2-DG induces UPR-mediated cell death in ALL

Inhibition of Akt potentiates 2-DG-induced apoptosis via downregulation of UPR in acute lymphoblastic leukemia

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Keywords: Leukemia, AMPK, Akt, ER stress, UPR, GRP78, CHOP, 2-DG, glycolysis, N-linked glycosylation, D-mannose, apoptosis, combination targeted therapy.

Abbreviation list: ALL – Acute Lymphoblastic Leukemia; AMPK – AMP-activated protein kinase; ACC – Acetyl-CoA Carboxylase; 2-DG – 2-deoxy-D-glucose; CHOP – C/EBP homologous protein; ER stress – endoplasmic reticulum stress; UPR – unfolded protein response; GRP78 – 78kDA glucose regulated protein precursor.
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ABSTRACT

The ability to pair the regulation of metabolism and cellular energetics with oncogenes and tumor suppressor genes provides cancer cells with a growth and survival advantage over normal cells. We investigated the mechanism of cell death induced by 2-deoxy-D-glucose (2-DG), a sugar analogue with dual activity of inhibiting glycolysis and N-linked glycosylation, in acute lymphoblastic leukemia (ALL). We found that, unlike most other cancer phenotypes in which 2-DG only inhibits cell proliferation under normoxic conditions, ALL lymphoblasts undergo apoptosis. Bp-ALL cell lines and primary cells exhibited sensitivity to 2-DG while T-ALL cells were relatively resistant, revealing phenotypic differences within ALL sub-types. Co-treatment with D-mannose, a sugar essential for N-linked glycosylation, rescues 2-DG-treated ALL cells, indicating that inhibition of N-linked glycosylation and induction of ER stress and the unfolded protein response (UPR) is the predominant mechanism of 2-DG’s cytotoxicity in ALL. 2-DG-treated ALL cells exhibit upregulation of P-AMPK, P-Akt and induction of ER stress/UPR markers (IRE1α, GRP78, P-eIF2α, CHOP), which correlate with PARP cleavage and apoptosis. Additionally, we find that pharmacological and genetic Akt inhibition upregulates P-AMPK, downregulates UPR, and sensitizes ALL cells to remarkably low doses of 2-DG (0.5 mM), inducing 85% cell death and overcoming the relative resistance of T-ALL. In contrast, AMPK knockdown rescues ALL cells by upregulating the pro-survival UPR signaling. Therefore, 2-DG induces ALL cell death under normoxia by inducing ER stress, and AKT and AMPK, traditionally thought to operate predominantly on the glycolytic pathway, differentially regulate UPR activity to determine cell death or survival.
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INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is the most common malignancy in children and adolescents and is a leading cause of cancer-related deaths in these patients (1). Current clinical practices have had only minimal impact on cure for patients with resistant phenotypes or after relapse (2, 3). A number of ALL phenotypes exhibit mutations that lead to inactivation or constitutive activation of oncogenic pathways such as LKB1, PTEN, PI3K/Akt and RAS, which have been linked to the regulation of energy metabolism in general, and glucose metabolism in particular (4-6). T-ALL is known to have a high rate of PTEN mutations that lead to constitutive activation of Akt (7). Our laboratory has previously demonstrated that the master energy regulator AMPK has significant feed-forward and feedback crosstalk with these pathways and that AMPK is a suitable target for ALL therapy (8, 9). Consequently, although targeting glucose metabolism in ALL has not been explored, the above-described biology of ALL lymphoblasts suggests a significant therapeutic potential.

The increased glycolytic rate exhibited by cancer cells compared to their nonmalignant counterparts, even in the presence of sufficient oxygen, was first described by Otto H. Warburg (10). This unique alteration in glucose metabolism provides a biochemical basis for targeting glycolysis as a selective anticancer treatment strategy. Indeed, the glucose analogue 2-deoxy-D-glucose (2-DG) has shown activity against solid tumors in pre-clinical models and early phase human clinical trials (11), but its role as an anti-leukemic agent has not been explored. 2-DG can induce cell growth arrest and cell death by inhibiting two key glycolytic enzymes, hexokinase (HK) and phosphoglucose isomerase (PGI) (12). Following facilitated diffusion into cells through glucose
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transporters, 2-DG is converted to 2-DG-6-phosphate by HK, but unlike glucose-6-phosphate, 2-DG-6-phosphate cannot be further metabolized by PGI, and its accumulation inhibits the glycolytic pathway (13). Inhibition of glycolysis has been demonstrated to be a main mechanism of 2-DG’s cytotoxicity in hypoxic cells of solid tumors (14). However, due to its structural similarity with mannose, 2-DG has also been shown to be a potent inhibitor of N-linked glycosylation (12, 15). Indeed, 2-DG can be incorporated in place of mannose into lipid-linked oligosaccharide (LLO) chains (15), but unlike mannose, 2-DG cannot accommodate the required complex branching structure of these molecules, leading to premature termination of LLO synthesis, and accumulation of misfolded, nascent ER-synthesized proteins (16).

