Glutathione promotes the synergistic effects of venetoclax and azacytidine against myelodysplastic syndrome-refractory anemia by regulating the cell cycle

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Abstract. Azacitidine is a DNA methyltransferase inhibitor that has been used as a singular agent for the treatment of myelodysplastic syndrome-refractory anemia with excess blast-1 and -2 (MDS-RAEB I/II). However, recurrence and overall response rates following this treatment remain unsatisfactory. The combination of azacitidine and venetoclax has been used for the clinical treatment of a variety of hematological diseases due to the synergistic killing effect of the two drugs. Venetoclax is a BCL-2 inhibitor that can inhibit mitochondrial metabolism. In addition, azacitidine has been shown to reduce the levels of myeloid cell leukemia 1 (MCL-1) in acute myeloid leukemia cells. MCL-1 is an anti-apoptotic protein and a potential source of resistance to venetoclax. However, the mechanism underlying the effects of combined venetoclax and azacitidine treatment remains to be fully elucidated. In the present study, the molecular mechanism underlying the impact of venetoclax on the efficacy of azacitidine was investigated by examining its effects on cell cycle progression. SKM-1 cell lines were treated *in vitro* with 0-2 μM venetoclax and 0-4 μM azacytidine. After 24, 48 and 72 h of treatment, the impact of the drugs on the cell cycle was assessed by flow cytometry. Following drug treatment, changes in cellular glutamine metabolism pathways was analyzed using western blotting (ATF4, CHOP, ASCT2, IDH2 and RB), quantitative PCR (*ASCT2* and *IDH2*), liquid chromatography-mass spectrometry (α-KG, succinate and glutathione) and ELISA (glutamine and glutaminase). Venetoclax was found to inhibit mitochondrial activity though the alanine-serine-cysteine transporter 2 (ASCT2) pathway, which decreased glutamine uptake. Furthermore, venetoclax partially antagonized the action of azacitidine through this ASCT2 pathway, which was reversed by glutathione (GSH) treatment. These results suggest that GSH treatment can potentiate the synergistic therapeutic effects of venetoclax and azacitidine combined treatment on a myelodysplastic syndrome-refractory anemia cell line at lower concentrations.

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Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous clonal disease that affects hematopoietic stem cells leading to dysplasia and ineffective hematopoiesis in the bone marrow (1). Low-risk MDS progresses slowly in patients, who typically have superior prognoses. By contrast, patients with high-risk MDS, particularly those with MDS-refractory anemia with excess blast-1 and -2 (MDS-RAEB I/II), have a 3-year disease-free survival rate of only 30% after hematopoietic stem cell transplantation (HSCT) due to recurrence (2). Therefore, it is important to explore novel therapeutic methods for MDS. Guadecitabine (3) (a second-generation hypomethylating agent), rigosertib (4) (a multiple kinase inhibitor) and inhibitors of programmed cell death protein 1 (PD-1) (5)/programmed death ligand 1 (PD-L1) (6)/cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (7) are among the agents used for treating MDS.

Azacitidine (Vidaza, Pharmion) is a DNA methyltransferase inhibitor that was first marketed in the United States in July 2004. It is a cell cycle-specific drug acting on cells in the S phase, and as a nucleoside analogue of cytidine, it affects cellular DNA methylation by inhibiting DNA methyltransferases; it may also incorporate into RNA and exert direct cytotoxicity (8). Azacitidine is mainly used for the clinical treatment of MDS with refractory anemia (9). However, patients with MDS treated with azacitidine monotherapy typically have a high recurrence rate, low overall response rate (28-48%) and prolonged response times (8-10 months) (10). The BCL-2 inhibitor venetoclax (VEN) is used in combination with azacitidine for treating adult

patients with acute myeloid leukemia (AML) who are ineligible for intensive induction chemotherapy in the USA (11). Previous clinical studies on VEN have been extended to include hematological tumors, such as chronic lymphocytic leukemia (CLL) and relapsed/refractory multiple myeloma (R/R MM). Venetoclax monotherapy has demonstrated antimyeloma activity in patients with R/R MM positive for t(11;14); the overall response rate was 21% (12,13). However, the full extent of the effects of the azacytidine/VEN remains to elucidated.

In previous studies, VEN was shown to inhibit mitochondrial reactive oxygen species (ROS) metabolism through BCL-2; the characteristic feature of most functionally defined leukemia stem cells is relatively low levels of reactive oxygen species, and these LSCs exhibit abnormal overexpression of BCL-2 (14,15). It has also been reported that regulating cellular mitochondrial ROS metabolism can affect the efficacy of VEN (16,17). However, another study previously found that VEN can exert inhibitory effects on mitochondrial metabolism through the activating transcription factor 4 (ATF4)-pathway, independent of BCL-2 (18). In addition, a study has suggested that azacitidine can reduce the levels of myeloid cell leukemia-1 in older patients with AML (19), which is an anti-apoptotic protein and a potential source of resistance to VEN (20). In particular, the effects of VEN on the efficacy of azacitidine remain unclear.

VEN treatment has been shown to inhibit mitochondrial reactive oxygen species metabolism in various cells, such as primary leukemia cells, MCF7 breast cancer cells and the CT26 colorectal cancer cell line (14,15,18). It has been previously demonstrated that altering mitochondrial glutamine metabolism can promote drug sensitivity to tumor cells, such as HeLa and HCT116 cells (21). DNA damage can inhibit mitochondrial glutamine metabolism. During glutamine metabolic inhibition, the tricarboxylic acid cycle is blocked, which is essential for the DNA damage response. This eventually leads to delayed DNA repair and aggravates DNA damage in primary mouse embryonic fibroblasts (22). Glutamine is important for the maintenance of the cellular redox balance by removing ROS and supporting the synthesis of glutathione (GSH), an antioxidant that serves a key regulatory role against oxidative DNA damage (23). It has previously been shown that increasing intracellular GSH levels can impair DNA methylation (24). This phenomenon can be explained by the fact that the GSH precursor cysteine is also synthesized from the homocysteine pool necessary for the synthesis of S-adenosine methionine, a co-factor for DNA and histone methylation (25). However, glutamine is a non-essential amino acid but can serve various physiological functions. α-ketoglutarate is generated through glutaminolysis and directly enters the tricarboxylic acid cycle in the mitochondria, which feeds into the aerobic respiratory pathway and anabolism (26). Furthermore, glutamate is the metabolite of glutamine and is directly involved in the synthesis of GSH (27).

