MV4-11 and MOLM-14 cell lines were most sensitive to growth inhibition with the dual drug combination. For further experiments, MV4-11 and MOLM-14 were used as ‘combination-sensitive’ cell lines and OCI-AML3 was used as a ‘combination-resistant’ cell line. Importantly, the drug combination was not cytotoxic to normal umbilical cord blood CD34⁺ cells using the same dose ranges (Figure 1C).

6-BT can induce differentiation and growth arrest in HL60 cells however its mechanism of cytotoxicity remains unclear [26]. To study the mechanism of cell death, we examined the effects of 6-BT and metformin treatment on caspase-3 cleavage at 42 hours following drug exposure in MV4-11 and MOLM-14 cell lines (Figure S1). Low levels of caspase-3 cleavage were observed in both lines and the effects were more pronounced in the MV4-11 cells. To further characterize these effects, we treated both cell lines with 6BT and metformin in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK). We found that the presence of Z-VAD-FMK partially reduced the cytotoxicity seen in MV4-11 cells induced by 6-BT and metformin, whereas their effect in MOLM-14 cells was largely unchanged (Figure S2). Apoptosis was determined by flow cytometry using Annexin V and DAPI (Figure 1D) at 48 hours. We also examined myelomonocytic differentiation in these cells following drug treatment and found no evidence that drug synergy was caused by differentiation (Figure S3). Drug synergy was defined by CompuSyn data analysis (Figure S4).

Metformin and 6-BT exhibit partial inhibition of mTOR pathway

AMPK activation following ATP depletion by metformin results in inhibition of mTOR phosphorylation [24]. To understand the mechanism of action of these two drugs, we next

examined mTOR pathway activation in both the sensitive and resistant cell lines. Cells were treated with 6-BT (10 μ M) and metformin (10 mM) for 24 hours and the effect on mTOR pathway activation was examined by Western blotting (Figure 2). AZD-8055, a specific mTORC 1/2 inhibitor was used as a positive control for inhibition of mTOR substrate phosphorylation [29]. In the sensitive lines, there was no effect noted with either drug alone and moderate (MV4-11) to complete (MOLM-14) inhibition of phosphorylation was noted in 4EBP1 – Ser 65 and p70S6 Kinase – Thr 389 following dual drug combination. No inhibition was observed in either cell line at site 4EBP1 - Thr 37/46, thus exhibiting incomplete inhibition of the mTOR axis. The resistant cell line OCI-AML3 demonstrated no inhibition of 4EBP1 or p70S6 kinase phosphorylation following treatment with single drug or the combination.

To further define the role of the mTOR pathway in the dual drug cytotoxicity, we tested AZD-8055 in combination with 6-BT in the sensitive AML cell lines. Both lines were treated with AZD-8055; singly and in combination with 6-BT (10 μ M) and cell death was examined at 48 hours (Figure 3A). Even at supra-physiologic concentrations of AZD-8055 there was no synergistic effect noted in both cell lines following dual drug treatment. Since Western blotting data was suggestive of incomplete mTOR inhibition following dual drug treatment, we hypothesized that an active mTOR pathway might be necessary for drug synergy. All cells were pre-treated with AZD-8055 followed by exposure to 6-BT and metformin for 48 hours (Figure 3B). Cell death increased in the MOLM-14 cell line with no effect on MV4-11 cells, consistent with the complete mTOR inhibition seen in MOLM-14 in Figure. 2. Given its structural similarity to 6-Mercaptopurine (6-MP), we hypothesized that 6-BT can inhibit ATP production by inhibiting nucleotide synthesis [26]. However, combination of metformin with 6-MP showed no effect on cytotoxicity in either cell lines (Figure 3C). Thus it appears that mTOR inhibition may play a minor role in drug synergy with 6-BT and metformin, but nucleotide synthesis likely has no role.

Metformin and 6-BT suppress reactive oxygen species and STAT5 activation

Metformin has been shown to effectively inhibit ROS production in cells by suppressing oxidative phosphorylation [30, 31]. We examined levels of ROS in the cells and found that the baseline levels differed between them. However, a significant decrease ($p < 0.05$) in levels of ROS was seen following 24 hours of treatment with metformin (10 mM) in all cells, and this remained consistent even with dual drug treatment (Figure 4A). ROS levels appear to strongly correlate with pSTAT5 levels in FLT3-ITD⁺ AML cells [8]. Therefore, we determined whether 6-BT and metformin treatment impacted upon pSTAT5 levels and found a notable reduction in pSTAT5, especially in MV4-11 cells (Figure 4B).

