Hexosamine Biosynthetic Pathway Inhibition Leads to AML Cell Differentiation and Cell Death

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Abstract

Treatment for acute myeloid leukemia (AML) has remained unchanged for past 40 years. Targeting cell metabolism is a promising avenue for future cancer therapy. We found that enzymes involved in metabolic hexosamine biosynthetic pathway (HBP) are increased in patients with AML. Inhibiting GFAT (the rate-limiting enzyme of HBP) induced differentiation and apoptosis in AML cells, sparing normal cells. UDP-GlcNAc, the end product of HBP, is the substrate for O-GlcNAcylation, a posttranslational modification. O-GlcNAc transferase (OGT) is the enzyme which transfers GlcNAc from UDP-GlcNAc to target proteins. Inhibition of O-GlcNAcylation, using OGT inhibitors as well as genetic knockdown of OGT, also led to cell differentiation and apoptosis of AML cells. Finally, HBP inhibition in vivo reduced the tumor growth in a subcutaneous AML xenograft model and tumor cells showed signs of differentiation in vivo. A circulating AML xenograft model also showed clearance of tumor load in bone marrow, spleen, and blood, after HBP inhibition, with no signs of general toxicity. This study reveals an important role of HBP/O-GlcNAcylation in keeping AML cells in an undifferentiated state and sheds light into a new area of potential AML therapy by HBP/O-GlcNAc inhibition. Mol Cancer Ther; 17(10); 2226–37. ©2018 AACR.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disorder characterized by differentiation arrest of myeloid cells. This leads to uncontrolled clonal proliferation of immature myeloid blasts (1, 2). It is the most common acute leukemia in adults. AML can be divided into eight different subclasses (M0–M7) based on the differentiation status, according to the French–American–British (FAB) classification (3–7). The incidence of AML increases with age and over 20,000 cases per year is reported in the United States (8, 9). Current long-term survival rates in older patients (5%–15%) is worse compared with younger patients (25%–50%; ref. 10). Toxicities associated with current treatment strategies and failure to tolerate those side effects, especially for older patients, are the two main reasons for this poor outcome. Although a number of targeted therapeutics have been proposed for treating AML, chemotherapy and allogeneic bone marrow transplantation still remain the first-line standard treatment options for most of the patients with AML (11, 12).

Differentiation arrest and uncontrolled proliferation of myeloid cells are the two main features of AML, which needs to be targeted together for a better treatment efficacy. Current AML treatment strategies including All-trans retinoic acid (ATRA) and chemotherapy often fail due to their inefficacy to target both key issues. Chemotherapeutic drugs cytarabine and Adriamycin mostly target the highly proliferative cells, and relapse is a common problem associated with it. Unfortunately, ATRA is successful as a differentiation therapy for only one rare subtype of AML, acute promyelocytic leukemia (APL; 5%–10% of AML; refs. 13–16). ATRA leads to long-term survival in 75% to 85% of patients with APL and is more tolerable for older patients. Therapeutic progress for AML has been very slow when compared with other cancers. Therefore, more effective and less toxic therapy strategies to target both differentiation arrest and proliferation represent an unmet need in the management of AML.

Targeting of cellular metabolism has emerged as an attractive strategy in the treatment of different cancers including AML. Inhibition of isocitrate dehydrogenase (IDH) has shown promising results in clinical trials in patients with AML with IDH mutations (17, 18). Moreover, inhibiting PI3K/AKT/mTOR signaling pathway in AML cells received much attention as this pathway is overexpressed in AML cells. Anticancer potential of direct inhibitors of this pathway and inhibitors of mediators upstream in this pathway are also extensively studied (19, 20). Cancer cells depend on less efficient glycolysis known as Warburg effect. This results in increased uptake of glucose (21–25). Cancer cells also consume large amounts of glutamine, a precursor amino acid for the synthesis of glucosamine, initiator in the hexosamine biosynthetic pathway (HBP; refs. 26, 27). Glutamine→fructose-6-phosphate amidotransferase (GFAT), a rate limiting enzyme of HBP combines fructose-6-phosphate from the glycolytic pathway with glutamine to produce glucosamine-6-phosphate (28–30). UDP-GlcNAc is the end-product of HBP, which serves as the substrate for O-GlcNAcylation. OGT and OGA are enzymes with opposite functions, OGT adds.
O-GlcNAc residue to the hydroxyl groups of serine and/or threonine residues and OGA removes O-GlcNAc from target proteins (31). O-GlcNAcylation is a rapidly cycling posttranslational modification similar to phosphorylation. Because it happens primarily at serine and threonine residues or occurs at sites adjacent to phosphorylation, O-GlcNAcylation often competes with phosphorylation (32–34). Unlike many different types of kinases and phosphatases existing in a cell, there is only one OGT and OGA controlling O-GlcNAcylation of all cellular proteins.