The 2-DG-induced accumulation of misfolded glycoproteins in the ER lumen leads to ER stress and induction of the UPR, a protective physiologic response aimed at reducing the presence of unfolded proteins and consequently ER stress (11, 12). The UPR is mediated via three ER transmembrane receptors: protein kinase dsRNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), which are activated upon dissociation from the main ER chaperone protein GRP78 to fully engage the UPR (17). In addition to its role in the removal of excess misfolded proteins from the ER lumen for proteosomal degradation, GRP78 also functions to suppress pro-apoptotic pathways triggered by ER stress (18). However, the protective capacity of the UPR is limited, and prolonged ER stress will eventually trigger irreversible pro-apoptotic signals such as increased expression of CHOP (CCAAT/enhancer binding protein homologous transcriptional factor) (19). In most malignant cell types, CHOP induction is considered sufficient to induce ER stress/UPR-
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mediated cell death, whereas in B-lineage lymphocytes, apoptotic death is determined by the homeostatic balance between multiple UPR proteins rather than changes in any single UPR protein (20).

In this study, due to the dual nature of 2-DG as an inhibitor of glycolysis and an inducer of ER stress, we evaluated its effects on these pathways in B-precursor and T-cell ALL cell models and primary cells. These studies uncovered that unlike most cancer phenotypes, which do not undergo cell death when treated with relatively low doses (<8mM) of 2-DG under normoxic as opposed to hypoxic conditions, ALL cells undergo apoptosis primarily through activation of ER stress. Moreover, we found differential sensitivity to inhibition of N-linked glycosylation vs. aerobic glycolysis within ALL subtypes, where Bp-ALL cells show significantly greater sensitivity to 2-DG than T-ALL cells. We demonstrated that the decreased sensitivity of T-ALL is conferred by Akt, and consequently co-targeting Akt signaling enhances cell death in 2-DG treated ALL cells. Finally, we establish that Akt and AMPK differentially modulate the ability of ALL cells to effectively activate UPR, which buffers the pro-apoptotic effects of 2-DG-induced glycoprotein misfolding and ER stress.
MATERIAL AND METHODS

Cell culture and reagents

The ALL cell lines CCRF-CEM (T-ALL), Jurkat (T-ALL), and NALM6 (B precursor-ALL) were maintained in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO) and antibiotics (penicillin, 100 I.U./ml; streptomycin, 100 µg/ml) (Cellgro). SupB15 cells (Bp-ALL, BCR-ABL [t(9;22)]) were maintained in Iscove’s DMEM plus heat-inactivated FBS and antibiotics. Primary ALL cells were obtained from patients with ALL at the University of Miami following IRB-approved informed consent. Primary cells were co-cultured using a human bone marrow stromal cell feeder layer (2.5 x 10^6 cells/75 cm²) immortalized by human telomerase reverse transcriptase (hTERT) transfection (provided by Dr. D. Campana, SJCRH, Memphis, TN). 2-deoxy-D-glucose, 2-fluorodeoxy-D-glucose, and D-mannose were from Sigma-Aldrich, and Akt Inhibitor X from Calbiochem (Gibbstown, NJ).

Cell viability and apoptosis assays

Cell viability was assayed by trypan blue exclusion using the Vi-CELL XR analyzer (Beckman Coulter, Brea, CA). Cell death was determined either using the Vi-CELL XR or by flow cytometry analysis of Annexin V-FITC/propidium iodide (PI) staining using BD Pharmigen FITC Annexin V apoptosis detection kit (CA, USA). Labeled cells were then analyzed by BDTM LSR II flow cytometer in the appropriate channel and FACSDiVa software version 4.1.2 (BD Biosciences). Events positive for
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annexin V and PI staining were gated and used to define the population of dead / dying cells. The level of cell death is expressed as the percentage of death measured in untreated cells. Apoptosis was expressed as a percentage (%) of cells stained with Annexin V-FITC/PI in the population (mean±SEM, n=3). Synergy analysis was determined using Chou’s combination index (CI) analyses using the following equation: CI = [(D1-combination/D1-single) + (D2-combination/D2-single)]. Assessment of synergy was performed by quantitating drug interaction with the CalcuSyn computer program (Biosoft, Ferguson, MO). (CI) values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively (21). Statistical differences in cell proliferation or cell death were assessed by one-way ANOVA followed by the Newman-Keuls multiple comparison test, or by unpaired Student’s t test using GraphPad PRISM (San Diego, CA).

