

Antileukemic Activity of 2-Deoxy-D-Glucose through Inhibition of N-Linked Glycosylation in Acute Myeloid Leukemia with *FLT3-ITD* or *c-KIT* Mutations

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Abstract

We assessed the antileukemic activity of 2-deoxy-D-glucose (2-DG) through the modulation of expression of receptor tyrosine kinases (RTK) commonly mutated in acute myeloid leukemia (AML). We used human leukemic cell lines cells, both *in vitro* and *in vivo*, as well as leukemic samples from AML patients to demonstrate the role of 2-DG in tumor cell growth inhibition. 2-DG, through N-linked glycosylation inhibition, affected the cell-surface expression and cellular signaling of both *FLT3-ITD* and mutated *c-KIT* and induced apoptotic cell death. Leukemic cells harboring these mutated RTKs (MV4-11, MOLM-14, Kasumi-1, and TF-1 *c-KIT* D816V) were the most sensitive to 2-DG treatment *in vitro* as compared with non-mutated cells. 2-DG activity was also demonstrated in leukemic cells harboring *FLT3-TKD* mutations resistant to the tyrosine

kinase inhibitor (TKI) quizartinib. Moreover, the antileukemic activity of 2-DG was particularly marked in *c-KIT*-mutated cell lines and cell samples from core binding factor-AML patients. In these cells, 2-DG inhibited the cell-surface expression of *c-KIT*, abrogated STAT3 and MAPK-ERK pathways, and strongly downregulated the expression of the receptor resulting in a strong *in vivo* effect in NOD/SCID mice xenografted with Kasumi-1 cells. Finally, we showed that 2-DG decreases Mcl-1 protein expression in AML cells and induces sensitization to both the BH3 mimetic inhibitor of Bcl-xL, Bcl-2 and Bcl-w, ABT-737, and cytarabine. In conclusion, 2-DG displays a significant antileukemic activity in AML with *FLT3-ITD* or *KIT* mutations, opening a new therapeutic window in a subset of AML with mutated RTKs. *Mol Cancer Ther*; 14(10); 2364–73. ©2015 AACR.

Introduction

Acute myeloid leukemia (AML) is characterized by the clonal expansion and accumulation of immature blasts in the bone marrow (1). Among the molecular pathways involved in leukemogenesis, including alterations in intracellular signal transduc-

tion, cell differentiation, DNA methylation, spliceosome machinery or in the cohesin complex, constitutive activation of receptor tyrosine kinases (RTK) is a crucial step for full blown leukemia development in a large subset of AML. In fact, the classical two-hit model proposed several years ago postulated that the combination of activating mutations of RTKs, driving cell survival and proliferation, with lesions in transcriptional factors, leading to a maturation block, were sufficient to induce AML (2).

FLT3 (Fms-like tyrosine kinase 3) and *c-KIT* are two transmembrane glycoprotein members of the class III RTKs expressed in early hematopoietic progenitors involved in cell proliferation, differentiation, and survival (3–5). Their role in leukemogenesis has been extensively studied, setting them as attractive therapeutic targets in some subsets of AML (6, 7). Gain-of-function mutations of the *FLT3* gene, mostly represented by internal tandem duplications (ITD) mutations in exon 11 or 12 encoding the juxtamembrane domain of the protein, are found in about 30% of patients with AML and are associated with poor outcome (8, 9). Point mutations in the tyrosine kinase domain (TKD) are also found in about 5% of AML patients, commonly at the D835 codon (10). *FLT3-ITD* and *FLT3-TKD* have been shown to induce ligand-independent cell proliferation through activation of canonical cell survival pathways, such as MAPK/ERK, PI3K/AKT, or STAT5, although these two mutations display differences in their signaling properties (11–14). For instance, *FLT3-ITD*, but not *FLT3-TKD*, strongly activates the STAT5 signaling pathway. In addition,

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FLT3-ITD has been shown to differentially activate those signaling pathways according to its cellular localization, driving STAT5 from the endoplasmic reticulum (ER) and MAPK as well as PI3K pathways from the plasma membrane (15). Small-molecule tyrosine kinase inhibitors (TKI) targeting FLT3 have been a matter of intense clinical research for the past decade in AML patients with FLT3-ITD. However, first-generation TKIs induced, at best, peripheral blood blast clearance without complete response. Several mechanisms of primary and secondary resistance, such as other FLT3 mutations, including FLT3-D835, autocrine stimulation, FLT3 overexpression, and poor pharmacokinetics, have been proposed to explain these disappointing results (7). More recently, quizartinib, a second-generation TKI with a higher selectivity for FLT3 and better pharmacologic properties, demonstrated a much more convincing efficacy (16). c-KIT mutations are found in about 20% to 40% of patients with core binding factor (CBF) AML and are associated with a higher incidence of relapse (17). The most frequent c-KIT mutations in CBF-AML are point mutations, insertions, or deletions in exon 17 and 8 (18), which encode the activation-loop in the kinase domain and an extracellular region of c-KIT, respectively (17, 19). Mutated c-KIT induces constitutive activation of PI3K-AKT and STAT3 pathways (20). In addition, the murine interleukin-3-dependent cell line BaF/3 modified to constitutively express the c-KIT D816V mutation acquires growth factor independence, supporting the mutation's transforming activity in CBF-AML (21). The dual BCR-ABL/Src kinase inhibitor, dasatinib, which also potently targets c-KIT, is currently being assessed in clinical trials for CBF-AML patients.

As a potent inhibitor of the glycolytic pathway, the anticancer activity of 2-deoxy-D-glucose (2-DG), has been extensively studied in solid tumors (22). Much less is known in hematologic malignancies and particularly in AML (23, 24). However, several cellular effects induced by 2-DG deserve further assessment of its activity in the context of AML biology. Inhibition of glucose metabolism through caloric restriction or 2-DG treatment has been shown to downregulate the Bcl-2 family member, myeloid cell leukemia 1 (Mcl-1), thereby restoring sensitivity to apoptosis induction (25, 26). Overexpression of Mcl-1 has been demonstrated in AML cells, and removal of Mcl-1, but not other member of the Bcl-2 family, including Bcl-xL, Bcl-2, or Bcl-w, induced leukemic cell death, setting Mcl-1 as a valuable therapeutic target in AML (27, 28). Independently of its effect on glycolysis, 2-DG can also affect protein glycosylation by interfering with N-linked glycosylation, leading to accumulation of misfolded proteins and an ER stress response (29–32). This mechanism can be alleviated by D-mannose supplementation without affecting the inhibition of glycolysis induced by 2-DG. It has been shown that 2-DG-induced N-linked glycosylation inhibition led to cell death independently of the glycolysis inhibition in some tumor types (30). In addition, inhibition of N-linked glycosylation can prevent cell-surface expression of immune receptors (29). Similar to other membrane receptors for growth factors, FLT3 and c-KIT undergo a complex maturation process, in which they undergo N-linked glycosylation in the ER before being transported to the Golgi apparatus where they are modified by further complex glycosylations and subsequently transported to the cell surface (33). This maturation process is affected by constitutive tyrosine phosphorylations induced by mutations of these receptors. Indeed, FLT3-ITD and mutated c-KIT are predominantly expressed as an immature, underglycosylated form compared with their wild-type

counterparts (34). Although 2-DG has been shown to induce the dephosphorylation of FLT3 in BaF/3 cells engineered to express both FLT3 and oncogenic mutated Cbl (35), its impact on mutated receptors has not been explored.

