

Role of glucose metabolism in the differential antileukemic effect of melatonin on wild-type and FLT3-ITD mutant cells

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Abstract. The FMS-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) mutation represents the most frequent genetic alteration in acute myeloid leukemia (AML) and is associated with poor prognosis. The mutation promotes cancer cell survival and proliferation, and shifts their glucose metabolism towards aerobic glycolysis, a frequent alteration in cancer. In the present study, the impact of melatonin on the viability of AML cell lines with (MV-4-11 and MOLM-13) or without the FLT3-ITD mutation (OCI-AML3 and U-937) was evaluated. Melatonin induces cell death in AML cells carrying the FLT3-ITD mutation, but only inhibits the proliferation of AML cells without this mutation. Consistently, melatonin decreases tumor growth and increases animal survival in a xenograft model of FLT3-ITD AML. Toxicity is related to a decrease in glucose uptake, lactate dehydrogenase activity, lactate production and hypoxia-inducible factor-1 α activation. Melatonin also regulates the expression of glucose metabolism-related genes, impairing the balance between anaplerosis and cataplerosis, through the upregulation of the expression of phosphoenolpyruvate carboxykinase 2 (PCK2). Collectively, the present findings highlight the regulation of glucose metabolism, currently considered a possible therapeutic target in cancer, as a key event in melatonin-induced cytotoxicity, suggesting its potential as a therapeutic tool for the treatment of patients with AML, particularly those carrying the FLT3-ITD mutation that results in low basal expression levels of PCK2.

Introduction

Acute myeloid leukemia (AML) is the most common myeloid malignancy in adults, and is considered to be the result of several genetic aberrations leading to irreversible dysregulation of genes critical for proliferation, differentiation and apoptosis (1). These genetic alterations are used by clinicians for both the diagnosis and prognosis of AML (2). Internal tandem duplications (ITD) or kinase domain mutations of FMS-like tyrosine kinase 3 (FLT3) represent the most frequent genetic alterations, occurring in ~30% of cases and being associated with poor disease outcome (3). Reports indicate that the FLT3-ITD mutation shifts the metabolism of glucose from oxidative phosphorylation towards aerobic glycolysis, also known as the Warburg effect, and increases the resistance of AML cells to chemotherapy (4,5), making aerobic glycolysis a potential therapeutic target for FLT3-ITD AML cells (6).

Melatonin is a natural indoleamine with diverse antitumoral properties (7). It inhibits the proliferation of most types of tumor, but only kills certain specific tumor types, such as Ewing sarcoma or hematological malignancies (8). Several mechanisms have been proposed for the antiproliferative effects of melatonin, but mechanisms underlying the proapoptotic effects of melatonin remain to be completely unraveled. The regulation of tumor cell metabolism has received increasing attention in relation to this; studies have demonstrated the ability of this indoleamine to regulate several metabolic pathways, mainly glycolysis and lipid metabolism (9-11).

The present study aimed to evaluate the hypothesis that melatonin specifically kills tumor cells with an altered glucose metabolism, using AML cells with or without the FLT-ITD mutation.

Materials and methods

Cell culture and reagents. MV-4-11 (FLT3-ITD), MOLM-13 (FLT3-ITD), U-937 [wild-type (wt)] and OCI-AML3 (wt) cell lines were purchased from the Leibniz Institute GSMZ-German

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Collection of Microorganisms and Cell Cultures GmbH and aliquoted to prevent phenotypic drift once received. Cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture containing 5,000 U/ml penicillin and 5,000 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Culture cells have been monitored to ensure that they are mycoplasma-free using a LookOut[®] mycoplasma quantitative (q)PCR detection kit (Sigma-Aldrich; Merck KGaA). All experiments were performed between passage 5 and 20. Cell culture reagents were purchased from Sigma-Aldrich (Merck KGaA) except for FBS, which was obtained from Gibco (Thermo Fisher Scientific, Inc.). Culture flasks and dishes were acquired from Thermo Fisher Scientific, Inc. The primary antibodies used for western blotting were hydroxylated hypoxia-inducible factor (HIF)1 α (1:1,000; cat. no. 3434; Cell Signaling Technology, Inc.), phosphorylated (p)-mTOR (1:1,000; cat. no. 2976; Cell Signaling Technology, Inc.), p-S6 (1:1,000; cat. no. 5364; Cell Signaling Technology, Inc.), p-AKT (1:1,000; cat. no. 9271; Cell Signaling Technology, Inc.), p-STAT5 (1:1,000; cat. no. 9314; Cell Signaling Technology, Inc.), dihydroliipoamide dehydrogenase (DLD; 1:250; cat. no. ab182146; Abcam), hexose-6-phosphate dehydrogenase (H6PD; 1:500; cat. no. ab72183; Abcam), phosphoenolpyruvate carboxykinase 2 (PCK2; 1:1,000; cat. no. ab70359; Abcam) and GAPDH (1:5,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.). EC-70124 was provided by EntreChem, S.L. Melatonin (cat. no. M5250) and mitochondrial complex I inhibitor rotenone (cat. no. R8875) were purchased from Sigma-Aldrich (Merck KGaA), while hexokinase II inhibitor 3-bromopyruvate (3-BrPA; cat. no. sc-260854), and PCK2 inhibitor 3-mercaptopicolinic acid (3-MP; cat. no. sc-206655) were purchased from Santa Cruz Biotechnology, Inc.