Construction of stable shRNA-expressing cell lines

To down-regulate the expression of Akt1 and GRP78, we used lentiviral particles generated from co-transfection of p8.2vpdeltaR, pVSVG-pseudotyped lentiviral and pLKO.1 plasmids encoding specific hairpin RNA sequences in HEK-293 cells (Clontech, Mountain View, CA). The hairpin oligonucleotides for shRNA-Akt1 (22) and GRP78 (23) were hybridized and the duplex ligated into pLKO.1puro (Addgene plasmid #10878). Negative control lentiviral shRNAs (sc-108080) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA). To knockdown AMPKα1, lentiviral particles containing up to 5 different constructs encoding target-specific 19-25nt shRNA were used (sc-296730V; Santa Cruz Biotechnology). Stable ALL cell lines expressing each one of these shRNAs were developed by transducing CCRF-CEM and NALM6 cells by spinoculation
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and selection with 1 µg/ml puromycin as described elsewhere (24). Each stable shRNA-expressing cell line was validated for knockdown by immunoblotting.

**Protein extracts and Immunoblots**

Cells were harvested at the specified time points, washed with 1X PBS, and sonicated in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (Thermo). Proteins (50 µg/lane) were resolved by SDS-PAGE electrophoresis and transferred onto PVDF membranes. β-actin was used as a loading control. The immunoblots presented are representative of 3 independent experiments.
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RESULTS

2-DG induces growth inhibition and cell death in ALL cells under normoxia

To evaluate the clinical relevance of glycolytic inhibitors in childhood ALL, we first determined the effects of 2-DG and 2-FDG on ALL proliferation and cell death under normoxic (pO2 = 21%) and hypoxic (pO2 = 0.5%) conditions. For this, ALL cell models for T-ALL (CCRF-CEM and Jurkat, both PTEN mutants), Bp-ALL (NALM6 and SupB15, a chemotherapy-resistant BCR-ABL[t(9;22)]-expressing cell line), and representative primary ALL cells (PATIENT) were treated with a range of doses of 2-DG or 2-FDG for up to 72 h. As shown in Figure 1, 2-DG (0.5 - 4 mM) induced growth inhibition (Fig. 1A) and cell death (Fig. 1B) under normoxic conditions in all ALL cell models and primary ALL cells tested. Significantly lower cytotoxicity was observed when cells were treated under hypoxic conditions (not shown). Although growth inhibition was comparable across T- and B-lineage phenotypes, T-ALL cells (CCRF-CEM and Jurkat) exhibited relative resistance to 2-DG-induced cell death compared to B-lineage ALL cells (Fig. 1B). Primary Bp-ALL cells tested exhibited sensitivity to 2-DG under normoxic conditions in the same range as observed in the representative cell models. In contrast, treatment with equimolar concentrations of the more selective glycolytic inhibitor 2-FDG had negligible effects on CCRF-CEM cells, and induced less cell death than 2-DG in NALM6 and SupB15 cells under normoxia (Fig. 1C).

It has been reported that in cancer phenotypes in which low dose treatment with 2-DG (<4mM) induces cell death under normoxia, 2-DG acts through inhibition of N-
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linked glycosylation (12, 14). D-mannose has been shown to reverse both the growth inhibitory and cytotoxic effects associated with inhibition of N-linked glycosylation, but cannot reverse the cytotoxic effects triggered by inhibition of glycolysis (25, 26). In addition, 2-FDG is known to preferentially inhibit glycolysis with minimal effects on N-linked glycosylation. Based on the profile of 2-DG and 2-FDG-induced cytotoxicity in ALL cells, we postulate that inhibition of N-linked glycosylation by 2-DG leads to cell death in T-ALL and although it appears to be the predominant mechanism leading to cytotoxicity in Bp-ALL, inhibition of glycolysis also contributes to cell death in the latter phenotype. Consequently, we co-treated CCRF-CEM, NALM6, and SupB15 cells with 2-DG and D-mannose, and evaluated the effects on cell death. Our results indicate that mannose completely reverses the cytotoxic effects of 2-DG in CCRF-CEM at 4 and 8 mM doses of 2-DG, but only at 4 mM 2-DG dose in NALM6 (Fig. 1D). Partial reversal was seen in NALM6 at higher dose of 2-DG (8 mM) and in SupB15 cells at both doses (Fig. 1D). Therefore inhibition of N-linked glycosylation is the predominant mechanism by which low-dose 2-DG induces cell death in ALL cells. However, phenotypic differences exist, and it is possible that in Bp-ALL, inhibition of aerobic glycolysis also contributes to 2-DG-induced cytotoxicity.