Higher amounts of glucose or glutamine flowing through the HBP result in increased production of UDP-GlcNAc, which is substrate for O-GlcNAcylation. Increased O-GlcNAcylation is implicated in many cancers especially in transcriptional regulation, cell proliferation, and survival (35–37). Recent studies have shown increased levels of O-GlcNAcylation in both synergetic and solid cancers (36, 38–42). Clinical behavior of chronic lymphocytic leukemia cells is reported to correlate with intensity of O-GlcNAcylated protein levels in those cells and O-GlcNAcylation is increased in these cells after cytokine stimulations and Toll-like receptor activation (41). Conversely, inhibition of HBP has been recently reported to promote castration-resistant prostate cancer (43). Thus, O-GlcNAcylation could be involved in the pathogenesis of several types of cancers.

In this study, we found elevated levels of HBP enzymes and O-GlcNAcylation in AML cells compared with normal cells. We demonstrated that HBP or OGT inhibition leads to AML cell differentiation and apoptosis. Finally, using two different xenograft AML mouse models, we show inhibition of growth and differentiation of tumor cells after HBP inhibition. Our findings suggest that targeting HBP or OGT is an attractive potential novel therapeutic strategy for all subtypes of AML.

Materials and Methods

Supplementary information provides a detailed account of all materials and methods used in this study.

Patient samples

Human samples from normal donors and patients with AML were obtained from Hematopoietic Stem Cell Core Facility located in Case Western Reserve University (Cleveland, OH).

Cell culture

OCI-AML3 (DSMZ), HL-60 (ATCC), and HEK293T (ATCC) cells were maintained according to standard protocols. Cell lines were obtained from Hematopoietic Stem Cell Core Facility located in Case Western Reserve University (Cleveland, OH).

Cell death assay

Cell death was estimated either by staining cells with propidium iodide (PI) followed by flow cytometry analysis or by trypan blue stain–based cell counting. In an experiment to determine PBMCs versus AML patient samples sensitivity toward 6-diazo-5-oxo-L-nor-leucine (DON) treatment, cell death was analyzed by gating cells on a forward-scatter versus side-scatter plot in a flow cytometer.

Apoptosis assay

Cells were stained with Annexin V and PI stain and analyzed by flow cytometer.

Cell proliferation assay

The proliferation was monitored by measuring the cell confluence in an IncuCyte ZOOM (Essen Bioscience) instrument or trypan blue stain–based cell counting.

Cell differentiation

Cells were plated at a density of $10^5$ cells per mL and treated as indicated till 5 days. DON was added every 24 hours. On day 5, cells were imaged for changes in the cell morphology, NBT reduction assay [NBT (0.25 mg/mL) and PMA (500 ng/mL)] and surface expression of differentiation markers CD11b and CD14.

shRNA interference and lentiviral packaging

The shRNA against OGT was validated for the knockdown efficiency. Lentiviral particles were produced using HEK293T cells and transduced by spinfection.

Ethics

All mice experiments were done according to Institutional Animal Care and Use Committees guidelines and approved protocol.

In vivo AML xenograft models

In solid AML xenograft tumor model, NSG mice were subcutaneously injected with $5 \times 10^6$ HL-60 cells and intraperitoneally administered either with PBS or DON (0.65 mg/kg bodyweight) as shown in Fig. 6A. Tumor volume was calculated using formula (width $\times$ width $\times$ length $\times$ 0.5). Tumor cells were analyzed for surface expression of differentiation markers CD11b and CD14. In another liquid AML xenograft model, sublethally irradiated (200 cGy) NSG mice were intravenously injected with $3 \times 10^6$ OCI-AML3 cells, followed by intraperitoneal administration of PBS or DON (0.65 mg/kg bodyweight) or cytarabine (50 mg/kg bodyweight) as shown in Fig. 6F. Bone marrow, spleen, and blood were analyzed for the tumor burden by staining with anti-human CD45.