In the present study, metabolic activity in the mitochondria and cell cycle progression were assessed in an MDS cell line after treatment with both VEN and azacitidine.

Materials and methods

Cell culture and treatment. The leukemic cell line SKM-1 (cat. no. CCL-95) was purchased from American Type Culture Collection. SKM-1 cells were cultured in an RPMI-1640 medium (cat. no. 10-040-CV; Corning, Inc.) containing 10% FBS (cat. no. 04-001-1ACS; Biological Industries) at 37˚C in 5% CO_2. SKM-1 cells were treated with 0-2 μM venetoclax and 0-4 μM azacytidine or a combination of the two for 24, 48 and 72 h of treatment. In some specified experiments, 1 mM GSH (cat. no. S4606; Selleck Chemicals) was added at the beginning of venetoclax and azacytidine treatment at 37˚C for 24 and 48 h.

Reverse transcription-quantitative (q)PCR. Total RNA was extracted using the TRIzol® method (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using Hifair® III 1st Strand cDNA Synthesis SuperMix (cat. no. 11137ES10; Shanghai Yeasen Biotechnology Co., Ltd.) following the manufacturer's instructions. qPCR was used to analyze isocitrate dehydrogenase 2 (IDH2) expression using the ABI 7500 system (Thermo Fisher Scientific, Inc.) and the Hieff® qPCR SYBR Green Master Mix (cat. no. 11201ES08; Shanghai Yeasen Biotechnology Co., Ltd.). The thermocycling conditions used for qPCR were as follows: 95˚C for 5 min; followed by 95˚C for 10 sec and 60˚C for 30 sec for 40 cycles; the melting curve stage was set to the instrument's default settings. β-actin was used as the reference gene and $2^{-\Delta\Delta Cq}$ method (28) was used for data analysis. The primer sequences are listed below: IDH2 forward, 5'-CGCCACTAT GCCGACAAAAG-3' and reverse, 5'-ACTGCCAGATAATAC GGGTCA-3'; alanine-serine-cysteine transporter 2 (ASCT2) forward, 5'-GTGGCGCTGCGGAAGCT-3' and reverse, 5'-GGCGTACCACATGATCCAG-3' and β-actin forward, 5'-GTCATCACCATTGGCAATGAG-3' and reverse, 5'-CGT CATACTCCTGCTTGCTG'-3.

Flow cytometry. Cell cycle analysis was conducted using the fixed cell staining method. The treated cells were first washed twice in PBS, before ice-cold 70% ethanol was used to fix pelleted cells at 4˚C for 40 min. After fixation, cells were washed twice in PBS, before being centrifuged at 850 x g at 4˚C for 5 min. RNAse (100 μg/ml; cat. no. 9001-99-4; Beijing Solarbio Science & Technology Co., Ltd.) was then added to the cells and incubated at 37˚C for 15 min. In total, $1x10^6$ cells/200 μl (50 μg/ml) propidium iodide (PI; cat. no. P4170; MilliporeSigma) were added and incubated at 4˚C for 15 min in the dark before the sample was analyzed using Cytoflex LX (Beckman Coulter, Inc.). Data analysis was conducted using FlowJo 10.8.1 (Becton Dickinson & Company). An example of the gated strategy used is shown in Fig. S1.

Western blotting. Western blotting was performed to detect the protein expression of IDH2 (1:1,000 dilution; cat. no. 60322; Cell Signaling Technology, Inc.), ASCT2 (1:1,000 dilution; cat. no. ab187692; Abcam), CCAAT/enhancer-binding protein homologous protein (1:1,000 dilution; CHOP; cat. no. 5554; Cell Signaling Technology, Inc.), Retinoblastoma protein (1:1,000 dilution; Rb; cat. no. 9309; Cell Signaling Technology, Inc.) and GAPDH (1:5,000 dilution; cat. no. 3683; Cell Signaling Technology, Inc.) following VEN and/or azacitidine treatment. Prepare protein standard solutions at concentrations of 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. A total of 20 μl of each standard or lysate was added to each

well of a 96-well plate. Next, 200 μl BCA working solution was added to each well and incubated at 37˚C for 30 min. The absorbance was measured at 562 nm using a spectrophotometer (iMARK; Bio-Rad Laboratories, Inc.), and the protein concentration of the samples was calculated based on the standard curve. Proteins (20 μg/well) were analyzed on a 12% gel using SDS-PAGE, and the proteins were transferred onto PVDF membranes. The membranes were then blocked with 5% BSA (cat. no. V900933; Vetec™; MilliporeSigma) at 26˚C for 1 h, before primary antibodies were added for incubation overnight at 4˚C with shaking. The membranes were then washed with TBST (0.1% Tween 20) for 5 min for four times. Subsequently, HRP-conjugated goat anti-mouse (1:10,000 dilution; cat. no. 7076; Cell Signaling Technology, Inc.) or anti-rabbit (1:10,000 dilution; cat. no. 7074; Cell Signaling Technology, Inc.) IgG secondary antibodies were added at 26˚C for 60 min. After washing with TBST for four times, membranes were incubated for 3 min in Supersignal™ West Pico Plus Chemiluminescent Substrate (cat. no. 34580; Thermo Fisher Scientific, Inc.) and visualized using the iBright FL1000 Imaging System (Thermo Fisher Scientific Inc.). iBright Analysis Software (Desktop Version 5.1.0; Thermo Fisher Scientific Inc.) was used for image quantification.

Cell Counting Kit-8 (CCK-8) assay. The cells (2,500 cells/100 μl) were first cultured in 96-well plates for 24 h at 37˚C and 5% CO_2. After the aforementioned treatments for 12, 24, 48 and 72 h at 37˚C, cells were washed with PBS, before 10 μl CCK-8 solution (cat. no. CA1210; Beijing Solarbio Science & Technology Co., Ltd.) was added into the wells and incubated for 4 h at 37˚C. Finally, the optical density at 490 nm was measured using a microplate reader (iMARK; Bio-Rad Laboratories, Inc.).