Evaluation of cell proliferation and cell viability. Cell viability was evaluated using an MTT reduction assay. Cells were seeded onto 96-well plates at a density of 5,000 cells/ml and treated with increasing melatonin concentrations (100-1,000 μ M) for 48 h. Once the treatments were completed, 10 μ l MTT solution diluted in PBS (5 mg/ml) was added. After 4 h of incubation at 37°C, one volume of the lysis solution [SDS (20%) and dimethylformamide pH 4.7 (50%)] was added and the mixture was incubated at 37°C overnight. Samples were measured using an automatic microplate reader (μ Quant; Bio-Tek Instruments, Inc.) at a wavelength of 540 nm.

For cell death evaluation, propidium iodide (PI) was used. It is a membrane-impermeant fluorescence dye that cannot enter the cells unless there are membrane breaks. Thus, dead cells can be determined as cells with PI uptake. Cells were seeded onto 6-well plates at a density of 5,000 cells/ml and treated with 1 mM melatonin, 5 μ M 3-BrPA or 500 nM rotenone for 48 h. For combination experiments, 250 μ M 3-MP or 10 mM glucose were added at the same time as melatonin. Once the treatment was completed, cells were harvested and resuspended in 500 μ l of PBS. PI staining solution (10 μ l; 10 μ g/ml in PBS) was added at room temperature for 10 min and the fluorescence of 10,000 cells/group was measured in a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Inc.). Data was analyzed using Flowing Software v2.5 (<http://flowingsoftware.btk.fi/>).

Measurement of glucose uptake activity using 2-NBDG. Glucose uptake activity was measured using a fluorescent D-glucose analogue 2-NBDG. Cells were seeded in 96-well plates at a density of 5,000 cells/ml and treated with 1 mM melatonin or 10 nM EC-70124 for 8 h. After treatment, cells were washed with PBS and incubated with 10 μ M 2-NBDG at 37°C for 35 min. Fluorescence was measured in an FLX-800 microplate fluorimeter (Bio-Tek Instruments, Inc.) at an excitation wavelength of 467 nm and an emission wavelength of 542 nm.

Measurement of lactate dehydrogenase (LDH) activity. For this assay, cells were seeded onto 24-well plates at a density of 5,000 cells/ml. Determination of total LDH activity was performed after 24 h treatment with 1 mM melatonin or 10 nM EC-70124 according to the specifications of a Lactate Dehydrogenase Activity Assay kit (cat. no. MAK066; Sigma-Aldrich; Merck KGaA). Absorbance was determined using a μ Quant automatic microplate reader at 490 nm and enzyme activity was related to protein content on each sample.

Measurement of intracellular lactate levels. Cells were seeded in 150-mm plates at a density of 5,000 cells/ml. After 24 h treatment with 1 mM melatonin or 10 nM EC-70124, the lactate concentration was determined using a Lactate Assay kit (cat. no. MAK064; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Absorbance was measured at 570 nm using a μ Quant automatic microplate reader.

Assessment of apoptosis by flow cytometry. Apoptosis was evaluated using an Annexin V-FITC Apoptosis Detection kit (cat. no. APOAF; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols. Briefly, cells were seeded in 6-well plates at a density of 5,000 cells/ml and treated with 1 mM melatonin for 24 h. Once the treatment was completed, cells were harvested and resuspended in 500 μ l of 1X binding buffer. Annexin V-FITC conjugate (5 μ l; 50 μ g/ml) and 10 μ l PI solution (100 μ g/ml) were added to each cell suspension and after 10 min of incubation, the apoptosis percentage was determined in 10,000 cells/group using a Beckman Coulter FC500 flow cytometer. Data was analyzed using Flowing Software v2.5 and represented as the sum of early apoptotic cells (PI-negative, Annexin V-positive cells) + late apoptotic cells (PI-positive, Annexin V-positive cells).