2-DG induces changes in the expression and phosphorylation/activation of Akt, AMPK and ER stress/UPR-associated signaling proteins in ALL cell lines

To further understand the molecular mechanisms by which 2-DG induces cell death in ALL, we examined the expression of critical signaling proteins associated with energy metabolism in general, glucose metabolism in particular, and cell proliferation.
2-DG induces UPR-mediated cell death in ALL shown in Figure 2A, 2-DG induced significant activation of P-AMPK (T172) in both cell lines. Consistent with increased P-AMPK expression, P-mTOR (S2448) and its downstream target P-p70S6K (T389) were down-regulated by 2-DG through the inhibitory effect of AMPK on mTOR activity (27), and the downregulation of mTOR signaling was greater in NALM6 cells as compared to CCRF-CEM cells (Fig. 2A). The PTEN-mutant CCRF-CEM cells exhibited both high basal expression and sustained increased expression of P-Akt (S473) (6 h vs. 24 h), whereas in NALM6 cells, the basal expression of P-Akt (S473) was lower and the strong upregulation of P-Akt (S473) observed at 6 h subsided by 24 h. Our data show a strong correlation between the high basal and sustained expression of P-Akt following treatment with 2-DG in CCRF-CEM cells and their relative resistance to this agent (Fig. 1B). We postulate that PTEN inactivation in CCRF-CEM cells and subsequent hyperactivation of the Akt pathway, leads to relative resistance of T-ALL cells to 2-DG. Due to the high prevalence of inactivating mutations in patients with T-ALL (7), this mechanism of resistance is of clinical relevance.

Inhibition of N-linked glycosylation by 2-DG has been shown to induce accumulation of misfolded nascent glycoproteins in the ER lumen, leading to ER stress and UPR activation (25, 28). We analyzed the expression of ER stress/UPR markers in CCRF-CEM and NALM6 cells treated with 2-DG. In both cell lines, 2-DG (4 mM) increased the expression of GRP78, GRP94, IRE1α, P-eIF2α (S51), and CHOP, confirming induction of ER stress/UPR, and their increased expression correlated with PARP cleavage, a marker for apoptosis (Fig. 2B). These data suggest that 2-DG induces ALL cell death through ER stress/UPR-mediated mechanisms. The changes in
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Akt/mTOR, and UPR signaling observed in these cell lines were replicated using representative primary ALL cells, validating their clinical relevance (Fig. 2C; representative Bp-ALL sample). To confirm that the putative inhibition of N-linked glycosylation by 2-DG led to induction of ER stress/UPR, we treated the cell line models with 2-DG in the presence or absence of mannose. Western blots revealed that the addition of mannose (10 mM) resulted in downregulation of the UPR markers GRP78, P-eIF2α (S51), and CHOP in both CCRF-CEM and NALM6 cells (Fig. 2D), suggesting that mannose relieves ER stress-induced cytotoxicity in ALL cells by reversing 2-DG-induced inhibition of N-linked glycosylation.

Inhibition of Akt sensitizes ALL cells to 2-DG-induced cell death via a UPR-mediated mechanism

Based on the upregulation of P-Akt (S473) in response to 2-DG and the relative resistance of cells, harboring inactivating PTEN mutations and constitutive activation of Akt, we hypothesized that Akt plays a central pro-survival role in ALL cells treated with 2-DG. To test this, we treated CCRF-CEM cells with a pharmacological inhibitor of Akt and examined its effects on 2-DG-mediated cell death. Figure 3A shows that inhibition of Akt signaling using the Akt inhibitor X (AIX, 10 µM), sensitized CCRF-CEM cells to even a very low dose of 2-DG (0.5 mM). This combination led to over 85% apoptotic death as compared to very modest effects with each agent alone (Figure 3A) (2-DG - 8.1%; AIX - 6.9%, p<0.001). In addition, we found this combination exhibited synergism (CI = 0.36) as determined using the equation by Chou (21). Similar effects, albeit of a lesser magnitude, were observed in NALM6 (CI = 0.60) (data not shown). Western blot
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analysis of CCRF-CEM cells treated with both agents (2-DG - 0.5 and 10 mM; AIX - 10 µM) showed significant downregulation of P-Akt (S473), and the UPR markers GRP78, IRE1α, P-eIF2α (S51), and CHOP, whereas the level of P-AMPK (T172) was significantly increased (Fig. 3B).

To confirm these results, we used a genetic approach based on the lentiviral delivery of shRNAs to specifically knock down Akt1 expression in ALL cells. We found that shAkt1-expressing ALL cells treated with 2-DG (0.5, 1.5, and 5.0 mM) exhibited significant increase in apoptotic death compared to shRNA scramble controls (shSCR) (p<0.001 for CCRF-CEM; p<0.01 for NALM6) (Fig. 3C). As expected, Western blotting showed downregulation of Akt1 in shAkt1-transduced cells compared to shSCR control cells. Moreover, as was the case in the AIX-treated cells, genetic downregulation of Akt lead to increased expression of P-AMPK (T172) and decreased expression of UPR markers IRE1α, GRP78, P-eIF2α (S51), and CHOP, compared to cells treated with 2-DG alone (Fig. 3D). Taken together, our results demonstrate that Akt signaling is critical for ALL cell proliferation and survival in response to 2-DG treatment, and supports the role of Akt as a positive regulator of the pro-survival arm of the UPR (29).