Statistical analysis

Applicable data were analyzed using unpaired Student’s t test. All experiments were done in triplicates ($n = 3$) unless mentioned otherwise. *P values assigned were ns = not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. In vivo experiments has 5 animals in each experimental group ($n = 5$).

Results

AML cells express higher levels of HBP enzymes and are hyper-O-GlcNAcylated

We checked the levels of O-GlcNAcylated proteins in AML patient cells versus normal PBMCs and found that overall protein O-GlcNAcylation was significantly elevated in AML patient subtypes (M1, M2, M4, and M5) and one relapsed AML-M1 patient cells compared with normal PBMCs (Fig. 1A). To understand the molecular events regulating O-GlcNAcylation in AML, we used the web-based microarray database called Oncomine to assess the relative gene expression levels of enzymes involved in O-GlcNAcylation. We analyzed 74 normal and 542 AML patient samples...
endogenous control. OGT in normal PBMCs, OCI-AML3 and HL-60. GAPDH was used as an
inhibitor on AML cell growth. DON inhibits GFAT1 (the enzyme that converts fructose-6-P to glucosamine-6-P), and thereby inhibits HBP and O-GlcNAcylated in AML cells. We performed a dose and time kinetics study to determine the concentration and duration at which DON inhibits the proliferation of AML cells with minimal toxic effects on normal cells. We found a dose-dependent increase in the killing potential of DON with 1 μmol/L DON causing 15% cell death, while 50 μmol/L killing up to approximately 60% of AML cells post 72 hours of incubation (Fig. 2A). Next, we incubated OCI-AML3 cells in presence of 50 μmol/L DON for 0 to 72 hours of treatment and found that at 24 hours about 30% of cells were killed and a plateau is achieved around 72 hours (Fig. 2B). We treated normal PBMCs and monocytes cells also with 50 μmol/L DON for 24 hours to study the survival response of these cells to HBP inhibition (Fig. 2C). Surprisingly, DON had only minor effects on the viability of normal cells, whereas AML patient cells belonging to different subtypes M1, M4, and M5 showed significant killing (Fig. 2D). Significant cell death (~35%) was also observed in OCI-AML3 and HL-60 cell lines at 24 hours post-DON treatment (Fig. 2E). We confirmed the decrease of O-GlcNAcylation after DON treatment in normal PBMCs and AML cells (Fig. 2F).

To gain insights into the effect of DON on AML proliferation, we used IncuCyte ZOOM technology for automation of imaging and quantification of cell confluence and nuclear count data. Cell confluence decreased about 90% in both OCI-AML3 cells and HL-60 cells after 72 hours of DON treatment, confirming the inhibition of cell proliferation in DON-treated AML cells (Supplementary Fig. S2A and S2B). Exposure of AML cells to DON (50 μmol/L) induced apoptosis as evidenced by Annexin V positivity of these cells (Supplementary Fig. S2C and S2D). DON-treated cells also showed an increase in the cleaved caspase-3 and cleaved PARP proteins, confirming DON-induced apoptosis in AML cells (Supplementary Fig. S2E). We further confirmed this finding using alternate methods. We used OGT inhibitors OSMI-1 (44, 45) and BADGP (45) to inhibit O-GlcNAcylated in AML cells. Both OSMI-1 and BADGP inhibited cell proliferation of OCI-AML3 and HL-60 (Fig. 3A and B) as evident by viable cell count.