Metabolite measurement and analysis. The glutamine concentration in the media was measured using a colorimetric method with a Glutamine (Gln) Colorimetric Assay Kit (cat. no. E-BC-K853-M; Elabscience Biotechnology, Inc.) according to the manufacturer's instructions, using a 50-kDa ultrafiltration filter (cat. no. UFC905096; MilliporeSigma). The culture medium was centrifuged at 3,000 x g at 4˚C for 40 min. and the filtrate collected for analysis. Prior to testing, the reagents were equilibrated to room temperature. Working solutions and reaction working solutions were prepared according to the manufacturer's instructions, kept on ice and then the prepared reaction working solution was used within 1 h. A 0-2 mM glutamine standard solution was prepared. A total of 30 μl working solution was added to each well of a 96-well plate, and then 50 μl of the standard or test sample was added to each well. The plate was incubated at 37˚C in the dark for 20 min, and then 140 μl reaction working solution was added to each well. The absorbance was measured at 450 nm (OD A1) for each well using a microplate reader. The plate was incubated at 37˚C in the dark for an additional 30 min. The absorbance was measured at 450 nm (OD A2) for each well using a microplate reader. The change in OD was then calculated (ΔA=A2-A1). The ΔA values from the standard solutions were used to construct a standard curve and the glutamine concentration of each sample was calculated based on the standard curve. In total, $1x10^6$ to $2x10^6$ cells/well of a 6-well plate were seeded and glutamine concentration was normalized to the number of cells in each well. Glutaminase activity was measured using a glutaminase (GLS) activity assay kit (AKAM007M; Beijing Box Biotechnology Co., Ltd.) and a spectrophotometer at 630 nm.

Mitochondrial membrane potential ($\Delta\psi$m) was measured using the fluorescent tetramethylrhodamine methyl (TMRM) ester probe (Sigma-Aldrich; Merck KGaA). After drug treatment, the cells were washed once with PBS, before TMRM at a working concentration of 100 nM and 1 μg/ml Hoechst 33342 (cat. no. H3570; Invitrogen; Thermo Fisher Scientific, Inc.) were added for nuclear staining. Cells were incubated for 30 min at 37˚C, followed by another wash with PBS. The cells were then plated on a confocal culture dish and observed using a confocal microscope (LSM880; Carl Zeiss AG). The TMRM excitation wavelength was set to 488 nm, whereas the emission wavelength was set to 573 nm. The Hoechst excitation wavelength was 350 nm, with an emission wavelength of 461 nm.

Liquid chromatography (LC)-mass spectrometry (MS). Cells, washed with cold PBS, were treated with the extraction buffer [5:3:2, methanol: acetonitrile: water; V/V]. After treatment with extraction buffer, the pellet was discarded after centrifugation at 20,000 x g at 4˚C for 20 min. The supernatant was analyzed using LC-MS.

A Millipore™ ZIC-pHILIC (2.1x150 mm, 5 μm) LC column (cat. no. 150454; MilliporeSigma) was coupled to a Nexera XR system (Shimadzu Corporation). In total, 20 μl were transferred into LC vials containing glass inserts for analysis. Samples were then subjected to an LC-MS analysis to detect and quantify known peaks. The column oven temperature and flow rate were set to 25˚C and 100 μl/min, respectively. Mobile phase compositions were as follows: A, 10 mM ammonium carbonate in water (pH 9.0); and B, acetonitrile hypergrade for LC/MS, ≤100% (cat. no. 100029; MilliporeSigma). The following gradient elutions were used: 80-20% B, 0-30 min; 20-80% B, 30-31 min; and hold at 80% B, 31-42 min. The LC system was coupled to a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific, Inc.) operating in heated electrospray ionization mode (ESI) for LC-MS analysis. Negative ion mode was used in mass spectrometry. The following default settings for ESI source were used: Nebulizer gas pressure, 15 psi; drying gas flow rate, 7 l/min; and drying gas temperature, 300˚C. The capillary voltage between the MS and nebulizer was ±3,500 V. All remaining ion transport parameters were determined using the Target Mass (TM) parameter, set by the operator. The TM was set as the closest value rounded to the nearest 50 of the expected mass:charge ratio (m/z). Target metabolites measured in the study: Glutathione (theoretical m/z, 307.084355); succinate (theoretical m/z, 118.027157); and α-KG (theoretical m/z, 146.022072).

Commercial standards, glutathione (cat. no. YZ-140706), succinate (cat. no. SS9520) and α-KG (SK8210) (all Beijing Solarbio Science & Technology Co., Ltd.), were run on the system prior to analysis as a quality control. For the preparation of standards, a 1-mM stock solution was first prepared for each metabolite standard and stored at -80˚C. On the day of the LC/MS run, the 1-mM stock solution and extraction solution was used to prepare fresh standard curves at the following concentrations: 1, 10 and 100 nM, and 1, 10 and 30 μM. For