Metformin and 6-BT treatment results in depletion of intracellular ATP without accompanied increase in glucose uptake

Metformin causes a significant decrease in cellular ATP production and an increase in glucose transport; thus subsequent glycolysis is required to maintain viable intracellular ATP levels [19, 20, 23]. We treated all cells for 24 hours with vehicle, 6-BT and metformin alone or in combination and measured the intracellular ATP and extracellular glucose levels simultaneously (Figure 5A, 5B). As expected, 6-BT treatment resulted in significant ATP

depletion in the MV4-11 and MOLM-14 cells with no change in OCI-AML3 cells as compared to vehicle controls [26]. Metformin alone and the combination resulted in significant ATP depletion in all three cell lines. Metformin by itself, caused a significant decrease in glucose levels in the media, indicating increased uptake by the cells as expected. However, treatment with 6-BT and metformin caused minimal glucose depletion (10–15%) within the extracellular media in the sensitive cell lines (MV4-11, MOLM-14) but significantly depleted the levels (60%) in the resistant cell line (OCI-AML3). It is likely that within the sensitive cells, the expected increase in glucose transport with metformin is blocked by 6-BT thereby providing no substrate for energy production and resulting in subsequent cell death.

6-BT reduces rescue glycolytic flux in response to metformin mediated inhibition of oxidative phosphorylation

To measure changes in mitochondrial oxidative respiration, we focused these analyses on one FLT3-ITD⁺ cell line (MV4-11) and one control cell line (OCI-AML3). We treated both lines with 6-BT and metformin alone or in combination for 24 hours and subsequently examined changes in the oxygen consumption rate (OCR) over 30 minutes using the Seahorse XF24 extracellular flux analyzer. Treatment with metformin alone or in combination caused a significant decrease in OCR as expected compared to vehicle controls in all cell lines uniformly (Figure 6A). [32]. With the combination demonstrating low glucose uptake in MV4-11 cells (Figure 5B), we hypothesized that 6-BT might be either affecting the rate of glycolysis or directly impacting glucose transport. Rate of glycolysis was measured (extra-cellular acidification rate-ECAR) using the Seahorse analyzer (Figure 6B). The baseline rate of glycolysis varied significantly between the two cell lines. In MV4-11 cells, 6-BT treatment alone resulted in a 50% decrease in ECAR which was negated when combined with metformin. OCI-AML3 cells did show a decrease in the rate of glycolysis with 6-BT alone, though this was not significant from baseline. 6-BT by itself was able to decrease cellular oxygen consumption and glycolytic flux in FLT3-ITD positive cells, an effect that has not been previously described.

6-BT suppresses GLUT-1 and c-Myc expression

To further understand the anti-glycolytic mechanism of 6-BT, we examined the effects of 6-BT on glucose transporter 1 (GLUT-1). Overexpression of GLUT-1 in prostate, lung, brain, and breast cancers is associated with adverse prognosis [33, 34]. We treated MV4-11 and OCI-AML3 cells under different drug conditions for 24 hours and measured the effects on GLUT-1 transcription. 6-BT alone reduced the GLUT-1 mRNA transcription by 50% and this effect was maintained even with the dual drug combination (Figure 7A), specifically in the sensitive MV4-11 cells. The short lived oncoprotein c-Myc has been shown to directly activate genes involved in glycolysis such as GLUT-1 and phosphofructokinase [14]. Since it was known that 6-BT inhibited c-Myc transcription in HL-60 cells, we examined the effects on c-Myc mRNA and protein levels (Figure 7B and C). Metformin decreases c-Myc levels in mouse models of prostate and breast cancer [35, 36]. c-Myc mRNA and protein levels were significantly decreased following 6-BT monotherapy as expected and the effect was more pronounced following 6-BT and metformin combination therapy. In hyperglycemic mice, reduction of c-Myc expression correlates with metformin sensitivity and could explain the

synergy seen with 6-BT in FLT3-ITD AML lines [17]. The reduction in c-Myc protein was not due to increased degradation since the half-life of the protein remained unchanged as determined by cycloheximide treatment (Figure 7D), but may be related to decreased pSTAT5 activation.