Inhibition of HBP pathway leads to AML cell death

Inhibition of HBP leads to AML cell death

The significant increase in expression of HBP enzymes and O-GlcNAcylation in AML cells prompted us to study the effect of HBP inhibitor on AML cell growth. DON inhibits GFAT1 (the enzyme that converts fructose-6-P to glucosamine-6-P), and thereby inhibits HBP and O-GlcNAcylated in AML cells. We performed a dose and time kinetics study to determine the concentration and duration at which DON inhibits the proliferation of AML cells with minimal toxic effects on normal cells. We found a dose-dependent increase in the killing potential of DON with 1 μmol/L DON causing 15% cell death, while 50 μmol/L killing up to approximately 60% of AML cells post 72 hours of incubation (Fig. 2A). Next, we incubated OCI-AML3 cells in presence of 50 μmol/L DON for 0 to 72 hours of treatment and found that at 24 hours about 30% of cells were killed and a plateau is achieved around 72 hours (Fig. 2B). We treated normal PBMCs and monocytes cells also with 50 μmol/L DON for 24 hours to study the survival response of these cells to HBP inhibition (Fig. 2C). Surprisingly, DON had only minor effects on the viability of normal cells, whereas AML patient cells belonging to different subtypes M1, M4, and M5 showed significant killing (Fig. 2D). Significant cell death (~35%) was also observed in OCI-AML3 and HL-60 cell lines at 24 hours post-DON treatment (Fig. 2E). We confirmed the decrease of O-GlcNAcylation after DON treatment in normal PBMCs and AML cells (Fig. 2F).

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Inhibition of HBP pathway leads to AML differentiation

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AML cells undergo terminal differentiation and apoptosis when treated with ATRA and is used as a therapeutic strategy in patients with APL. We studied whether DON-induced apoptosis of AML involves cell differentiation. We found that low-dose DON (12.5 μmol/L) treatment for 5 days induces differentiation of AML cells, similar to as seen after ATRA treatment, as evidenced by the morphology changes (Fig. 4A) and Nitroblue Tetrazolium (NBT) staining (Fig. 4B). ATRA was used as a positive control and a combination of DON and ATRA caused differentiation similar to DON or ATRA alone (Fig. 4A). Morphologically, DON induced monocytic differentiation results in an increased cytoplasmic to nuclear ratio. In addition, these cells exhibited a condensed and indented nucleus along with significant enlargement in cell size.

using Haferlach leukemia statistics and it showed a significant fold increase in GFAT1 (1.156; P = 4.11E−7) and OGT (1.360; P = 2.01E−16) gene expression in AML cells compared with normal samples (Fig. 1B and C). Analysis of fold change of different enzymes involved in O-GlcNAcylation was performed using datasets available at Oncomine database (Supplementary Fig. S1). Furthermore, we found that HBP pathway enzymes including GFAT1, PGAM3, UAP1, GNAI, and OGT were overexpressed in AML cell lines OCI-AML3 and HL-60 compared with normal PBMCs (Fig. 1D).
Figure 2.
Blocking protein O-GlcNAcylation kills AML cells. DON treatment blocks O-GlcNAcylation and subsequent cell death in OCI-AML3 cells was monitored in a dose-dependent manner after 72-hour treatment (A) and in a time-dependent manner with DON (50 μmol/L) treatment (B). C, Cell viability of normal PBMCs and primary monocytes 24 hours after DON (50 μmol/L) treatment compared with the untreated control. D, Cell viability of PBMCs and AML patient blast samples treated with DON or untreated control after 24 hours. E, Effect of DON (50 μmol/L) on the cell viability of OCI-AML3 and HL-60 cells after 24 hours treatment. F, Western blot analysis showing O-GlcNAc profile of PBMCs, OCI-AML3, and HL-60 using O-GlcNAc (RL2) antibody. Cells were incubated (16 hours) as indicated. C-untrated control or D-DON (50 μmol/L). Actin was used as an endogenous loading control. Statistical significance was calculated using unpaired Student t test (N = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
OCI-AML3 cells undergo monocytic differentiation, whereas HL-60 cells typically undergo granulocytic or monocytic differentiation. CD11b is used as a marker for myelomonocytic differentiation and CD14 as a marker for monocytic differentiation. Both OCI-AML3 and HL-60 cells showed increase in CD11b expression from 5% to 65% and 7% to 35%, respectively, on 5th day after DON treatment (12.5 μmol/L), whereas CD14 expression increased from 4% to 80% in OCI-AML3 and from 3% to 30% in HL-60 cells (Fig. 4C and D). Besides morphologic and immunophenotypic changes, DON exhibits high respiratory activity in OCI-AML3, a characteristic of differentiated cells as measured by NBT reduction (Fig. 4B). Alternatively, OSMI-1 and BADGP treatments also show increase in CD11b and CD14 expression on 5th day. OCI-AML3 cells exhibit approximately 40% and HL-60 approximately 12% CD11b expression upon O-GlcNAc inhibition using OSMI-1 10 and 25 μmol/L, respectively (Fig. 3C and D). BADGP (2 mmol/L) also induced CD11b expression approximately 32% in OCI-AML3 and approximately 22% in HL-60 cells (Fig. 3E and F). CD14 expression increased to 45% in OCI-AML3 and 16% in HL-60 upon O-GlcNAc inhibition using OSMI-1 (10 μmol/L; Fig. 3C and D) and up to 35% in OCI-AML3 and HL-60 upon inhibition with BADGP (2 mmol/L; Fig. 3E and F). As inhibition of HBP pathway and O-GlcNAcylation by DON pushed AML cells to differentiation, we also tested what happens to O-GlcNAcylation status when AML cells are induced to differentiate. We used ATRA to induce differentiation and found that AML cells after ATRA treatment have decreased O-GlcNAcylation (Fig. 4E). These experiments demonstrate that AML cell differentiation is associated with a decreased O-GlcNAcylation.