In this study, we assessed the potential anti-AML activity of 2-DG through the modulation of RTKs expression and signalization as well as Mcl-1 expression, focusing on AML models in which mutated RTKs are intimately linked to leukemogenesis.

Materials and Methods

Cell lines and AML samples

Leukemic cell lines U-937, TF-1, OCI-AML3, Kasumi-1, MOLM-14, RS4-11, and MV4-11 were purchased in 2013 from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz, Germany). MOLM-14 cell line was used to generate a FLT3-ITD-D835Y cell line (MOLM-14/TKD). The *FLT3-ITD* gene was cloned into the pLKO.1-blast lentiviral expression vector (Addgene Plasmid 26655). Mutation producing a D835Y amino-acid substitution within FLT3 kinase domain (FLT3-ITD-D835Y) was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), in accordance with the manufacturer's instructions using the following (5'–3') primer: CTTTGATTGGCTCGATATATCATGAGTGATTCCAAC. We used 293-T packaging cells to produce FLT3-ITD and FLT3-ITD-D835Y lentivirus through cotransfection of FLT3-containing plasmids with lentiviral protein-encoding plasmids. Supernatants were collected over 3 consecutive days beginning 48 hours after transfection, and stored at –80°C. We plated 10⁶/mL MOLM-14 cells in 100 μL of α-MEM medium and added 5 μL of lentiviral supernatant to the culture. After 3 hours, culture medium was supplemented with 10% FBS. Puromycin selection started 48 hours after lentiviral infection and allowed enrichment for FLT3-ITD or FLT3-ITD-D835Y-expressing MOLM-14 cells. TF1 c-KIT D816V and BaF/3 c-KIT-D816V were a kind gift from Patrice Dubreuil in 2013 (CRCM, Marseille, France). Leukemic cell lines were not authenticated in our laboratory. AML samples were obtained from patients at the Hematology Department of Toulouse, after consent in accordance with the Declaration of Helsinki (HIMIP collection of Inserm-U1037, n1DC-2008-307-CPTP1 HIMIP).

Antibodies and reagents

Antibodies anti-phospho-Flt-3 (Y591), anti-phospho-Stat-5 (Y694), anti-Stat-5, anti-phospho-Stat-3 (Y705), anti-Stat3, anti-phospho-c-KIT (Y719), anti-phospho-Akt (S473), anti-Akt, anti-phospho-p44/42 MAPK Erk1/2 (T202/Y204), anti-p44/42 MAPK Erk1/2, and anti-Mcl-1 were obtained from Cell Signaling Technology; C-20 anti-Flt-3/Flk-2 and C-19 anti-c-KIT were from Santa Cruz Biotechnology; mouse monoclonal anti-Flt3 was used for immunofluorescence analysis (MAB812; R&D Systems); flow cytometry antibodies anti-hCD117, anti-hCD135 were from BD Pharmingen. 2-DG was from Sigma.

Western blot analysis

Proteins were resolved using 4% to 12% n-polyacrylamide gel electrophoresis Bis-Tris gels (Life Technology) and electrotransferred to nitrocellulose membranes. After blocking in PBS–0.1% Tween 20% to 5% bovine serum albumin, membranes were immunostained with appropriate antibodies and horseradish peroxidase-conjugated secondary antibodies and visualized with an enhanced chemoluminescence detection system.