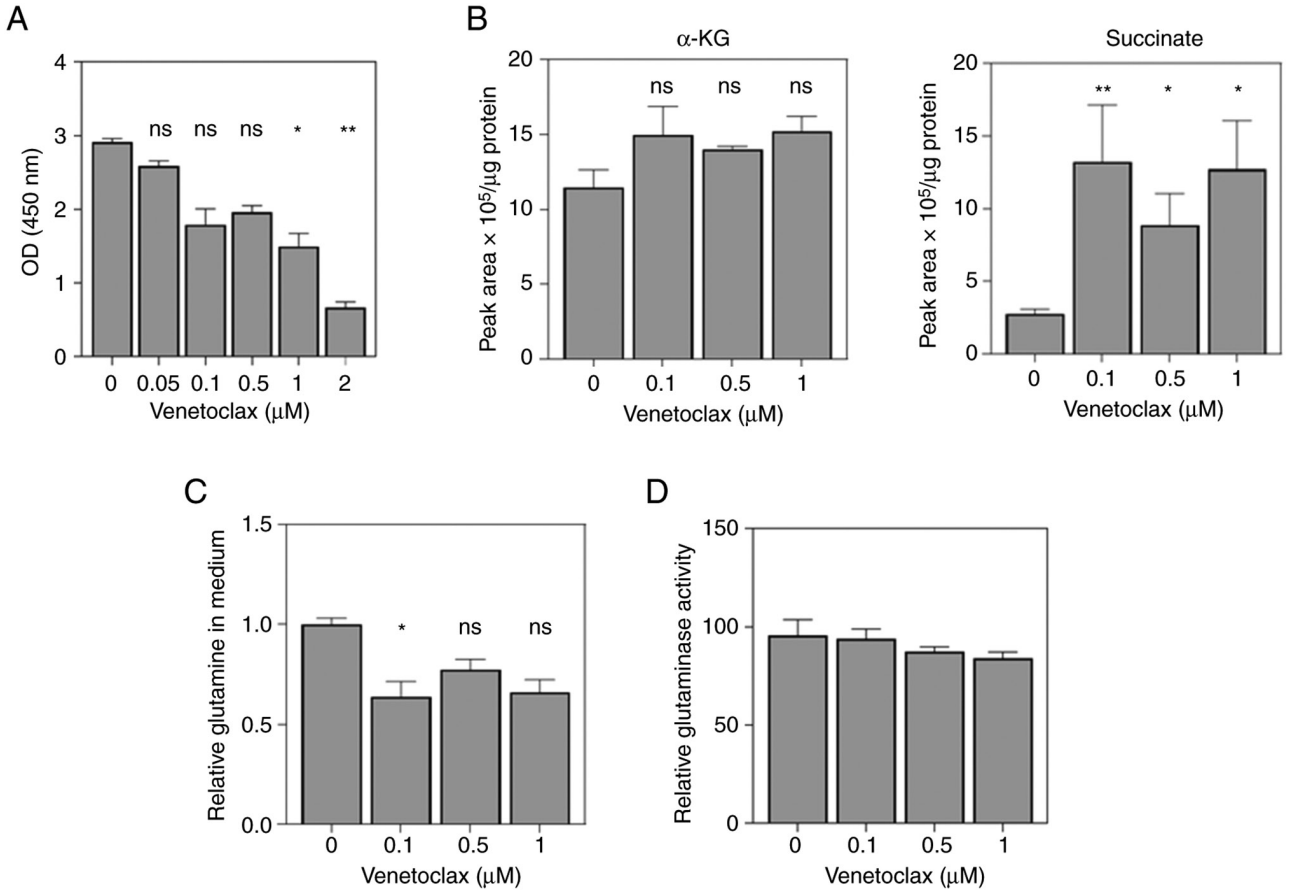

Figure 1. Effects of venetoclax on mitochondrial glutamine metabolism in SKM-1 cells. (A) Indicated concentrations of venetoclax were added to the SKM-1 culture medium, before cell viability was assessed using the Cell Counting Kit-8 assay 48 h after treatment. (B) SKM-1 cells were treated with the indicated concentrations of venetoclax for 24 h, before they were harvested and α-KG and succinate levels were measured using liquid chromatography-mass spectrometry. (C) The levels of glutamine in the culture medium were measured 24 h after SKM-1 cells were treated with venetoclax. Glutamine in the media was normalized to that of the control group. (D) Intracellular glutaminase activity was measured using a glutaminase activity assay kit. *P<0.05 and **P<0.01 vs. 0 μM venetoclax. α-KG, α-ketoglutarate; OD, optical density.

each metabolite, authentic standards were first run on the LC/MS instrument to confirm that they produced stable peaks at the correct m/z ratio and the retention time of the correct molecule. Peak areas were normalized to the amount of protein used. The BCA method was used to quantify protein content according to the manufacturer's protocol.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experimental repeats. An unpaired Student's t-test was used to assess the differences between two groups, one-way ANOVA was used for multiple comparisons followed by a Holm-Šídák's multiple comparisons test. One-way ANOVA (parametric) was used for multiple comparisons for the rest of the results. Dunnett's test was used for each other treatment group compared with the control group. Graphpad Prism 9.0 software (Graphpad Software. Inc.; Dotmatics) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

VEN treatment inhibits mitochondrial glutamine metabolism in the SKM-1 cell line. The potential effects of VEN treatment on metabolism in SKM-1 cells were first evaluated. The SKM-1 cell line, which was originally established from a patient with myelomonocytic leukemia, has been frequently used for MDS research (29,30). The clinical diagnosis and disease progression records of the patient, who was initially diagnosed with MDS, refractory anemia with an excess of blasts in transformation according to the French-American-British criteria (31), are well documented (29,32). Over the following 7 months, low-dose cytarabine therapy was administered multiple times until the patient developed resistance to treatment and subsequently developed AML (32). Therefore, SKM-I cell line was considered suitable for the present study on MDS-RA. Different concentrations of VEN were used for cell viability analysis, where the results showed that VEN began to exert an inhibitory effect at a concentration of 0.1 μM. In particular, there was little difference in the inhibitory effects at concentrations of between 0.1 and 1 μM (Fig. 1A). Furthermore, the extent of intracellular metabolism in the cells treated with 0.1-1 μM VEN was assessed. The results showed that the levels of α-ketoglutarate (α-KG) were increased but not significantly so, suggesting that VEN can regulate α-KG metabolism upstream of the IDH2 pathway (Fig. 1B). In addition, a significant accumulation of succinate levels was also observed, suggesting that mitochondrial glutamine metabolism was affected by VEN treatment (Fig. 1B). The consumption of glutamine and