O-GlcNAcylation and phosphorylation are two posttranslational modifications that often occur on the same protein competing for the same serine/threonine site(s) and bear a reciprocal relationship.
Figure 4. DON induces AML differentiation. A, Image of OCI-AML3 cells treated with DON (12.5 µmol/L), ATRA (1 µmol/L) either alone or in combination at day 5. Cells show a change in morphology and cytoplasm to nuclear ratio. ATRA is known to induce APL differentiation. B, OCI-AML3 cells were treated with or without DON till day 5, as indicated. Differentiated cells reduce nitroblue tetrazolium (NBT) in the assay, indicated by the development of bluish/purple color. Surface expression of differentiation marker CD11b and CD14 was determined in either control or DON (12.5 µmol/L) treated OCI-AML3 (C) and HL-60 cells (D) on day 5 by flow cytometry. E, Western blot analysis of differentiating AML cells shows reduction in O-GlcNAc (RL2) protein modification. OCI-AML3 and HL-60 cells were treated with indicated compounds for 3 days and probed for O-GlcNAc. GAPDH served as an endogenous loading control. ATRA was used to induce differentiation and DON was used to inhibit protein O-GlcNAcylation. Statistical significance was calculated using unpaired Student’s t test (n = 3; ***; P < 0.001).
relationship. The interplay between O-GlcNAc and O-phosphate has been implicated in regulating protein function and plays an important role in cancer. It has been previously shown that inhibiting O-GlcNAcylation enhances phosphorylation of kinases such as Akt and GSK3β (46, 47). These proteins are extensively studied in AML cells (48, 49) and are shown to play an important role in survival of AML cells. However, the cell type-specific regulation of protein phosphorylation by O-GlcNAcylation in AML cells has not been explored. Hence, we checked the phosphorylation of these proteins in AML cells after DON treatment. It has been reported that O-GlcNAcylation of Akt at Thr 305 and 312 inhibits its phosphorylation at Thr 308. We found that DON treatment and thereby inhibition of O-GlcNAcylation, increased phosphorylation of Akt at Thr308 and Ser473, and GSK3 phosphorylation at Ser9 (Supplementary Fig. S3A). These results suggest that alteration in cellular O-GlcNAcylation levels could affect phosphorylation of Akt and GSK-3β in AML cells.

OGT downregulation also leads to terminal cell differentiation in AML cells

Because transfer of GlcNAc to proteins is mediated by a single enzyme OGT and blocking OGT will inhibit O-GlcNAcylation, OCI-AML3 cells were transiently transfected with OGT-siRNA or scrambled siRNA. Percent cell confluence showed significant drop after 96 hours when compared with scrambled siRNA transfected cells (Supplementary Fig. S4A). Western blot analysis of these siRNA-transfected cells showed inhibition of OGT and O-GlcNAcylated proteins compared with scrambled siRNA-transfected cells (Supplementary Fig. S4B), and these cells gradually underwent apoptosis as evidenced by caspase-3 cleavage and PARP cleavage (Supplementary Fig. S4B).