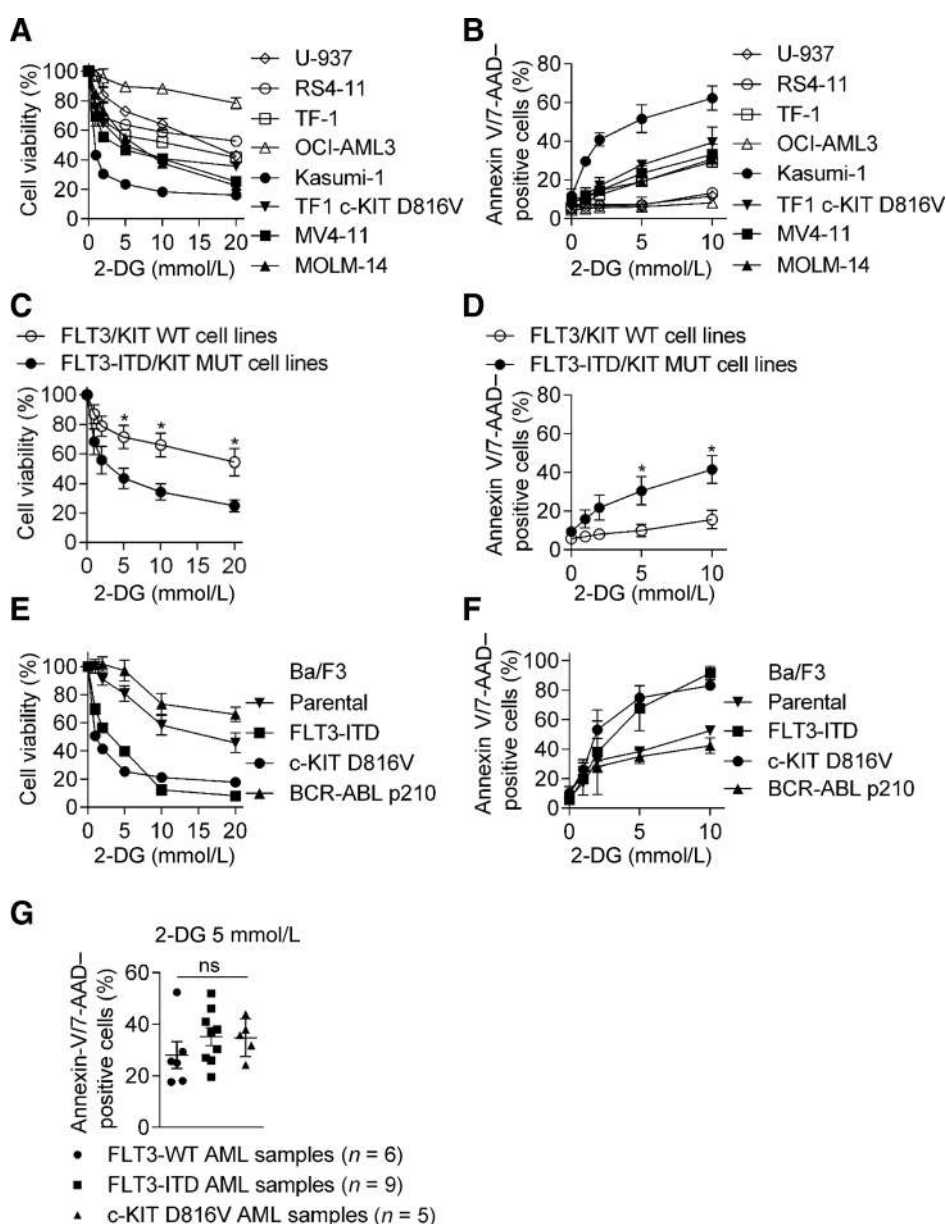


Figure 1. 2-DG inhibits cell proliferation and induces various apoptotic responses in AML cells. MV4-11, MOLM-14, Kasumi-1, RS4-11, TF1, TF-1 c-KIT-D816V, OCI-AML3, and U-937 cells were cultured 24 hours alone or with 2-DG at increasing concentrations before analysis. A, cell viability was quantified by the MTS assay. Proliferation indices were normalized to untreated controls. Results are mean \pm SEM of three independent experiments performed in triplicate. B, apoptosis was studied using Annexin V/7-AAD staining and flow cytometry. Results shown are mean \pm SEM and are representative of three independent experiments. Results were pooled for comparison between RTKs mutated and RTK unmutated cell lines viability (C) and apoptosis (D). Parental BaF/3 or BaF/3 harboring FLT3-ITD, c-KIT-D816V, or BCR-ABL were treated 24 hours with 2-DG at different concentrations before cell viability (E) or apoptosis (F) analyses as previously described. Patient samples harboring FLT3-ITD mutations, c-KIT-D816 or wild-type for these RTK (G) were plated at 5×10^5 cells/mL and treated 24 hours with 2-DG at indicated concentrations before apoptosis analysis using Annexin V/7-AAD staining and flow cytometry. Results shown are mean \pm SEM. *, $P < 0.05$; ns, not significant.

ATP analysis

ATP was measured using the Promega CellTiter-Glo Kit. After treatment, 50,000 cells were resuspended in 80 μ L and distributed in a 96-well plate. Cells were treated in quadruplicate with PBS, oligomycin A, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), or sodium iodoacetate both alone or in combination with oligomycin A or FCCP. After 1 hour of incubation, 100 μ L of CellTiter-Glo reaction mix was added to each well for a final volume of 200 μ L. Plates were then analyzed for luminescence. Global ATP and percentages of both glycolytic and mitochondrial ATP were determined by comparing the different conditions.

Tumor xenograft in NOD/SCID mice

Xenograft tumors were generated by injecting subcutaneously 5×10^6 Kasumi-1 or MOLM-14 cells in 100 μ L of PBS cells on both

flanks in NOD/SCID mice. Once the tumors reached 50 to 100 mm^3 in size, animals were treated daily with 2-DG (500 mg/kg/d, intraperitoneally) or cytarabine (15 mg/kg/d, intraperitoneally) or vehicle (PBS). Treatment with 2-DG was well tolerated. Tumors were measured with a caliper and volume calculated using the formula: $v = A \times B^2/2$, where A is the larger diameter and B is the smaller diameter. All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Methods for *FLT3* and *cKIT* RNA expression are available in supplementary files.

Statistical analysis

Data from three independent experiments were reported as mean \pm SEM. Statistical analyses were performed using unpaired

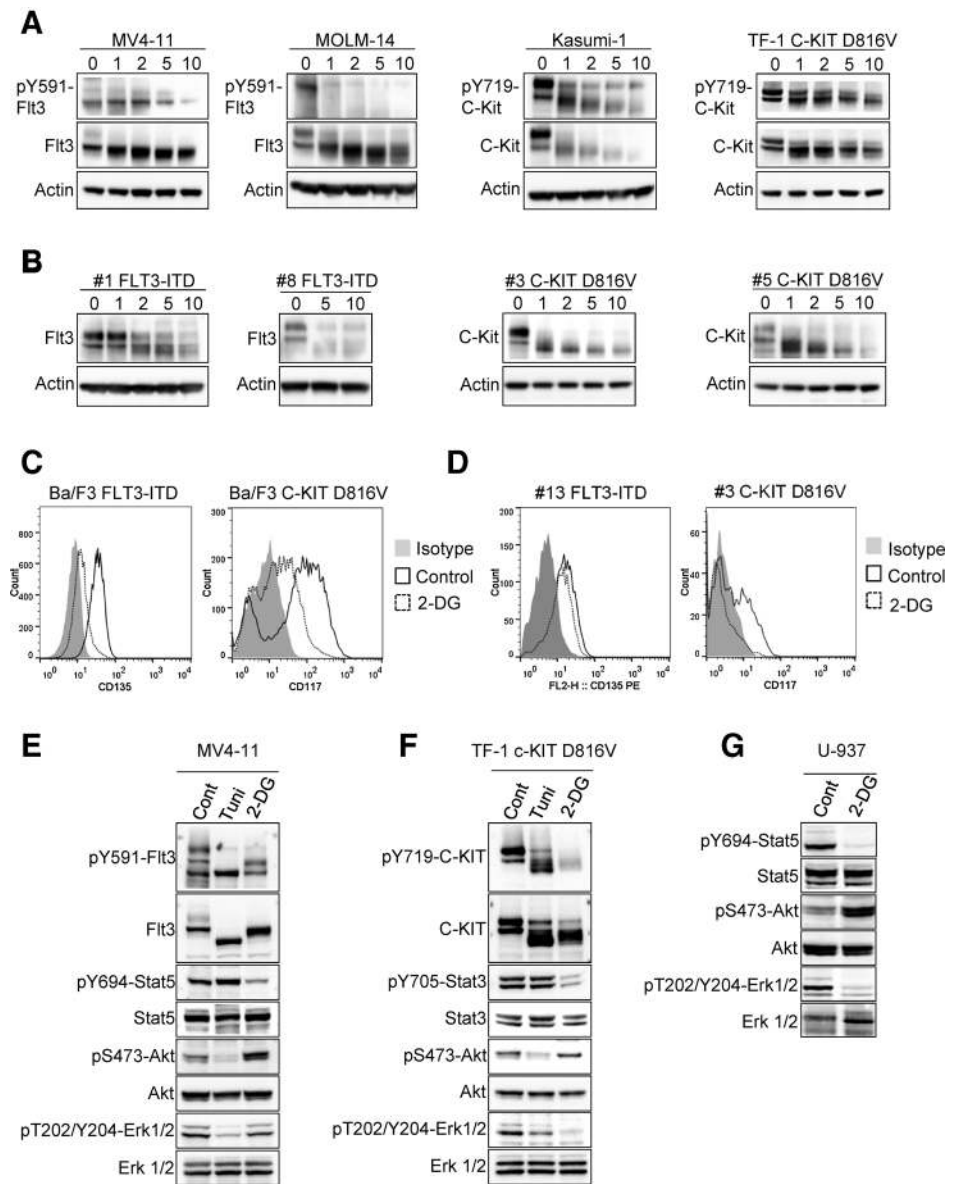


Figure 2.