Inhibition of AMPK restores UPR function and rescues ALL cells from ER stress-mediated cell death following treatment with 2-DG alone or in combination with Akt downregulation

During the course of our investigations, we uncovered that 2-DG induced P-AMPK (T172) in ALL cells (Fig. 2A), and that inhibition of Akt further enhanced AMPK activation (Fig. 3). To determine the role of AMPK in 2-DG treated ALL cells, we used
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shRNAs to down-regulate AMPKα1 in CCRF-CEM and NALM6 cells. Cells were treated with 2-DG (0.5 and 2.5 mM) ± AIX (10 µM), and the expression of AMPK, Akt, UPR markers, and cell death were examined. We found that downregulation of AMPK rescued CCRF-CEM and NALM6 cells from cell death induced by the combination of 2-DG + AIX (Fig. 4A and 4B; \( p<0.0001 \) and \( p<0.001 \) for shAMPK vs. shSCR cell death in CCRF-CEM and NALM6, respectively). Importantly, these effects were accompanied by increased induction of UPR markers in both AMPK knock-down cell lines treated with 2-DG + AIX (Fig. 4C and 4D), indicating that AMPK knock-down restored the ability of ALL cells to effectively engage the UPR.

**Inhibition of GRP78 sensitizes ALL cells to 2-DG-induced cell death**

To definitively establish that the ability of ALL cells to effectively engage the UPR is central to the ability of ALL cells to buffer 2-DG-induced cytotoxicity, we used a genetic approach to inhibit the main effector of UPR signaling – GRP78 (17, 18) and evaluated apoptotic death in 2-DG-treated ALL cells. Downregulation of GRP78 sensitized ALL cell models to equimolar concentrations of 2-DG, leading to a significant increase in cell death compared to shSCR-expressing cells (Fig. 5B, \( p<0.001 \) for CCRF-CEM/shSCR vs. CCRF-CEM/shGRP78; Fig. 5C, \( p<0.01 \) for NALM6/shSCR vs. NALM6/shGRP78). These data confirm that the ability of ALL cells to effectively engage the UPR is essential to buffer the pro-apoptotic effects of 2-DG.
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DISCUSSION

Reprogramming tumor metabolism has recently been proposed as an emerging hallmark of cancer (30). This includes reprogramming of glucose metabolism in general, and the glycolytic pathway in particular, to allow cancer cells to generate ATP through aerobic glycolysis, whereas this switch is limited in normal cells (31). Although there is substantial evidence to support increased glycolysis in tumor vs. normal tissue, recently there have been reports indicating that glycolysis is closely linked with the cell cycle and that increased glucose metabolism is a consequence of growth signaling within a cell (normal or tumor), and not necessarily as a result of “rewiring” of a cancer cell (32). Nonetheless, increased glucose metabolism appears to be a hallmark of cancer, which is likely due to their uncontrolled and constant movement through the cell cycle as well as other factors associated with oncogenic transformation (30, 33).

Recently it has been shown that ALL lymphoblasts exhibit increased glycolytic rate even in the presence of sufficient oxygen (34). On this basis we examined the effects of targeting glucose metabolism, and found that unlike in most carcinomas, 2-DG induced significant apoptosis in ALL cell models and primary ALL cells under normoxic conditions. Nevertheless, phenotypic differences in sensitivity were seen between Bp-ALL and T-ALL, with the latter exhibiting relative resistance due to the hyperactivation of Akt signaling in this PTEN-mutant-prevalent phenotype. Most cancer phenotypes that exhibit resistance to 2-DG under normoxia, buffer the downstream pro-apoptotic signals induced by this glycolytic inhibitor by alternatively using other energy sources, fueling oxidative phosphorylation via the tricarboxylic acid cycle (TCA) to generate ATP (35, 36). Therefore, our data suggest that ALL cells may be either unable to compensate via
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oxidative phosphorylation or that 2-DG interferes with pathways critical for cell survival independent of glycolysis. Using a sugar essential for N-linked glycosylation (D-mannose), we demonstrated that 2-DG’s known ability to inhibit the N-linked glycosylation pathway contributes significantly to ALL cell death under normoxia, and that in T-ALL cells, inhibition of this pathway appears to be the preferential event leading to cell death. In contrast, in Bp-ALL cells, D-mannose only partially reversed the 2-DG cytotoxicity, suggesting that 2-DG induces cell death in Bp-ALL cells via inhibition of both N-linked glycosylation and glycolysis. Conversely, the more selective glycolytic inhibitor 2-FDG failed to induce cell death in T-ALL and did only partially in Bp-ALL, consistent with increased dependence on glycolysis by B-ALL than T-ALL cells (37). Our data supports the notion that phenotypic differences in the regulation of glucose metabolism lead to the differential sensitivity of T- vs. Bp-ALL cells to 2-DG, and that further elucidation of these phenotypic differences may lead to lineage-specific therapeutic strategies.