OCI-AML3 cells transfected with three different OGT shRNAs showed decrease in OGT levels directly proportional to the decrease in the overall levels of O-GlcNAcylation of cellular proteins (Fig. 5A). The decrease in the protein O-GlcNAcylation was maximum in shRNA-OGT3, followed by shRNA-OGT1 and minimal in shRNA-OGT2 transfected cells. At day 5, viable cell count shows decreased rate of cell proliferation in OGT-ShRNA OCI-AML3 cells (Fig. 5B). Moreover, staining with Annexin V/PI confirms apoptosis of these cells (Fig. 5C) in shRNA-OGT3 (~53% cells) and shRNA-OGT1 (~32% cells) transfected cells at day 5 (Fig. 5C and D). shRNA-OGT2 with minimal effect on OGT served as an internal control, show only approximately 7% apoptosis (Fig. 5C and D). Furthermore, increase in surface expression of differentiation markers CD11b (shRNA-OGT3 ~42%; shRNA-OGT1~12%) and CD14 (shRNA-OGT3 ~75%; shRNA-OGT1~30%) in OCI-AML3 suggests cells were undergoing differentiation upon downregulation of OGT (Fig. 5E and F). Interestingly, shRNA-OGT2 did not show increase in expression of differentiation markers CD11b and CD14.

Pharmacologic inhibition of HBP pathway eliminates AML cells in vivo

To determine whether DON can be utilized as a potential therapeutic agent for AML, we used an AML xenograft NSG mice model. HL-60 cells were subcutaneously injected in NSG mice. At day 4 tumors were palpable, mice were treated with DON as schematically represented in Fig. 6A. Following treatment significant decrease in AML tumor volume was observed in DON-treated mice compared with vehicle-treated xenograft mice (Fig. 6B and C). O-GlcNAc levels were found to be moderately reduced in tumor extract from mice administered with DON when compared with vehicle treated (Supplementary Fig. S5A). In addition to reduced tumor volume in DON-treated mice, increase in expression of differentiation marker CD11b and CD14 was observed (Fig. 6D and E).

In another circulating tumor in vivo xenograft model, NSG mice were intravenously injected with OCI-AML3 cells and followed by three intraperitoneal injections of DON (Fig. 6F). At day 18, control (PBS) and DON-treated mice were sacrificed to analyze the tumor burden in bone marrow, spleen, and blood as detected by human CD45-positive cells. As shown in Fig. 6G, a significant reduction of AML tumor load was observed in bone marrow, spleen, and blood in DON-treated mice compared with the control mice. We also compared the effect of DON with existing chemotherapeutic drug cytarabine and the effect was comparable. All vehicle-treated xenografts showed nearly 40% to 60% positivity for human CD45+ cells in bone marrow, 3% to 5% in spleen, and 0.5% to 2.5% in blood at 18 days posttransplantation, whereas DON-treated mice showed dramatic reduction in CD45+ cells, 1% to 15% in bone marrow, 0% to 0.5% in spleen, and 0% to 0.5% in blood (Fig. 6G). Mice weight did not significantly vary during the treatment regime (Fig. 6H).

To evaluate toxicity of DON, C57BL mice were similarly intraperitoneally injected with DON. Western blot analysis of harvested organs confirmed the decrease in levels of O-GlcNAc in spleen, liver, and kidney in DON-administered mice (Supplementary Fig. S5B) compared with control. However, bone marrow did not show any major alteration in O-GlcNAc levels. Their weight profile (Supplementary Fig. S5C) and H&E stain of kidney, liver, and spleen (Supplementary Fig. S5D) did not show any significant difference from PBS-injected control mice. Hematologic profile of DON-injected mice showed a marginal decrease in hemoglobin and hematocrit values compared with normal range. DON treatment also causes a decrease in platelet count suggesting thrombocytopenia. However, WBCs, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and RBCs were in normal range (Supplementary Fig. S6). Overall, these experiments show specific killing of AML cells with no major toxicity to normal cells.
Figure 5.
OGT downregulation leads to apoptosis and differentiation of OCI-AML3 cells. OCI-AML3 cells transduced with either control shRNA or shRNAs against OGT were analyzed on day 5. A, Western blot analysis for OGT and O-GlcNAc levels after lentiviral transduction with 3 different shRNAs directed against OGT in OCI-AML3 cells. GAPDH was used as an endogenous loading control. B, Cell count of OCI-AML3 cells on day 5 after lentiviral transduction with either control or shRNA against OGT. C, OCI-AML3 cells were stained with Annexin V and PI to determine apoptotic cell population, on day 5 after lentiviral transduction. D, Graph showing total population of early and late apoptotic OCI-AML3 cells under indicated condition on day 5. Surface expression of differentiation markers CD11b (E) and CD14 (F) in OCI-AML3 transduced with different shRNA at day 5. Statistical significance was calculated using unpaired Student t test (n = 3; ***, P < 0.001).
Figure 6.