Cell-surface expression of FLT3-ITD and c-KIT-N822K/D816V is affected by 2-DG. A, FLT3-ITD or c-KIT-mutated cell lines were treated 24 hours with increasing 2-DG doses from 1 mmol/L up to 10 mmol/L and then analyzed by Western blot analysis. B, Western blot analyses were performed to determine the impact of 2-DG on FLT3-ITD or c-KIT-D816V expression in primary AML samples. C, BaF/3 FLT3-ITD cells or BaF/3 c-KIT-D816V cells were incubated 24 hours with 5 mmol/L of 2-DG before cell-surface expression analysis of CD135 (FLT3) or CD117 (KIT), by flow cytometry. D, cell-surface expression of FLT3-ITD ($n = 4$) or c-KIT-D816V after 2-DG exposure was also assessed by flow cytometry as previously described in patient samples. MV4-11 (E) or TF-1 c-KIT-D816V (F) were treated overnight with 1 μ mol/L of tunicamycin or 24 hours with 5 mmol/L of 2-DG and then analyzed by Western blot analysis as previously described using the appropriate antibodies. U-937 cells were exposed 24 hours with 5 mmol/L 2-DG before Western blot analysis (G).

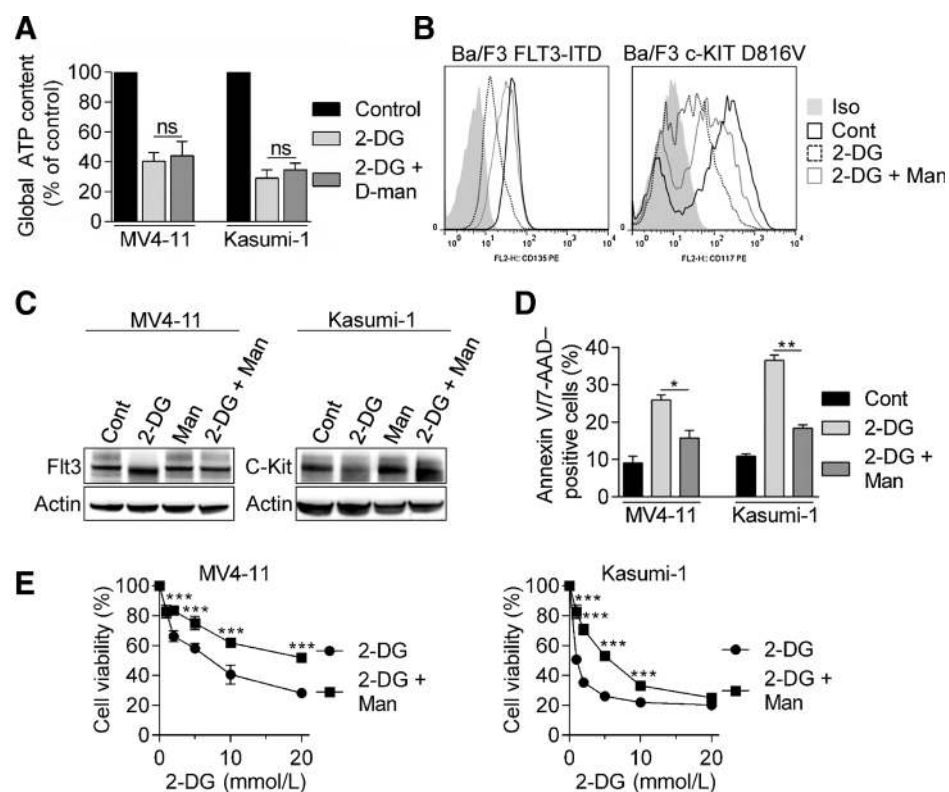
two-tailed Student *t* tests with Prism 5 software (GraphPad Software Inc.). $P < 0.05$ was regarded as significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, respectively.

Results

2-DG inhibits cell viability and induces apoptosis in leukemic cell lines and primary AML samples bearing FLT3-ITD or c-KIT mutations

We first assessed the impact of 2-DG exposure on cell viability and apoptosis in leukemic cell lines with different molecular backgrounds (Supplementary Table S1). After 24 hours, 2-DG inhibits cell viability in a dose-dependent manner in all tested cell lines with an IC_{50} ranging from 1.2 mmol/L in the most sensitive cell line Kasumi-1 to >20 mmol/L in OCI-AML3 (Fig. 1A). This effect was mainly due to apoptosis induction in Kasumi-1, TF-1 c-KIT-D816V, MV4-11, and MOLM-14 cell lines, whereas OCI-

AML3, U-937, and RS4-11 cells, which do not express oncogenic RTKs, displayed virtually no feature of apoptosis after 2-DG treatment (Fig. 1B). When we compared the overall sensitivity to 2-DG according to RTK mutations, cell lines with FLT3-ITD or c-KIT mutations were significantly more sensitive than nonmutated cell lines (Fig. 1C and D). Moreover, both FLT3-ITD and c-KIT-D816V mutations sensitized the BaF/3 murine cell line to 2-DG activity while the cytosolic oncogene BCR-ABL did not (Fig. 1E and F). Because the proapoptotic effects of 2-DG in leukemic cell lines appeared to correlate with the expression of membrane RTKs, such as FLT3-ITD and c-KIT, we assessed the activity of 2-DG in primary cells from patients according to these mutations (Supplementary Table S2). 2-DG induced apoptosis in primary AML samples with FLT3-ITD ($n = 9$) or c-KIT-D816V ($n = 5$) in a dose-dependent manner, whereas FLT3-wt samples were less sensitive although the difference was not statistically significant (Fig. 1G).