Discussion

Metformin has been tested as a new anti-cancer agent due to its ability to activate AMPK and suppress the mTOR pathway. In diabetic patients receiving metformin the incidence of cancer is reduced and major roles for mTOR in aging, protein synthesis, and cancer have become increasingly well documented in recent years [32, 37, 38]. In AML, metformin has been reported to effectively dephosphorylate 4EBP1, inhibit translation, and reduce growth in *in vitro* and xenograft experiments [24, 39]. Specifically in FLT3-ITD AML, metformin can synergize with FLT3-ITD inhibitors to enhance cell death via mTOR inhibition and induction of autophagy [40]. The concentration of glucose in *in vitro* and *in vivo* conditions modulates the efficacy of metformin, with low glucose conditions enhancing cytotoxicity due to impaired glycolysis [41]. Higher concentrations of metformin are often needed in *in vitro* systems to achieve equivalent efficacy since cell culture media glucose concentrations range between 10–20 mM, typically considered as pre-diabetic to diabetic range. A typical dose of metformin used in humans is 20 mg/kg and doses used in murine studies have averaged about 250 mg/kg. When taking into account the human equivalent dose based on body surface area, these are very comparable [42]. However, metformin induced toxicity is also rate-limited by induction of glycolysis [43], and there is a need to improve upon the efficacy of metformin monotherapy, especially under conditions of hyperglycemia.

In this study, we tested metformin in combination with 6-BT and found that the two drugs can synergize to promote cell death in FLT3-ITD AML cell lines. This effect was marginally dependent on caspase-3 activation and primarily independent of mTOR or nucleotide synthesis inhibition. Combination drug therapy caused significant intra-cellular ATP depletion accompanied by depletion of extra-cellular glucose. As expected, metformin alone caused a decrease in oxidative phosphorylation and increase in glycolysis as evidenced by reduced OCR and increased ECAR. 6-BT treatment interestingly showed a dramatic decrease in glycolytic flux as demonstrated by a significant decline in ECAR with simultaneous decrease in GLUT-1 mRNA. Impaired glycolysis by 6-BT in AML cells likely accounts for the strong synergy seen with metformin since glycolytic escape is a known mechanism of metformin resistance. Similar results have been obtained in solid tumors using 2-deoxyglucose combined with metformin [23, 44]. This is the first demonstration that 6-BT can regulate metabolism and synergize with another metabolic inhibitor to kill AML cells.

6-BT alone or in combination with metformin was remarkably non-toxic to human CD34⁺ umbilical cord blood cells. We know that phosphorylated 6-BT is structurally identical to the 6-mercaptapurine anti-metabolite 6-methylthioinosine monophosphate (MeTIMP). However, 6-BT unlike 6-MP was unique in terms of its ability to synergize with metformin. Our data is consistent with a role for 6-BT in blocking glucose uptake through a glucose transporter. NBTI (nitrobenzylthioinosine), an analog of 6-BT, has previously been reported

to bind to GLUT-1 and block its function [45]. This mechanism of action could possibly contribute to the observed glycolytic changes.

In our previous study [26], pSTAT5+ FLT3-ITD⁺ AML and HNT34 BCR-ABL⁺ CMML [46, 47] cells were most sensitive to 6-BT, compared to HL-60 and OCI-AML3. In spite of the low cytotoxicity in HL-60 and OCI-AML3 cells, ATP depletion and growth arrest was seen accompanied by a decrease in c-Myc mRNA. In the current study, we further explored the regulation of c-Myc at the protein level and found that it was dramatically decreased 24 hours following treatment. In addition to the metabolic effects on glycolysis and oxidative phosphorylation, the drug combination was an effective inhibitor of STAT5 and c-Myc but did not induce apoptosis or differentiation. Our data is consistent with a mechanism involving both STAT5-dependent and STAT5-independent regulation of c-Myc in FLT3-ITD⁺ cells treated with 6-BT and metformin. By impairing the ability of metformin to upregulate glycolysis, the combination of 6-BT and metformin has potentially synergistic effects that could directly and indirectly lead to cytotoxicity in AML cells with FLT3 mutations (Fig. S5). 6-BT has recently been shown to activate non-canonical Wnt signaling and inhibit canonical Wnt signaling in AML cells inducing differentiation [48]. FLT3-ITD mutations result in activation of the canonical Wnt signaling pathway by stabilizing β -catenin and upregulating Frizzled-4 [49]. It is possible that the switch to non-canonical Wnt signaling in FLT3-ITD cells by 6-BT may steer cells towards differentiation; making them sensitive to metformin-induced metabolic stress. Further studies with primary AML samples will be needed to determine whether the drug combination will be an effective new strategy for different subsets of myeloid leukemia patients with differing metabolic and pSTAT5 profiles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights for paper – Leukemia Research

- 1.** Metformin and 6-BT synergize to induce cytotoxicity in FLT3-ITD⁺ leukemia.
- 2.** 6-BT modulates c-Myc and GLUT-1 expression and inhibits glycolytic flux.
- 3.** Combined block of oxidative and anaerobic metabolism promotes AML cell death.