In vivo DON administration reduces AML. **A**, Schematic representation of in vivo administration of DON or PBS in HL-60-based AML mice xenograft model. NSG mice were subcutaneously injected with HL-60 cells to generate solid AML mice xenograft model, followed by intraperitoneal injection treatment as shown. **B**, Tumor volume was monitored on days 4, 7, 11, and 14. **C**, Representative image of harvested tumor is shown from control and DON-treated mice group at day 14. Number indicates the weight of tumor in grams. Harvested tumors were analyzed for the presence of surface expression of differentiation marker CD11b (**D**) and CD14 (**E**). **F**, Schematic representation of in vivo administration of DON/cytarabine/PBS in OCI-AML3-based AML xenograft mice model. Sublethally irradiated NSG mice were intravenously injected with OCI-AML3 cells to generate liquid AML xenograft mice model, followed by intraperitoneal injection treatment as shown. **G**, Bone marrow, spleen, and blood cells isolated from indicated experimental groups were analyzed for the presence of human CD45 surface expression. Control group was intraperitoneally injected with PBS and treated group was intraperitoneally injected with DON (0.65 mg/kg). **H**, Weight profile of animals from different experimental group during treatment regime at indicated days in AML mice model (n = 5; statistical significance was calculated using unpaired Student t test; ns = not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
Discussion

Cancer cells generally use higher amounts of glucose and glutamine, channeled to different pathways, to maintain energy generation pathways, cell survival, and proliferation. Glutamine metabolism has developed as an attractive target for the treatment of different cancers. Glucose and glutamine channeled to HBP is utilized for making UDP-GlcNAc, the substrate for O-GlcNAcylation (53). Increased O-GlcNAcylation of proteins has been reported in many cancers (38–42). We report here that there is an upregulation of key enzymes involved in HBP and it results in an increased O-GlcNAcylation of proteins in AML cells. Inhibition of this pathway resulted in AML cell death. Interestingly, the death of AML cells was preceded by their differentiation (Fig. 4). This shows that O-GlcNAcylation is a necessary event for AML cell survival.

Hyper-O-GlcNAcylation in AML cells points to a greater dependency of these cells on HBP for their survival compared with normal cells. We used DON, a GFAT inhibitor to inhibit HBP and O-GlcNAcylation in AML cells. In agreement with existing literature, we found that HBP inhibition leads to increase in specific phosphorylation of Akt and GSK3–β. To our surprise, moderate inhibition of HBP using low dose of DON led to AML cell differentiation, similar to ATRA, a commonly used differentiating agent for APL (16). It will take 4 to 7 days for the AML cells to die in presence of low concentration of DON, whereas they undergo apoptosis within 24 hours in presence of high concentration of DON. On the basis of this, we speculate that depending on degree of inhibition of HBP, AML cell fate mechanisms differ. ATRA binds to the retinoic acid receptor (RAR) and activates transcription of genes necessary for AML differentiation. ATRA treatment fails to induce differentiation in cells with dysfunctional RAR signaling (54). ATRA treatment is successful only in one subset of patients with AML with APL fusion protein as it induces activation and degradation of the APL fusion protein leading to reexpression of the myeloid differentiation program, terminal differentiation, and apoptosis of leukemic cells (55). Because HBP was found activated in all AML subtypes tested including AML-1, AML-2, AML-4, and AML-5, inhibition of this pathway might be promising to treat all AML subtypes. Other AML subtypes also need to be analyzed for HBP enzymes and O-GlcNAcylation status in future studies.