**Figure 3.**

Inhibition of N-linked-glycosylation but not glycolysis is responsible for inhibition of cell-surface expression of RTKs and induction of apoptosis after 2-DG treatment. MV4-11 and Kasumi-1 were treated 6 hours with 5 mmol/L or 2 mmol/L 2-DG, respectively, alone or in combination with 10 mmol/L D-mannose and then ATP was measured using the CellTiter-Glo luminescent assay (A). Ba/F3 expressing FLT3-ITD or c-KIT-D816V were treated 24 hours with 5 mmol/L 2-DG alone or in combination with 10 mmol/L D-mannose before cell labeling with CD135 or CD117 and flow cytometry analysis (B). MV4-11 or Kasumi-1 cells were incubated 24 hours with 5 mmol/L or 2 mmol/L 2-DG respectively, alone or in combination with 10 mmol/L D-mannose before immunoblotting analysis (C). Apoptosis (D) or cell viability (E) was measured after 24 hours 2-DG exposure at different concentrations, alone or in combination with 10 mmol/L D-mannose. Results shown are mean \pm SEM and are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2-DG inhibits cell-surface expression of FLT3 and KIT

FLT3 or c-KIT transport to the plasma membrane is dependent on their glycosylation status. As 2-DG is able to interfere with this process (29, 30), we assessed the expression of the two RTKs at the cell surface after 2-DG exposure. In leukemic cell lines, 2-DG induced an electrophoretic mobility suggestive of reduced glycosylation of the two RTKs (Fig. 2A). This shift was also observed in patient samples with FLT3-ITD or c-KIT-D816V mutations (Fig. 2B). Similar results were observed in cell line expressing wild-type FLT3 (RS4-11) or c-KIT (TF-1; Supplementary Fig. S1A). We also compared the effect of 2-DG with both tunicamycin (which completely inhibits glycosylation) and brefeldin A (BFA; which inhibits only mature glycosylation of receptors in the Golgi complex). BFA induced the expression of a partially glycosylated form of the receptor, whereas tunicamycin induced the expression of a nonglycosylated form as previously described (Supplementary Fig. S1B; ref. 15). The level of migration of FLT3 and c-KIT after 2-DG treatment was in between those obtained after tunicamycin and BFA, suggesting that 2-DG does affect their glycosylation status. Treatment with both the pan-caspase inhibitor ZVAD-fmk and the proteasome inhibitor bortezomib did not restore expression of the glycosylated full size form of each receptor, suggesting that proteolysis mediated by apoptosis or ubiquitin-proteasome pathway were not involved in this mechanism (Supplementary Fig. S1C and S1D). 2-DG exposure also inhibited the phosphorylation of FLT3-ITD. Interestingly, we observed a decrease of c-KIT protein levels in Kasumi-1 and primary AML samples after 2-DG treatment. A decrease of FLT3-ITD protein level was also observed in some patient samples but not in MV4-11 and MOLM-14 cell lines. 2-DG also affected the mRNA expression of *KIT* but not *FLT3* in Kasumi-1 and MV4-11, respectively (Supplementary Fig. S1E).

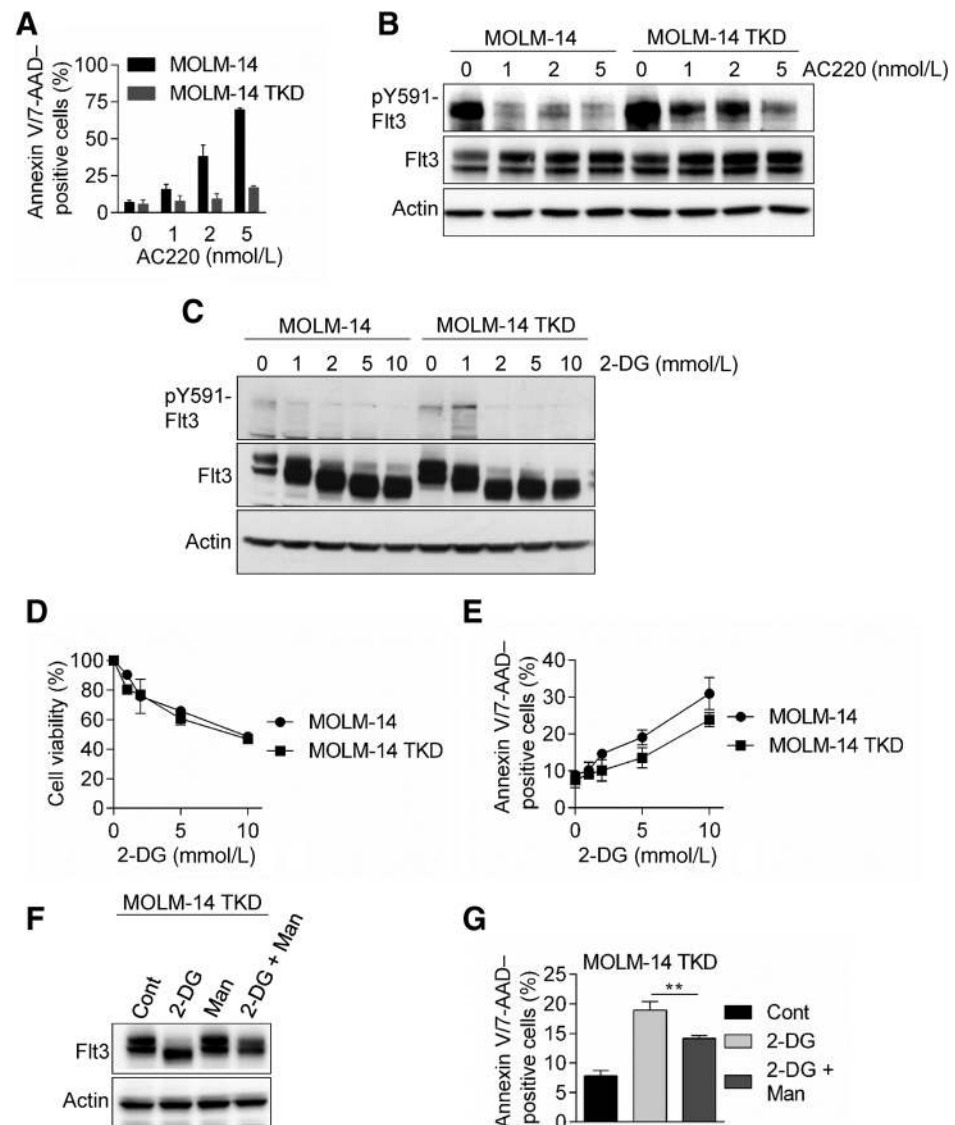
Flow cytometry analysis demonstrated that 2-DG decreased cell-surface expression of FLT3 and c-KIT in both murine Ba/F3-FLT3-ITD and Ba/F3-KIT-D816V cell lines (Fig. 2C) and in primary AML samples with FLT3-ITD or c-KIT-D816V mutations (Fig. 2D). Furthermore, immunofluorescence microscopy analysis showed differences regarding FLT3 localization between control and 2-DG-treated cells in which FLT3 was mainly cytoplasmic (Supplementary Fig. S1F).