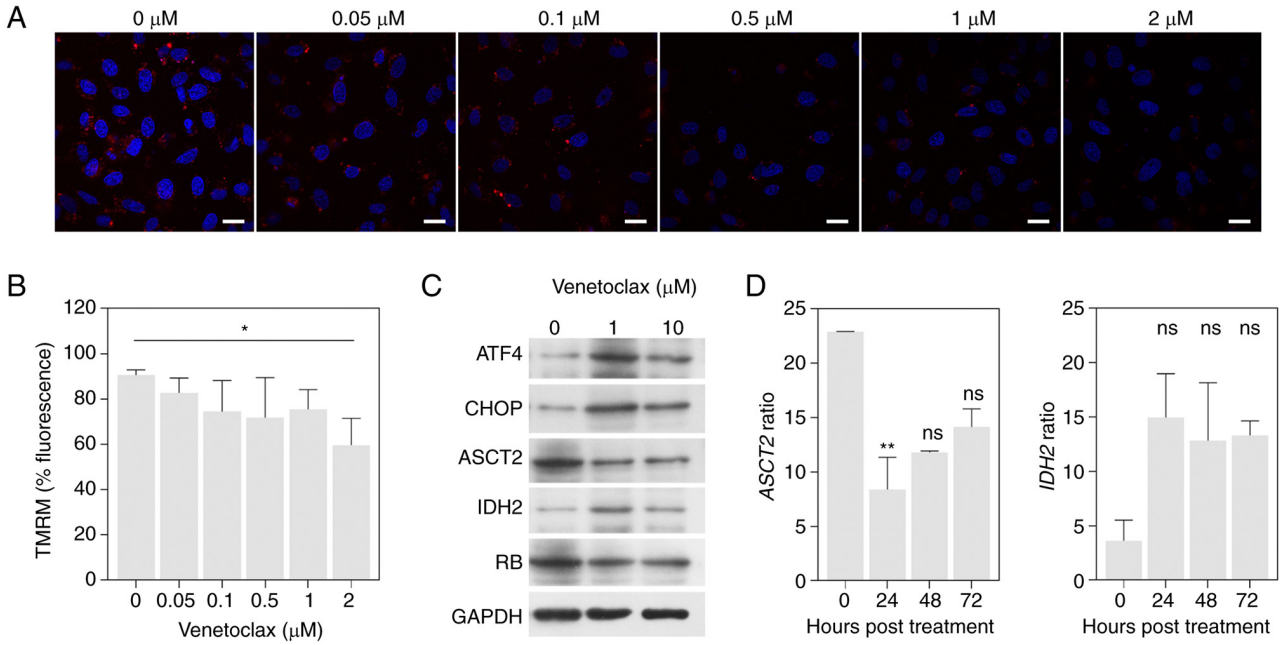

Figure 2. Venetoclax affects ASCT2-mediated glutamine uptake. (A) Representative TMRM fluorescence images of each group, with red fluorescence representing mitochondria and blue fluorescence representing the cell nuclei. Scale bar, 20 μm. (B) Detection of mitochondrial membrane potential after treatment with different concentrations of Venetoclax for 24 h. (C) After 24 h Venetoclax treatment *in vitro*, the expression of ATF4, CHOP, ASCT2, IDH2 and Rb was measured through western blotting. ATF4 and CHOP expression was markedly increased in the Venetoclax group compared with that in the control group. (D) The transcriptional levels of IDH2 and ASCT2 at different time points after drug administration were detected using reverse transcription-quantitative PCR (n=3). Results are presented as the means ± standard deviation. *P<0.05 vs. 0 μM venetoclax. **P<0.01 vs. 0 h post treatment. TMRM, tetramethylrhodamine methyl ester; CHOP, CCAAT/enhancer-binding protein homologous protein; ATF4, activating transcription factor 4; ASCT2, alanine-serine-cysteine transporter 2; IDH2, isocitrate dehydrogenase 2; Rb, retinoblastoma.

the activity of glutaminase were next examined. The results indicated that glutamine uptake was significantly inhibited by 0.1 μM VEN (Fig. 1C), whilst the activity level of glutaminase remained almost unchanged (Fig. 1D). This suggests that VEN can inhibit mitochondrial glutamine metabolism independent of glutaminase activity.

VEN inhibits the mitochondrial metabolism through ATF4 and ASCT2. According to the aforementioned results, the part of mitochondrial metabolism inhibited by VEN appears to be independent of glutaminase. Therefore, the expression levels of other key markers involved in mitochondrial metabolism were next assessed. Generic mitochondrial metabolic activity in the form of the membrane potential was first measured after VEN treatment, where the results showed that mitochondrial metabolism decreased to a certain extent, but remained non-significant until the concentration of VEN reached 2 μM (Fig. 2A and B). Western blotting results showed that the expression levels of ATF4 and CHOP were increased 24 h after VEN treatment, which was consistent with the previous results that mitochondrial metabolic function was inhibited (16,33) (Fig. 2C). In addition, the expression level of IDH-2 was also increased, which was consistent with the results shown in Fig. 1B, as IDH-2 is a key metabolic enzyme catalyzing the interconversion of isocitrate to α-KG (34). Notably, there was a decrease in the expression of ASCT2, a key transport protein required for glutamine uptake, at 24-h post treatment (Fig. 2D). ASCT2 inhibition has been shown to prevent HCC1806 breast cancer cell line proliferation by inhibiting glutamine uptake (35). This suggests that VEN may inhibit the uptake of glutamine by inhibiting ASCT2 expression. During the 72-h treatment, ASCT2 transcription levels were first decreased at 24 h before recovery. The transcription levels of IDH2 continued to increase; however, this was not statistically significant (Fig. 2D).

Low concentrations of VEN do not promote the cytotoxic activity of azacytidine. The synergistic mechanism between azacitidine, a DNA methyltransferase and VEN requires further evaluation. Therefore, the optimal concentration of the combination of the two drugs was first evaluated *in vitro* in the present study. At a lower concentration of VEN (0.1 μM) and 2 μM of azacitidine, the synergistic effect of VEN and azacitidine began to appear. By contrast, when 0.5 and 1 μM azacitidine were used, the two drugs could not produce a synergistic effect (Fig. 3A). When 0.5 and 1 μM VEN were used, a synergistic cytotoxic effect of VEN and AZA was observed in all concentration groups of AZA (Fig. 3B). Cell cycle progression after treatment with 0.1 μM VEN and 1 μM azacytidine was next tested, where the results showed that VEN partially inhibited the inhibitory effect of azacitidine on S-phase entrances (Fig. 3C and D). In addition, the proportion of cells in the G_2/M phase was significantly decreased in response to the combination treatment compared with that in cells treated with azacytidine alone (Fig. 3E). However, the use of 1 μM azacitidine had no effect on the VEN-induced inhibition of mitochondrial glutamine metabolism (Fig. 3F).