Because inhibiting O-GlcNAcylation results in AML cell differentiation, we hypothesized that hyper-O-GlcNAcylation keeps AML cells in undifferentiated state. To test this hypothesis, we analyzed the levels of O-GlcNAcylation in AML cells before and after ATRA-induced differentiation. Interesting enough, we found that ATRA-induced differentiation is associated with a decrease in O-GlcNAcylation, confirming our hypothesis that differentiation is connected with O-GlcNAcylation or HBP activation status of cells. Moreover, we found that similar to ATRA-induced differentiation, HBP inhibition by DON also leads to early down-regulation of c-myc and c-myb transcript levels, which is linked to directing AML cells toward differentiation. Further work needs to be done to explore the actual mechanism behind this phenomenon. It is intriguing to see that both DON and ATRA behave in a similar manner in causing AML cell differentiation in an O-GlcNAcylation–dependent manner. Little is known about the role of ATRA in O-GlcNAcylation. However, one previous study shows interaction between OGT and nuclear receptors including RAR-α. Biological effects of ATRA are considered to be mainly mediated through RAR-α (56). Thus, it is conceivable that both DON and ATRA yield similar phenotypic effect and suppression of O-GlcNAcylation. Detecting the specific O-GlcNAcylated proteins associated with differentiation and delineating their function has the potential to lead to new arenas of targeted AML therapy.

DON is a GFAT inhibitor and acts upstream in HBP. To confirm that the observed effects are indeed due to HBP inhibition and not an artificial outcome of DON treatment, we used alternate methods to confirm or refute our findings. Incubation of AML cells with small-molecule OGT inhibitors (OSMI-1 and BADGP) as well as OGT knockdown using shRNA inhibited AML cell proliferation and induced differentiation, confirming that the phenotypes we observe are indeed due to the inhibition of O-GlcNAcylation. Interestingly, DON was found most effective of all these strategies for antileukemic activity. These findings suggest that the development of pharmacologic agents that target HBP or OGT enzyme could have a significant therapeutic effect on AML. There are no existing OGT inhibitors tested in the clinic currently for treating patients with cancer, indicating a need to develop novel and more effective OGT inhibitors. However, we should not forget the fact that mammalian OGT is a single copy (X-linked) gene and its knockout has been shown to be lethal (57). Therefore, targeting OGT in cancer cells needs a detailed dosing study to define optimal dose of OGT inhibitors showing minimal toxicity to normal cells.

Because GFAT and OGT are both nutrient-sensing enzymes, it is plausible that inhibiting them may affect normal metabolic state of cell. Metabolic profiling of post-burn cancer cell model OGT inhibition decreases glucose consumption and lactate production, which results in the inhibition of proliferation of these cells (58). We analyzed oxidative phosphorylation indicator OCR and glycolysis indicator ECAR in AML cells after DON incubation and observed only some modest changes at the time points analyzed. A clear global picture of metabolic changes occurring in AML cells after HBP inhibition is essential to understand metabolic link and the mechanism behind AML cell differentiation and cell death post-HBP inhibition.

Finally, our in vivo data are a clear evidence of therapeutic potential of DON and opens door for the use of many other HBP inhibitors for AML therapy. Subcutaneous AML xenograft model shows reduction in tumor burden accompanied with in vivo cell differentiation. In yet circulating AML model, eradication of AML cells from blood, spleen, and bone marrow was noticed after DON treatment. Immunocompromised NSG mice did not show any weight loss, indicative of nontoxicity. AML killing was significant even after three DON injections, which made us to think about its toxic effect on all other hematopoietic populations. Because NSG mice do not possess an intact immune system, we injected DON in C57BL6 mice for toxicity studies. We sacrificed all mice 1 day after 3rd injection to include both immediate toxic effects as well as long-term effects. Besides reduced platelet count, DON-injected C57BL6 mice did not show any significant changes in hematopoietic cell populations or organ histology.

Altogether, pharmacologic targeting of HBP enzymes or OGT could have a significant therapeutic effect on patients with AML irrespective of their subtype. Because HBP inhibition targets both cell proliferation and differentiation of AML cells simultaneously, this strategy will be more effective than existing treatment methods. AML cells seem to be selectively sensitive to HBP inhibition.
without major toxicities to normal cells; hence, developing new HBP or OGТ inhibitors also will be a good bet for AML therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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