2-DG alters FLT3-ITD and KIT-D816V signaling

The oncogenic properties of FLT3-ITD and c-KIT-D816V are mediated by constitutive activation of STAT3/5 as well as MAPK and PI3K/Akt pathways. In addition, both mutated receptors drives MAPK and PI3K/Akt activation from the cell surface, whereas STAT3 and STAT5 signaling are induced from the ER (15). Accordingly, tunicamycin, which inhibits glycosylation of plasma membrane receptors thereby blocking their surface expression, induced the dephosphorylation of Akt and ERK while leaving intact the phosphorylation of STAT5 and STAT3 in MV4-11 and TF-1 c-KIT-D816V cell lines (Fig. 2E and F). On the other hand, 2-DG has been shown to activate the PI3K-Akt and MAPK pathways through IGF1-R signaling induction in cancer cell lines (36). Thus, we assessed the impact of 2-DG on these major signaling pathways in AML cells. In MV4-11 cells, 2-DG (5 mmol/L, 24 hours) reduced the phosphorylation of STAT5 and ERK1/2, whereas pAkt was increased (Fig. 2E). Consistent with the inhibition of STAT5 phosphorylation, 2-DG treatment was also associated with a decreased in phosphorylation of Lyn, an upstream activator of STAT5 in FLT3-ITD AML cells (Supplementary Fig. S2; ref. 37). In TF-1 c-KIT-D816V cells, 2-DG inhibited the phosphorylation of STAT3 and ERK1/

Figure 4.

2-DG overcomes resistance to quizartinib (AC220) induced by mutations in the TKD of FLT3. Resistance of MOLM-14/TKD to quizartinib-induced apoptosis was evaluated by treating parental MOLM-14 or MOLM-14/TKD cells 24 hours with 1 nmol/L, 2 nmol/L or 5 nmol/L quizartinib before Annexin V/7-AAD labeling and flow cytometry analysis (A). To assess the impact of AC220 and 2-DG on FLT3 expression and phosphorylation, cells were treated 24 hours with different doses of ACC220 or 2-DG and analyzed by Western blot analysis as previously described (B and C). Parental MOLM-14 or MOLM-14/TKD were exposed 24 hours to increasing concentrations of 2-DG before cell growth quantification by the MTS assay (D) or apoptosis quantification by Annexin V/7-AAD labeling (E). MOLM-14/TKD cells were incubated 24 hours with 2-DG, D-mannose, or in combination before immunoblotting analysis (F) and Annexin V/7-AAD labeling (G). Results are mean \pm SEM of three independent experiments. **, $P < 0.01$.



2 but not pAkt (Fig. 2F). In U937 cells, 2-DG abrogated STAT5 and ERK1/2 phosphorylation while inducing a robust phosphorylation of Akt (Fig. 2G). Altogether, these results show that 2-DG consistently downregulates STATs and ERK pathways, while activating the PI3K-Akt pathway in AML cell lines. However, the Akt inhibitor-VIII, which fully inhibited Akt phosphorylation, did not enhance cell death-induced by 2-DG, indicating that the phosphorylation of Akt following 2-DG exposure did not protect leukemic cells from apoptosis in these models (Supplementary Fig. S3).

Inhibition of N-linked glycosylation is responsible for the inhibition of cell-surface expression of RTKs and induction of apoptosis after 2-DG treatment

As 2-DG inhibits both glycolysis and N-linked glycosylation, we sought to determine which mechanism drive cell-surface expression of the two mutated RTKs and apoptosis. Inhibition

of N-linked-glycosylation by 2-DG can be effectively reversed by addition of exogenous D-mannose without affecting glycolysis (29). Accordingly, the inhibition of global ATP content induced by 2-DG in MV4-11 and Kasumi-1 cells was not affected by cotreatment with D-mannose (Fig. 3A). We then assessed the cell-surface expression of FLT3-ITD and c-KIT upon treatment with 2-DG plus D-mannose. D-mannose cotreatment was associated with plasma membrane expression of both receptors in the murine BaF/3 models (Fig. 3B). Moreover, Western blot analysis showed that the glycosylation status of FLT3-ITD and c-KIT was also restored after cotreatment with D-mannose in MV4-11 and Kasumi-1 cells (Fig. 3C). Finally, D-mannose blocked 2-DG-induced apoptosis and inhibition of cell growth (Fig. 3D and E). Altogether, these results indicate that the cytotoxic effects of 2-DG are mainly mediated by its ability to inhibit N-linked-glycosylation rather than glycolysis in AML cells.