Due to its structural similarity to mannose, a sugar central to N-linked glycosylation of proteins in the ER lumen, 2-DG can enter the glycosylation pathway and disrupt the elongation of polysaccharide chains linked to nascent proteins, resulting in accumulation of misfolded proteins in the ER lumen (12, 25) and induction of UPR (38). While the role of UPR is to alleviate sustained ER stress by inhibiting protein translation, activating chaperones and enhancing proteosomal degradation of misfolded proteins (17), the persistent ER stress will turn on the pro-apoptotic arm of UPR regulated by CHOP (19). Our data indicate that 2-DG induces ALL cell death via a mechanism mediated by ER stress/UPR as evidenced by increased expression of several UPR markers, including
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CHOP, which correlates with PARP cleavage and evidence of cell death demonstrated by Annexin V/PI staining. Moreover, we demonstrate that the addition of D-mannose not only rescues 2-DG-treated cells but correlates with the downregulation of UPR markers. Therefore, our data is consistent with published reports indicating that inhibition of glycolysis modulates prednisolone resistance in ALL and that very high doses of 2-DG (40 mM) induce cytotoxicity in lymphoma cell lines (39, 40).

Mammalian cells exhibit redundancy in the regulatory pathways that coordinate essential physiological processes aimed at buffering pro-apoptotic signals. This redundancy is significant in pathways that pair cellular energetics (AMPK/mTOR) with oncogenic (Akt) pathways related to growth/survival of ALL cells and glucose metabolism (41, 42). Indeed, the high frequency of PTEN inactivation in T-ALL results in constitutive activation of Akt (43), and resistance to TKI-induced apoptosis in BCR/ABL-positive leukemia has been associated with PTEN downregulation (44). Phosphorylation/activation of Akt results in upregulation of the glucose transporter GLUT1 and promotes the translocation of hexokinase into the mitochondria (45). It has also been reported that in addition to UPR, ER stress activates parallel endogenous cell survival mechanisms that directly buffer apoptotic signals through the Akt and ERK pathways (29) and via the anti-apoptotic proteins cIAP-2 and XIAP (46). We demonstrate that the inhibition of Akt in 2-DG-treated ALL cells results in synergistic cell death. Moreover, both the pharmacological and genetic inhibition of Akt led to significant upregulation of AMPK activity and concomitant downregulation of UPR factors in 2-
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DG-treated cells. It has been shown that Akt can negatively regulate AMPK activity in various cell models (47-49) and that AMPK can act as a suppressor of UPR activity (9).

We propose that it is the inhibition of the pro-survival UPR response by AMPK, that significantly potentiates apoptosis in response to 2-DG treatment, rather than CHOP-mediated apoptosis, induced in response to prolonged UPR and extensive ER stress. Indeed, shRNA-mediated inhibition of AMPK rescued ALL cells from 2-DG-induced cytotoxicity, and correlated with restoration of the essential UPR signaling factors. Therefore, restoration of effective UPR is needed for ALL cells to buffer and reduce the pro-apoptotic signals mediated by sustained 2-DG-induced ER stress. The pro-survival role of the UPR in determining the cellular response to 2-DG in ALL was further confirmed using shRNA inhibition of GRP78, which also sensitized ALL cells to 2-DG. Based on these data we propose a model for the mechanism of 2-DG-induced cell death via ER stress/UPR-mediated apoptosis in which AMPK and Akt modulate the UPR (Fig. 6)

In summary, we demonstrate for the first time that 2-DG (at low dose) induces cell death in ALL cells under normoxia by triggering ER stress/UPR-mediated apoptosis. The sensitivity of ALL cells to 2-DG under normoxia places them among the few cancer phenotypes that behave this way, which is important when considering 2-DG as a potential anti-leukemic agent since leukemia cells circulate freely through normoxic and hypoxic environments. The resistance to glycolytic inhibitors observed in PTEN-mutant T-ALL cells underscores the role of Akt signaling as a pro-survival pathway that buffers ER stress/UPR-mediated cell death. The crosstalk between the AMPK and Akt pathways and glucose metabolism represents synthetically lethal interactions and supports further
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consideration of 2-DG, alone or in combination with selected targeted agents, as an anti-leukemic agent for future translation into clinical trials for resistant/refractory or relapsed ALL

Grant Support:

This investigation was supported by STOP Children's Cancer Fund and the Women's Cancer Association of the University of Miami.
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References


8. Leclerc GM, Leclerc GJ, Kuznetsov JN, Barredo JC. A novel role for AMPK as a molecular switch regulating the activation of Akt and RAS signaling in acute
2-DG induces UPR-mediated cell death in ALL


2-DG induces UPR-mediated cell death in ALL


2-DG induces UPR-mediated cell death in ALL


2-DG induces UPR-mediated cell death in ALL


39. Zagorodna O, Martin SM, Rutkowski DT, Kuwana T, Spitz DR, Knudson CM. 2-Deoxyglucose-induced toxicity is regulated by Bcl-2 family members and is enhanced by antagonizing Bcl-2 in lymphoma cell lines. Oncogene 2011.