Figure 1. Synergistic cytotoxicity of 6-BT and metformin in FLT3-ITD⁺ AML cell lines
A) MV4-11, MOLM-14, OCI-AML3, Nomo-1 and THP-1 cell lines were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM) or 6-BT and metformin for 48 hours. Cell death was measured by trypan blue staining. **B)** Growth inhibition following treatment with 6-BT and metformin was measured in MV4-11, MOLM-14, OCI-AML3, Nomo-1 and THP-1 cell lines following treatment with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM) or 6-BT and metformin for 48 hours. Fold inhibition in growth was measured by counting viable cells using the trypan blue exclusion method. (** $p < 0.05$). **C)** Normal cord blood CD34⁺ cells were treated under similar conditions and the viability was assessed by flow cytometry with DAPI staining after 48 hours of treatment. **D)** MV4-11, MOLM-14, OCI-AML3, Nomo-1 and THP-1 cell lines were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM) or 6-BT and metformin for 48 hours. Annexin V and DAPI staining of cells followed by flow cytometry was used to measure percentage of early (annexin V+/DAPI-) and late (annexin V+/DAPI+) apoptosis/death.

Figure 2. 6-BT and metformin have partial effects on inhibition of mTOR activity as measured by phosphorylation of downstream substrates

MV4-11, MOLM-14 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM), 6-BT and metformin or with AZD-8055 (500 nM) for 24 hours and protein lysates were probed with antibodies to total and phosphorylated (Ser 65 and Thr 37/46) 4EBP1 and total and phosphorylated (Thr 389) p70S6 Kinase. GAPDH was used as the loading control.

Figure 3. mTOR inhibition by metformin or nucleotide synthesis inhibition by 6-BT does not account for the synergistic cytotoxicity of the combination

A) MV4-11 and MOLM-14 cells were treated with vehicle (DMSO), 6-BT (10 μ M), AZD-8055 (250 and 500 nM) or combinations and cytotoxicity was measured at 48 hours using trypan blue. **B)** MV4-11, MOLM-14 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 μ M) and metformin (10 mM) with or without pre-treatment with AZD-8055 (250 nM) for 2 hours and cell death was estimated using trypan blue. **C)** MV4-11 and MOLM-14 cells were treated with vehicle (DMSO), 6-mercaptopurine (6-MP) (100 and 1000 nM), 6-BT (10 μ M) or combinations and cytotoxicity was measured at 48 hours using trypan blue. (** $p < 0.05$)

Figure 4. 6-BT and metformin reduces reactive oxygen species (ROS) levels and expression of pSTAT5

A) MV4-11, MOLM-14 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 µM), metformin (10 mM), 6-BT and metformin for 24 hours and ROS levels within live cells were measured with H₂DCFDA staining via flow cytometry **B)** MV4-11, MOLM-14 and OCI-AML3 were treated with vehicle (DMSO), 6-BT (10 µM), metformin (10 mM) and 6-BT and metformin for 24 hours and protein lysates were probed with antibodies to total and phosphorylated (Tyr 694) STAT5. GAPDH was used as the loading control.

Figure 5. 6-BT and metformin reduces intracellular ATP levels and extracellular glucose uptake
A) MV4-11, MOLM-14 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM), 6-BT and metformin for 24 hours and intracellular ATP levels were measured in live cells using the Perkin Elmer ATP Bioluminescence kit. **B)** MV4-11, MOLM-14 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM), 6-BT and metformin for 24 hours and extracellular glucose levels were measured using the Life Technologies Amplex Red Glucose Assay. (** $p < 0.05$)

Figure 6. 6-BT and metformin combination reduces glycolytic flux in response to metformin inhibited oxidative phosphorylation

A) & B) MV4-11 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM), 6-BT and metformin for 24 hours and oxidative phosphorylation rate (oxygen consumption rate – OCR) and glycolytic rate (extracellular acidification rate – ECAR) was measured using the Seahorse XF24 extracellular flux analyzer.

Figure 7. 6-BT suppress GLUT-1 and c-Myc expression

A) MV4-11 and OCI-AML3 cells were treated with vehicle (DMSO), 6-BT (10 μ M), metformin (10 mM), 6-BT and metformin for 24 hours. GLUT-1 mRNA levels were measured using RT-PCR. **B) & C)** c-Myc mRNA and protein levels were measured by RT-PCR and Western blotting respectively (** $p < 0.05$). **D)** MV4-11 cells were treated with 6-BT (10 μ M) for 0–120 minutes in the presence of cycloheximide to measure the half-life of c-Myc.