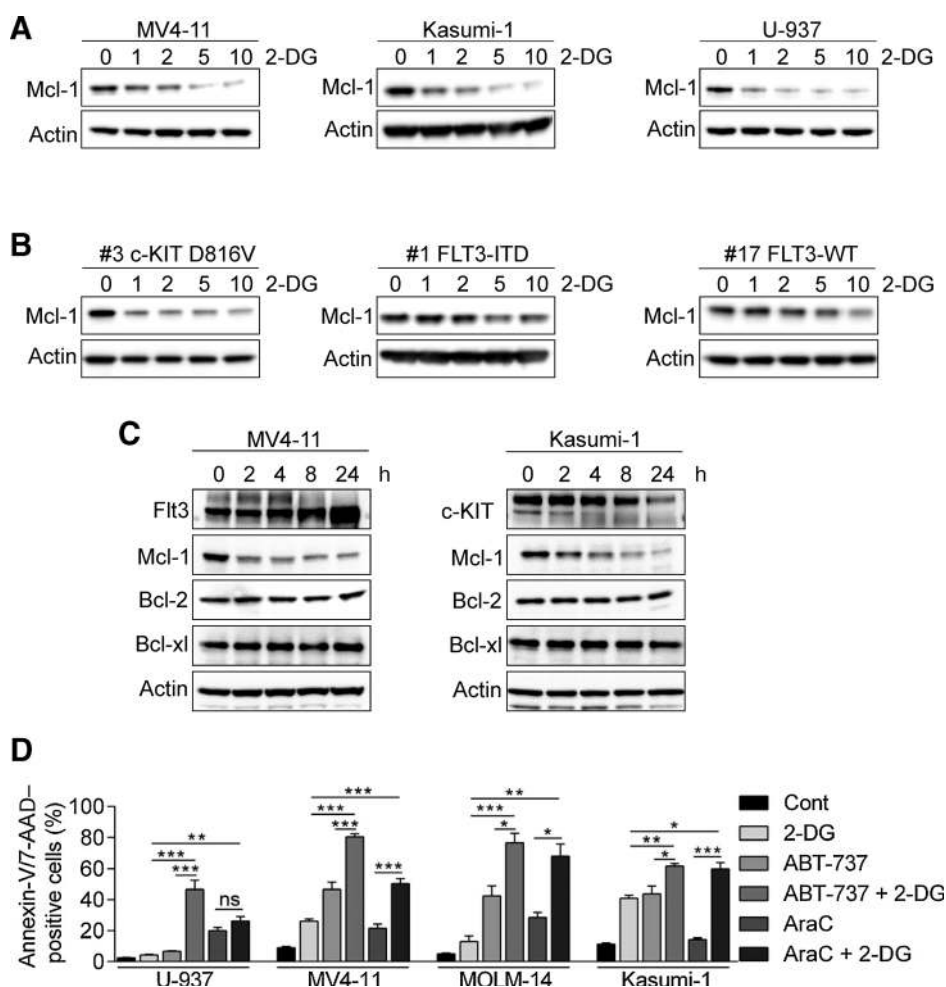


Figure 5. 2-DG targets Mcl-1 protein expression and sensitizes to ABT-737 and cytarabine. MV4-11, Kasumi-1, U-937 (A) or AML primary samples (B) were treated 24 hours with 2-DG at indicated concentrations before cell lysis and Western blot analysis as previously described. MV4-11 and Kasumi-1 were treated with 2-DG (5 nmol/L and 2 nmol/L, respectively) and collected at different time points (C). Combination assay between 2-DG, ABT-737, or cytarabine was performed by treating cells 24 hours with 5 mmol/L 2-DG alone or in combination with 100 nmol/L ABT-737 or 1 μ mol/L cytarabine, before Annexin V/7-AAD labeling and flow cytometry analysis (D). Results shown are mean \pm SEM and are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

2-DG is active in quizartinib-resistant leukemic cells

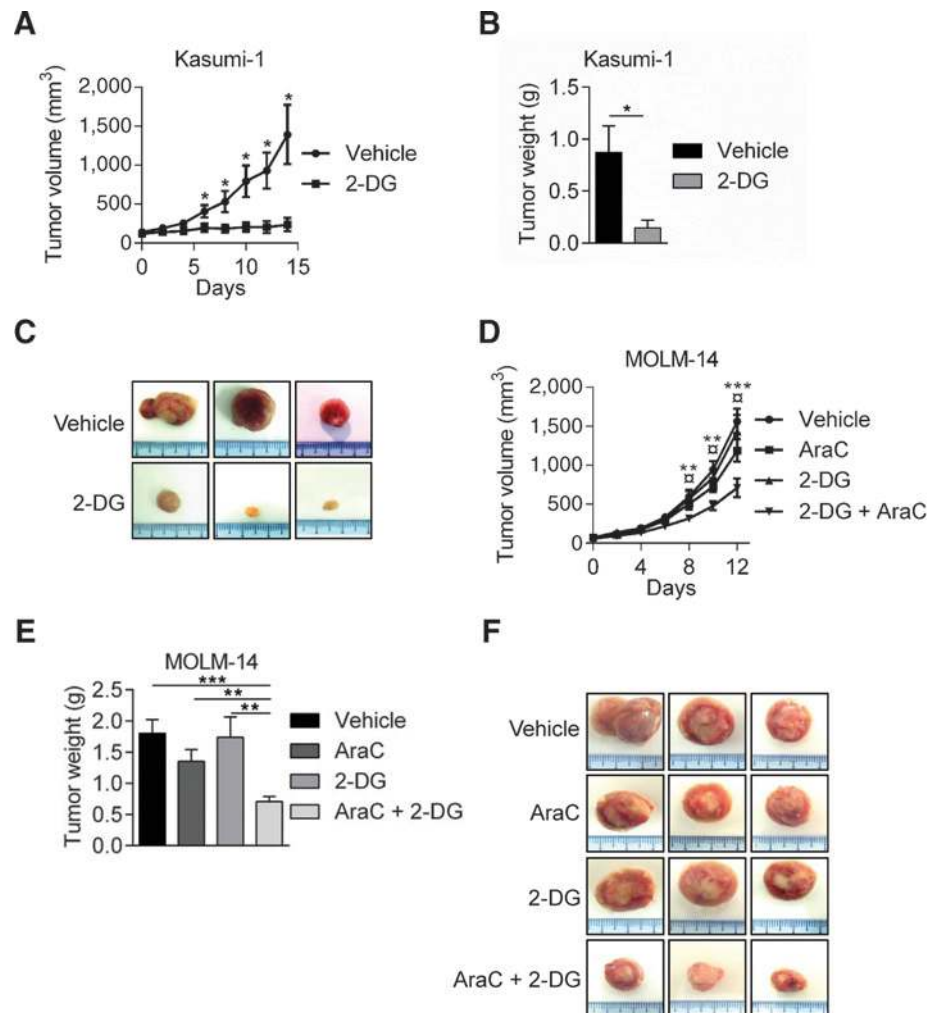
Secondary point mutations in the FLT3 TKD, including the activation loop at the D835 residue, are common causes of acquired clinical resistance to FLT3 inhibitors such as quizartinib or sorafenib (38). We hypothesized that 2-DG could be active in quizartinib-resistant cells. For this purpose, we used MOLM-14 cells harboring a double-mutant FLT3-ITD/TKD protein (MOLM-14/TKD) inducing a high level of resistance to quizartinib (AC220) as compared with the parental MOLM-14 (Fig. 4A). As shown in Fig. 4B, AC-220 inhibited the phosphorylation of FLT3 (to a lesser extent in MOLM-14/TKD cells) but did not affect the glycosylation of the receptor in both cell lines. In contrast, 2-DG treatment reduced the level of the fully glycosylated form of FLT3 and increased its immature form in the two cell lines (Fig. 4C). The phosphorylation of FLT3 was also downregulated. 2-DG treatment inhibited cell viability and induced apoptosis in MOLM-14/TKD to a similar level than in MOLM-14 (Fig. 4D and E). Moreover, D-mannose restored the glycosylation status of FLT3-ITD/TKD and reduced 2-DG-induced apoptosis in MOLM-14/TKD cells (Fig. 4F and G). In one AML sample bearing the FLT3-TKD mutation, 2-DG also affected the expression of FLT3 and induced cell death (Supplementary Fig. S4).