GSH can promote the synergistic effect of VEN and azacytidine. According to the aforementioned results, it is likely that

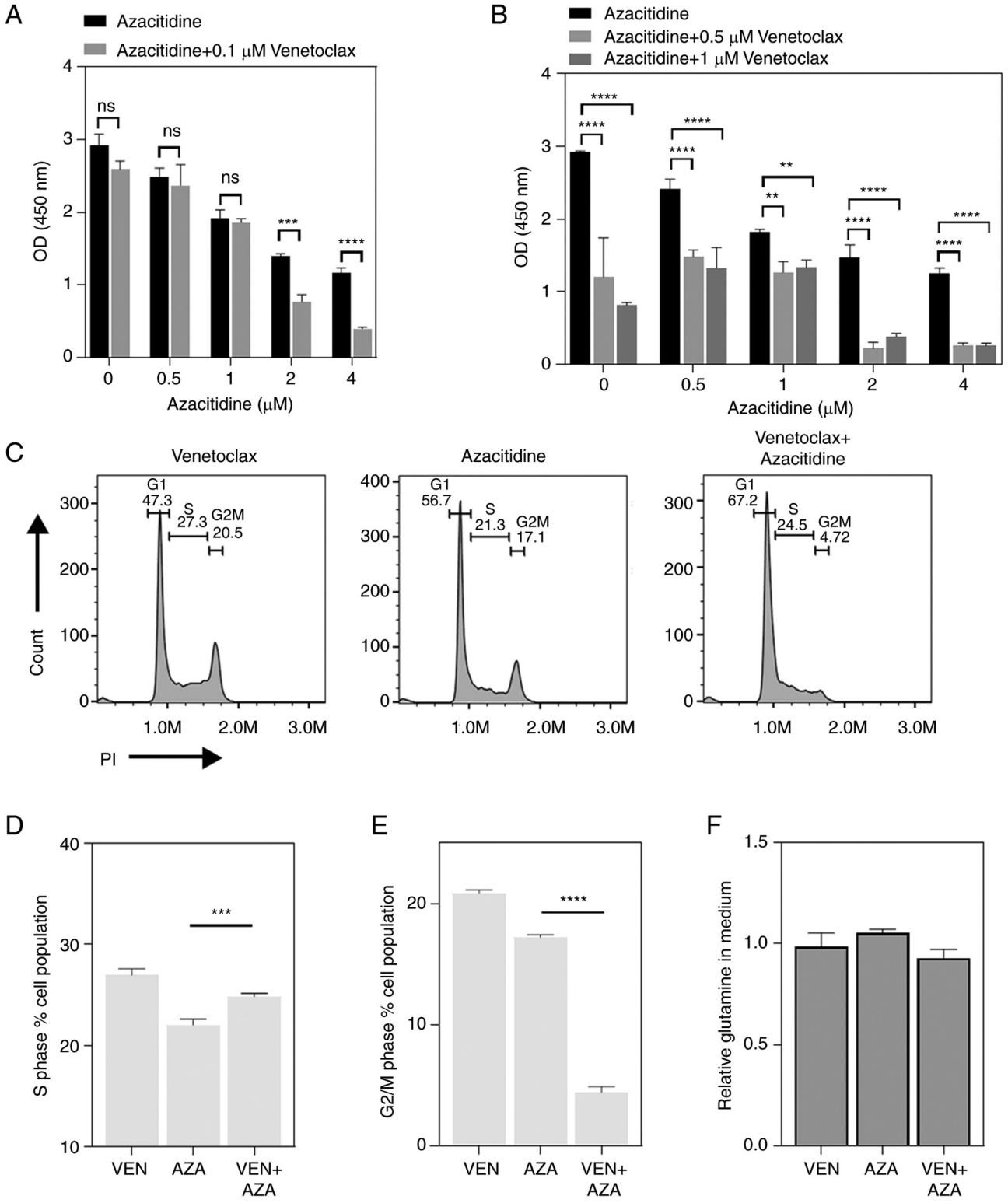

Figure 3. Evaluation of the synergistic effect of VEN and AZA. (A and B) Different concentrations of AZA with or without VEN were added to the SKM-1 culture medium before cell viability was evaluated using a Cell Counting Kit-8 assay 48 h after treatment. (C) Representative flow cytometry diagram of cell cycle analysis 48 h after VEN and/or AZA treatment. (D) S phase and (E) G_2/M cell ratio in each group treated with VEN or azacytidine, where recovery of S phase was observed in cells in the VEN + AZA group. (F) Glutamine was detected in the culture medium 24 h after the SKM-1 cells were treated with the indicated drugs. One-way ANOVA was used for multiple comparisons followed by Holm-Šídák's multiple comparisons test. **P<0.01, ***P<0.001 and ****P<0.0001 vs. Azacitidine alone. VEN, Venetoclax; AZA, azacytidine; OD, optical density.

azacytidine can 'compensate' for the effects of VEN on DNA repair to some extent. In previous reports, azacitidine was found to exert comparatively more potent cytotoxic effects on cells in S phase (36,37). When VEN and azacitidine were co-administered, there was a significant decrease in the proportion of S phase cells compare with that in the azacitidine monotreatment group. Further analysis of the proportion of S phase cells following VEN monotherapy revealed that as the concentration of VEN