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FIGURE LEGENDS

Figure 1.  **Cytotoxicity of 2-DG in ALL cells under normoxia.**  Growth inhibition (A) and cell death (B) in T-ALL (CCRF-CEM and Jurkat) and Bp-ALL (NALM6, SupB15, and primary Patient Bp-ALL) cells treated with 2-DG (4 mM) for 72 h under normoxia (pO2 = 21%). Cell death (C) in CCRF-CEM, NALM6 and SupB15 cells treated with 2-FDG (4 and 8 mM) for 72 h under normoxia. Cell death (D) in CCRF-CEM, NALM6, and SupB15 cells treated with 2-DG (4 and 8 mM) and either with (MAN, 2 and 4 mM) or without (CTRL) D-mannose for 72 h under normoxia. Growth inhibition and cell death were expressed relative to control values (mean ±SEM, n=3), or as a percentage (%) of cells in the population (mean ±SEM, n=3), respectively. * and # denote p<0.0001 and p<0.01, respectively for glycolytic inhibitor-treated cells vs. CTRL (panels A, B, C), or for 2-DG alone vs. 2-DG + MAN (panel D).

Figure 2. **2-DG activates AMPK, Akt, and UPR signaling proteins in ALL.**  A) Immunoblotting of AMPK and Akt/mTOR signaling factors in CCRF-CEM and NALM6 cells treated with 2-DG (4 and 8 mM) for 6 and 24 h under normoxia. B) Immunoblotting of UPR signaling factors in CCRF-CEM and NALM6 cells treated with 2-DG (4 mM) for 24 h. C) Western blots of Akt/mTOR and UPR signaling proteins in primary Patient Bp-ALL cells treated with 2-DG (4 mM) for 24 h. D) Immunoblotting of UPR factors in CCRF-CEM and NALM6 cells treated with 2-DG and either with (+) or without (-) D-mannose (MAN, 10 mM).
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**Figure 3.** Inhibition of Akt synergistically sensitizes ALL cells to 2-DG and down-regulates expression of UPR factors. A) Level of apoptosis in CCRF-CEM cells treated with 2-DG (0.5 mM) and the Akt inhibitor X (AIX, 10 µM) either alone or in combination for 24-72 h under normoxia. B) Immunoblotting of P-Akt (S473), P-AMPK (T172), and UPR markers in CCRF-CEM cells treated with 2-DG (0.5 and 10 mM) in presence or absence of AIX (10 µM) for 24 h. C) Effects of Akt1 downregulation on 2-DG-induced apoptosis in stably transduced CCRF-CEM and NALM6 cells expressing either shRNAs against Akt1 (shAkt1) or control/scrambled shRNAs (shSCR) and treated with 2-DG (0.5 - 5.0 mM) for 72 h under normoxia. D) Immunoblotting of Akt1, P-AMPK (T172), and UPR signaling proteins in the CCRF-CEM and NALM6 cells treated in (C) for 24 h. * and # denote p<0.001 and p<0.01, respectively for single drug vs. combination (panel A), or shSCR vs. shAkt1 (panels C, D).

**Figure 4.** Inhibition of AMPK rescues ALL cells from 2-DG-induced cell death. Apoptosis in CCRF-CEM (A) and NALM6 (B) cells expressing either scramble shRNAs (shSCR) or shRNAs against AMPK (shAMPK), and treated with 2-DG (0.5 - 2.5 mM) and with or without AIX (10 µM) for 72 h under normoxia. * and # denote p<0.0001 and p<0.001, respectively for shSCR vs. shAMPK. Western blot analysis of AMPK, Akt/mTOR and UPR signaling pathway proteins in the shSCR- and shAMPK-expressing CCRF-CEM (C) and NALM6 (D) cells treated as described in panels A and B for 24 h.

**Figure 5.** Downregulation of GRP78 sensitizes 2-DG-treated ALL cells. Western blot analysis of GRP78 (A) in stably transduced CCRF-CEM and NALM6 cells expressing
2-DG induces UPR-mediated cell death in ALL

shRNAs against GRP78 (shGRP78), and treated with 2-DG (0.5 - 2.5 mM) for 24 h under normoxia. Level of apoptosis in stably transduced CCRF-CEM (B) and NALM6 (C) cells expressing either scramble shRNAs (shSCR) or shRNAs against GRP78 (shGRP78), and treated with 2-DG (0.5, 1.0 and 2.5 mM) for 72 h under normoxia. * and # denote p<0.001 and p<0.01, respectively for shSCR vs. shGRP78.