2-DG decreases Mcl-1 protein expression in AML cells and induces sensitization to ABT-737 and cytarabine *in vitro*

Previous studies have shown that targeting Mcl-1 may overcome apoptotic resistance and sensitize AML cells to cytotoxic drugs (39, 40). In addition, glycolysis inhibition by caloric restriction or 2-DG treatment has been shown to downregulate Mcl-1 expression, thereby restoring sensitivity to ABT-737-induced apoptosis in lymphoma cells (25, 26). 2-DG downregulated the expression of Mcl-1 in MOLM-14, Kasumi-1, and U-937 cell lines as well as in primary AML samples (Fig. 5A and B). Mcl-1 downregulation occurred as early as 2 hours after 2-DG treatment, before alteration of RTK expression. Furthermore, 2-DG did not affect the expression of Bcl-2 and Bcl-xL (Fig. 5C). 2-DG (to reduce Mcl-1 level) was then combined with ABT-737 (to neutralize other antiapoptotic molecules, including Bcl-2 and Bcl-xL) or cytarabine (to induce a proapoptotic signal; Fig. 5D). Compared with single-agent treatment, the combination of 2-DG+ABT-737 showed a stronger effect in U-937, MV4-11, MOLM-14, and Kasumi-1 cell lines, whereas a stronger interaction between 2-DG and cytarabine was observed in MV4-11, MOLM-14, and Kasumi-1, but not U937 cell lines. These data suggest that 2-DG's ability to target both Mcl-1 and FLT3-ITD or c-KIT may be necessary to sensitize leukemic cells to cytarabine.

Figure 6.

2-DG inhibits tumor growth of AML cells *in vivo*. Kasumi-1 or MOLM-14 cells (5×10^6) were injected subcutaneously in NOD-SCID mice. When tumors reached 50 to 100 mm³, mice injected with Kasumi-1 were treated daily with 200 μ L PBS^{-/-} or 200 μ L 2-DG at 500 mg/kg/d (A–C). Mice injected with MOLM-14 were treated with injections of 200 μ L PBS^{-/-}, 100 μ L 2-DG at 500 mg/kg/d, 100 μ L cytarabine at 15 mg/kg/d, or 200 μ L mix of cytarabine and 2-DG (D–F). Tumors were measured on indicated days. On the last day of experiment, tumors were dissected and weighed. C (Kasumi-1) and F (MOLM-14) show representative photographs of xenografted tumors the day of dissection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



2-DG inhibits tumor growth and sensitizes leukemic cells to cytarabine *in vivo*

We established a subcutaneous xenograft model of AML using NOD/SCID mice, which were subcutaneously injected in the flank with MOLM-14 and Kasumi-1 cells. When tumors are established, mice were treated with daily intraperitoneal injections of 2-DG (500 mg/kg/d, both cell lines; ref. 41), cytarabine (15 mg/kg/d, MOLM-14 only), or 2-DG+cytarabine. Correlating with *in vitro* studies, 2-DG used as a single agent, strongly inhibited the growth of Kasumi-1 (Fig. 6A–C), whereas MOLM-14 growth was not affected at the doses used in this experiment (Fig. 6D). However, while neither cytarabine nor 2-DG affected MOLM-14 tumor growth as single agents, the combination had a synergistic anti-leukemic effect (Fig. 6D–F).

Discussion

The antileukemic activity of 2-DG has only recently been studied and mainly under the scope of its ability to inhibit glycolysis or shift energy metabolism to fatty acid oxidation (23, 24). We took advantage of another property of 2-DG, which affects protein glycosylation by inhibiting N-linked glycosylation, to describe its antileukemic activity in a subset of AML. Indeed, it

has been demonstrated that fluvastatin and others compounds of the statin family were able to impair FLT3 glycosylation, leading to a reduction in cell-surface expression and an increase in cell death, specifically in leukemic cell lines with RTK mutations (42). Another study dealing with acute lymphoblastic leukemia cells showed that inhibition of N-linked glycosylation as well as induction of ER stress and the unfolded protein response were also the predominant mechanism of 2-DG's cytotoxicity (43). In our study, we have demonstrated that 2-DG affected the cell-surface expression and cellular signaling of both FLT3-ITD and mutated c-KIT. This mechanism was associated with cell death induction. We have also observed that leukemic cells harboring mutated RTKs were the most sensitive to 2-DG treatment *in vitro*. Moreover, the antileukemic activity of 2-DG was particularly marked in the c-KIT-mutated cell line Kasumi-1 and in CBF-AML cells. In these cells, 2-DG inhibited the cell-surface expression of c-KIT, abrogated STAT3 and MAPK-ERK pathways and strongly downregulated the expression of the receptor. A decrease of FLT3 protein level has also been observed in primary AML samples, but not in MV4-11 or MOLM-14 cell lines. It has been shown that the transcription of both *FLT3* and *KIT* genes is under the control of the Sp1/NF- κ B dimer (44, 45). Interestingly, the glycosylation status of Sp1 plays a critical role in its activity (46, 47). Under

glucose starvation, Sp1 is completely deglycosylated and degraded by the proteasome (46). In addition, 2-DG inhibits Sp1 activity in HeLa cells through modulation of O-glycosylation (32). We can therefore reasonably speculate that 2-DG interfered with Sp1 activity in AML cells contributing to the decrease of c-KIT as shown in our study. Thus, in addition to small-molecule RTK inhibitors, modulating the glycosylation status of mutated RTKs to affect both signal transduction and protein expression could represent an alternative therapeutic strategy in AML with mutated RTKs. Accordingly, we have also shown that 2-DG is also active in quizartinib-resistant FLT3-ITD AML cells harboring TKD mutations.

Deregulation of cell survival programs is not only a crucial step in the leukemogenic process, but renders malignant cells resistant to various apoptotic triggers, including cytotoxic treatments, such as cytarabine. Compared with other Bcl-2 prosurvival family members, Mcl-1 is consistently expressed at higher levels in AML and is upregulated by FLT3-ITD (48). Thus, Mcl-1 represents an important therapeutic target in AML and therapeutic compounds that block Mcl-1 expression may improve clinical responses to cytotoxic agents. 2-DG has been shown to be synergistic with BH3 mimetic molecules through downregulation of Mcl-1 protein levels in lymphoma cells (26). We also observed a strong interaction between 2-DG and the BH3 mimetic ABT-737 in AML cell lines. However, 2-DG only induced sensitization to cytarabine toxicity in the FLT3-ITD-mutated cell lines, but not in U-937 cells. It is plausible that 2-DG increased sensitivity to cytarabine through downregulation of Mcl-1, but also through inhibition of FLT3-ITD or c-KIT cell-surface expression and downstream signaling pathways, a combination of events which led to massive cell death.

2-DG has been recently tested in phase I trials (49, 50). Pharmacokinetics studies have shown that mmol/L concentrations of 2-DG were hardly achievable at the maximal-tolerated doses and thus, although we do not observed significant toxicity in mice, achieving antitumoral concentrations in human could be challenging.

In summary, we have demonstrated that 2-DG alters RTKs expression and downstream signaling pathways, downregulates Mcl-1 expression and displays a significant antileukemic activity

in AML with FLT3-ITD or c-KIT mutations. 2-DG is also active on AML cells resistant to the most potent FLT3 inhibitor currently in clinical trials, namely quizartinib, and can restore sensitivity to cytarabine. These results open a new therapeutic window in a large subset of AML with mutated RTKs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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