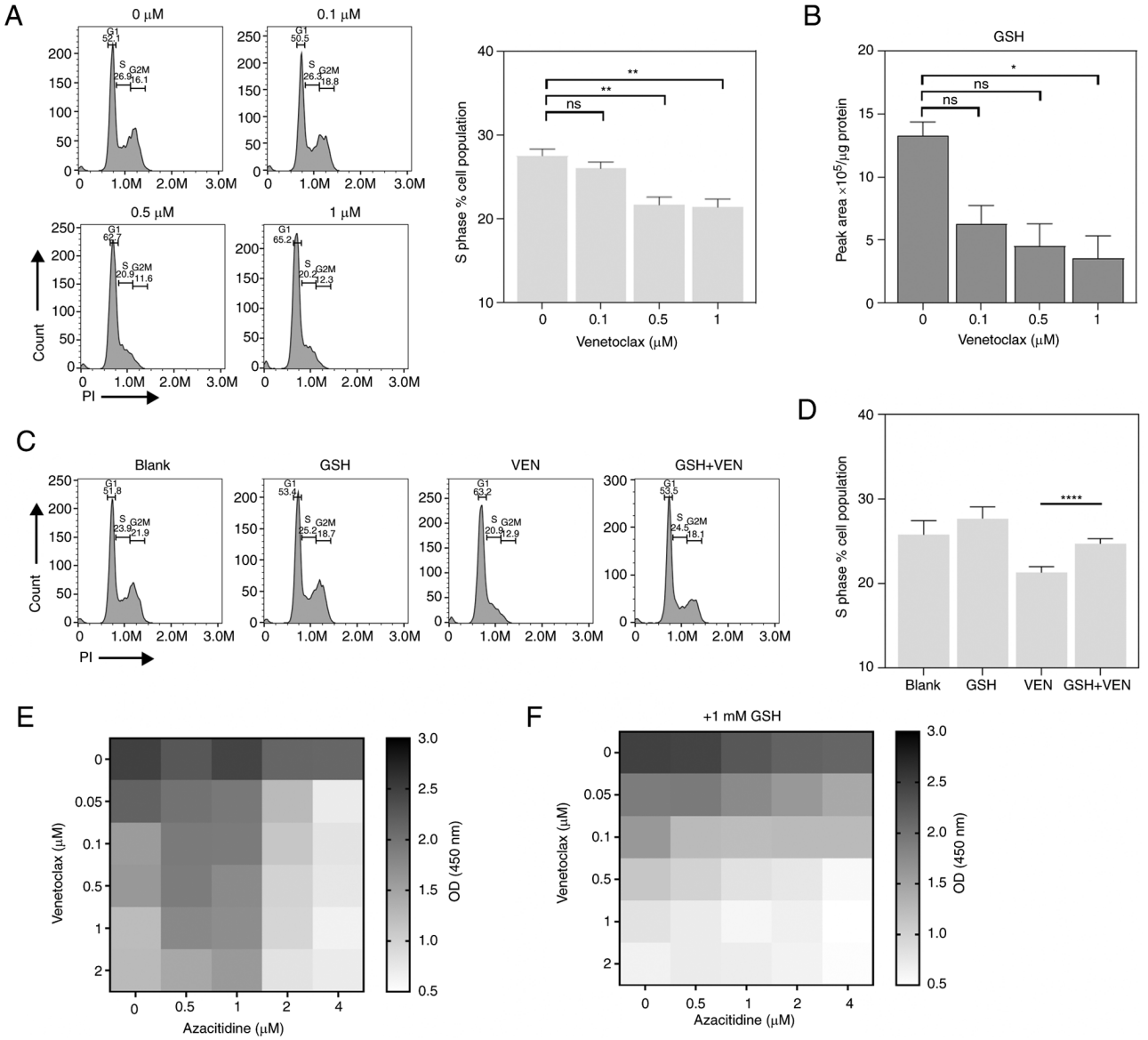

Figure 4. GSH can further promote the synergistic effect of VEN and azacitidine. (A) Representative flow cytometry histogram of cell cycle and S phase ratio after treatment with the indicated concentrations of VEN for 24 h. (B) SKM-1 cells were treated with the indicated concentrations of VEN for 24 h before being harvested, and had GSH concentration measured using liquid chromatography-mass spectrometry. (C) Representative flow cytometry histogram of cell cycle 24 h after treatment. (D) S phase ratio after treatment with indicated concentrations of VEN and 1 mM GSH for 24 h. (E) Different concentrations of VEN and azacitidine were added to the SKM-1 culture medium, whereas (F) 1 mM GSH was added during treatment, before cell viability was measured using a Cell Counting Kit-8 assay 48 h after treatment. The values in the heat map represent the mean values from three replicates; darker shades indicate a higher OD450 value. *P<0.05 and **P<0.01 vs. 0 μM venetoclax in Fig. 4A and B. ****P<0.0001. VEN, Venetoclax; GSH, Glutathione.

increased, the proportion of S phase cells also decreased, reaching significance at 0.5 and 1 μM (Fig. 4A). The reduction in S-phase was positively associated with the decrease in glutamine levels (Fig. 1C). The decrease in glutamine levels suggests that VEN may be influencing cellular glutaminolysis, where glutamine generates glutamate, which then maintains the intracellular redox state by producing GSH (38). Therefore, the level of intracellular GSH was further examined. The results showed that as the concentration of VEN increased, there was a corresponding decrease in intracellular GSH levels (Fig. 4B). This observation suggests that replenishing GSH may contribute to the efficacy of VEN + azacitidine treatment. Through supplementation experiments to increase the GSH levels in the culture environment, it was found that when 1 mM GSH was used, there was a significant recovery in the proportion of S phase cells compared with that in the VEN monotherapy group (Fig. 4C and D). This may be due to VEN affecting glutamine metabolism, thereby influencing the cell cycle. When 1 mM glutathione was added to the culture medium, the synergistic effect of azacitidine and VEN was also markedly improved (Fig. 4E and F). Treatment using 0.1 μM VEN with 1 μM azacitidine caused a notable inhibition of cell viability in SKM-1 cells in the presence of GSH compared with its absence.

Discussion

MDS frequently occurs in middle-aged and elderly patients, of whom only ~10% are eligible for HSCT (39). The safety and

efficacy of VEN + azacitidine as the initial treatment of AML have been previously proven (40). Tsao *et al* (41) previously reported that VEN combined with azacitidine exerted significant synergistic effects and inhibited the proliferation of AML cells *in vitro*. In another study, Pan *et al* (42) demonstrated that VEN rapidly killed AML cells in a murine primary xenograft model. Bogenberger *et al* (43) observed that VEN + azacitidine therapy directly inhibited tumor cell proliferation in patients with MDS. In addition, DiNardo *et al* (44) previously applied VEN + azacitidine to treat patients with R/R AML and MDS, where the efficacy and safety of VEN + azacitidine were identified. An objective response was observed in 9 (21%) patients, including 2 complete responses. However, the mechanism of this possible VEN/azacitidine synergism remains unclear.