**Figure 6. Proposed mechanism of action for 2-DG in ALL cells under normoxia.**

2-DG interferes with N-linked glycosylation, leading to accumulation of misfolded glycoproteins, which triggers ER stress and induction of unfolded protein response (UPR). Binding of the misfolded glycoproteins to the central UPR regulator GRP78 will result in its dissociation from the UPR effectors (IRE1α, ATF6, and PERK), allowing them to fully engage UPR. GRP78 also acts a major chaperone during protein folding, removes excess misfolded proteins from the ER lumen for proteosomal degradation, and functions to suppress pro-apoptotic pathways triggered by CHOP. Separately, 2-DG inhibits glycolysis, decreases ATP levels and leads to activation of AMPK. Our data demonstrate that AMPK activation blocks expression of UPR signaling and significantly potentiates apoptosis in 2-DG treated ALL cells. Akt promotes UPR signaling and cell survival via downregulation AMPK. Therefore we hypothesize that it is the inhibition by AMPK of the pro-survival GRP78-mediated UPR signaling that significantly potentiates apoptosis in response to 2-DG plus AIX treatment, rather than the classical CHOP-mediated apoptotic signaling induced by extensive ER stress and prolonged UPR.
### A

<table>
<thead>
<tr>
<th>2-DG (mM):</th>
<th>CCRF-CEM</th>
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<tr>
<td>0 4 8</td>
<td>6 h</td>
<td>6 h</td>
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<td>24 h</td>
</tr>
<tr>
<td>0 4 8</td>
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- **P-AMPKα** (Thr172)
- **AMPKα1**
- **P-Akt** (Ser473)
- **total Akt**
- **P-mTOR** (Ser2448)
- **P-p70S6K** (Thr389)
- **β-actin**

### B

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<th>2-DG (4 mM):</th>
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- **GRP78**
- **GRP94**
- **IRE1α**
- **P-eIF2α** (Ser51)
- **CHOP**
- **PARP**
- **β-actin**

### C

<table>
<thead>
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<th>2-DG (4 mM):</th>
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- **P-mTOR** (Ser2448)
- **IRE1α**
- **GRP78**
- **P-Akt** (Ser473)
- **β-actin**

### D

<table>
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<th>MAN (10 mM):</th>
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- **2-DG (mM):**
  - 0 0.5 10 0 10

- **GRP78**
- **P-eIF2α** (Ser51)
- **CHOP**
- **β-actin**

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Figure A: CCRF-CEM cell line showing apoptosis percentages at different time points (24h, 48h, 72h) after treatment with various concentrations of 2DG and AIX.

Figure B: Western blot analysis of CCRF-CEM cells treated with different concentrations of 2DG and AIX showing phosphorylation levels of Akt (Ser473), AMPKα (Thr172), GRP78, IRE1α, eIF2α (Ser51), CHOP, and β-actin.

Figure C: CCRF-CEM and NALM6 cell lines showing apoptosis percentages after treatment with 2DG and shRNA targeting Akt1. CCRF-CEM: shSCR vs shAkt1, NALM6: shSCR vs shAkt1.

Figure D: Western blot analysis of CCRF-CEM and NALM6 cell lines treated with 2DG and shRNA targeting Akt1 showing phosphorylation levels of Akt1, AMPKα (Thr172), GRP78, IRE1α, eIF2α (Ser51), CHOP, and β-actin.
A. Western blots showing the levels of GRP78 and β-actin in CCRF-CEM and NALM6 cells treated with 2DG. The data shows an increase in GRP78 levels in both cell lines with shGRP78 compared to shSCR.

B. Graphs showing the percentage of apoptosis in CCRF-CEM and NALM6 cells treated with 2DG. In CCRF-CEM, shGRP78 induces a significant increase in apoptosis at 2.5 mM 2DG compared to shSCR. In NALM6, shGRP78 shows a trend towards increased apoptosis at 1 mM 2DG.

C. Graph showing the percentage of apoptosis in CCRF-CEM and NALM6 cells treated with 2DG. CCRF-CEM displays a significant increase in apoptosis with shGRP78 at 2.5 mM 2DG. NALM6 shows a trend towards increased apoptosis with shGRP78 at 1 mM 2DG.
Molecular Cancer Research

Inhibition of Akt potentiates 2-DG-induced apoptosis via downregulation of UPR in acute lymphoblastic leukemia

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Mol Cancer Res  Published OnlineFirst June 12, 2012.