VEN is a selective inhibitor of BCL-2. In 2016, it was approved by the United States Food and Drug Administration for the treatment of CLL with chromosome 17p deletion (45). In recent years, the potential application of VEN for the treatment of AML/MDS has been extensively investigated in the USA and Europe, the consistency and efficacy of which have been widely verified (46,47). However, there have only been a small number of relevant reports from Asian countries (48,49) and to the best of our knowledge, no investigations involving patients of Han ethnicity in China have been performed. BCL-2 inhibition is a novel targeted therapy for the treatment of AML, which can activate the mitochondrial apoptotic pathway in AML cells (42). In patients with high-risk MDS, high BCL-2 expression has been reported (50). Previous studies have shown that BCL-2 inhibitors are effective against high-risk MDS or secondary AML (50,51).

Apart from targeting BCL-2, VEN has also been shown to act on ATF4 upstream of altering mitochondrial metabolism (18). Previous reports showed CD4 T cell function to depend on ATF4-mediated catabolic glycolysis and glutaminolysis metabolism (52). In a recent study, ATF4 was found to be highly expressed in a variety of tumors, such as breast cancer (53) and melanoma (54), where it can promote tumor growth through fibroblasts, as knockdown of ATF4 expression in fibroblasts can significantly inhibit tumor angiogenesis and tumor growth in melanoma and pancreatic tumors (54). This suggests that the ATF4 pathway is a key factor mediating mitochondrial metabolism. In the present study, VEN treatment was observed to not only promote ATF4 expression, but also inhibited ASCT2 and promoted IDH2 expression. ATF4 is a factor that can promote tumor growth in breast cancer and melanoma, but in the present results, ATF4 was highly expressed in SKM-1 cells following VEN treatment. This suggests that there are distinct molecular regulatory mechanisms between hematological malignancies and their solid tumor counterparts. However, this also suggests the potential of applying ATF4 inhibitors as a combination therapy with VEN.

IDH2 catalyzes the reversible oxidative decarboxylation of isocitrate into αKG in mitochondria whilst reducing $NADP^+$ to NADPH (55,56). *IDH2* is one of the genes showing the highest frequency of mutations among other metabolic genes associated with human cancers such as glioma (57) and cholangiocarcinoma (58). IDH2 is associated with cell metabolism and epigenetic regulation, where *IDH2* mutants have been reported to promote tumorigenesis (59). *IDH2* mutations redirect carbon metabolites and oxidative phosphorylation towards d-2-hydroxyglutarate (D-2HG) production, and elevated D-2HG may somehow promote formation and progression of AML (60), although the mechanism by which D-2HG promotes AML development remains unclear. In addition, lack of the wild-type IDH1/2 enzyme can lead to downstream vulnerabilities in gliomas (59), which can improve the effects of small molecule inhibitors, such as poly (ADP-Ribose) polymerase inhibitors, BCL-2 inhibitors and biguanides (such as metformin, an antidiabetic drug that can inhibit glutaminolysis) (61). The sensitivity of cancer cells to radiotherapy and chemotherapy can also be improved through knocking down wild-type *IDH1* (62-64). In a previous *in vitro* study on lung cancer cells, IDH2 knockdown by shRNA resulted in decreased HIF1α expression, leading to the attenuation of lung cancer cell proliferation and tumor growth (65). In the present study, the expression of IDH2 was increased to some extent after VEN treatment (Fig. 2C and D), whereas the level of α-KG was also increased to a certain extent, suggesting that VEN may interfere with IDH2-related metabolism.

In addition to its impact on α-KG, it has been reported that VEN can also affect mitochondrial metabolism (16). In the present study, VEN was found to inhibit the glutamine metabolic pathway. The products regulated by the glutamine metabolism pathway, such as GSH, are important for regulating the intracellular redox state (66). It has been previously shown that the intracellular redox state serves an important role in regulating the cell cycle (67,68). Low-level cellular oxidation triggered by superoxide and hydrogen peroxide activates proliferative cell signaling pathways, which are necessary for physiological mitotic signal transduction (68). Oxidation events occurring during the early G_1 phase of the cell cycle are critical regulatory steps for progression into the S phase (69). During the G_1 phase, cellular GSH levels are low, but the subsequent increases in total GSH is necessary for cells to transition from G_1 into the S phase (70). The intracellular levels of GSH were measured in the present study 24 h following VEN treatment, which were observed to be significantly decreased. This suggests that the application of VEN can lead to an imbalance in the intracellular redox levels.

By GSH compensation, the synergistic effect of VEN + azacytidine was enhanced. However, it is worth noting that deficiency in GSH, which is an important antioxidant, can lead to the excessive generation of ROS and subsequently cell apoptosis (71). Previous studies have found that GSH-mediated detoxification is involved in cisplatin resistance in several types of tumors (72,73). In addition, reducing GSH levels in cancer cells has been shown to enhance the therapeutic effect of cisplatin and even reverse drug resistance (74,75). Therefore, these results indirectly suggest that an increase in the oxidative-reductive level of cells does not induce VEN or AZA resistance.

In conclusion, results from the present study suggest that VEN can inhibit glutamine metabolism, leading to a reduction in intracellular GSH levels. This in turn causes cell cycle arrest and affects the efficacy of azacitidine. The addition of GSH was then found to promote the synergistic effects of VEN and azacitidine *in vitro*. The present study provides a new direction for the exploration into the synergistic effects of azacitidine

and VEN in clinical practice, by investigating the impact of VEN on the cell cycle and glutamine metabolism in MDS-RA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XX and DL contributed to the study conception and design. Material preparation was performed by XW, data collection was performed by XW and LY, and data analysis was performed by XW, LY and BL. The first draft of the manuscript was written by XW and LY and all authors commented on previous versions of the manuscript. XW and XX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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