Caspase-3 activity. To measure the activation of caspase-3, a fluorometric caspase-3 assay kit (cat. no. CASP3F; Sigma-Aldrich; Merck KGaA) was used according to the manufacturer's protocols. Briefly, cells were seeded in 100 mm plates at a density of 5,000 cells/ml and treated with 1 mM melatonin for 24 h. Once the treatment was completed, cells were harvested and processed according to manufacturer's protocol. After 2 h of incubation of the reaction mixture at room temperature in darkness, samples were analyzed in an FLX-800 microplate fluorimeter at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

RT² Profiler[™] PCR Array screening. Cells were seeded in 100 mm plates at a density of 5,000 cells/ml and treated with 1 mM melatonin for 8 h. Total cellular RNA was extracted

using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich; Merck KGaA) and reverse transcription was conducted using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RT² Profiler PCR Array screening of 84 glucose metabolism genes (cat. no. PAHS-006Z; SABiosciences Corporation; Qiagen, Inc.) was performed via qPCR using Green PCR Core Reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.) in an AB7700 Real-Time System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermal cycling parameters were 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec, with a final elongation step at 72°C for 5 min. Data analysis of relative levels of mRNA expression was based on the $\Delta\Delta C_q$ method (12), with normalization of the raw data to housekeeping genes included in the array (β -actin, β -2-microglobulin, GAPDH, hypoxanthine phosphoribosyltransferase and ribosomal protein) using the web-based software GeneGlobe Data Analysis Center (Qiagen, Inc.).

Western blot analysis. For protein expression analysis, cells were seeded on 100-mm plates and treated with 1 mM melatonin for 8 or 24 h. After treatments, cells were lysed with ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin-A, 110 nM NaF, 1 mM PMSF, 20 mM Tris-HCl pH 7.5). Protein concentration was determined using Pierce™ 660 nm protein assay reagent (cat. no. 10177723; Pierce; Thermo Fisher Scientific, Inc.) and 30 μg were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham; GE Healthcare). Blots were blocked using Pierce Clear Milk Blocking Buffer (cat. no. 13494209; Pierce; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and incubated overnight at 4°C with appropriate primary antibodies. Immunoreactive polypeptides were visualized using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:4,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) and enhanced chemiluminescence detection reagents (Amersham; GE Healthcare) according to the manufacturer's protocols.

In vivo xenograft assays. Female immunodeficient mice were purchased from Janvier Labs (CB17 SCID, 5 weeks old, weight range 19.05±0.3 g) and maintained under sterile and controlled conditions (25°C, 60% humidity, 12/12 light/dark cycle) with food and water *ad libitum*. All animal research protocols were approved by the Animal Research Ethical Committee of the University of Oviedo (permit no. PROAE 14/2017).

In order to evaluate tumor growth, MOLM-13 (FLT3-ITD) cells (5×10^6 cells with 25% Matrigel) were injected subcutaneously into the right flank of CB17 SCID mice. Alternatively, in order to evaluate animal survival, 2×10^6 cells were injected into the tail veins of CB17 SCID mice. At 10 days after injection, animals were randomized in the different experimental groups of 10 mice/group in both animal models (subcutaneous or intravenous) and melatonin administration via drinking water was initiated (10 mM melatonin as final concentration in the drinking water). Indolamine administration was well

tolerated by all mice, as no significant weight loss was detected (data not shown). The greatest longitudinal diameter (length) and the greatest transverse diameter (width) were measured by an external caliper every other day. Tumor volumes based on caliper measurements were calculated by the modified ellipsoidal formula [tumor volume = $1/2 \times (\text{length} \times \text{width}^2)$]. Animals were sacrificed using CO_2 once the tumor volume reached 2,000 mm^3 .

Statistical analysis. Results are presented as the mean \pm SEM of at least three independent experiments. Significance was tested by one-way ANOVA followed by Tukey test for multiple groups comparison, unpaired t-tests when only two groups are compared or two-way ANOVA followed by Bonferroni test when different drug concentrations in different cell types are compared. $P \leq 0.05$ was considered to indicate a statistically significant difference. The *in vivo* survival graphs were generated using the Kaplan-Meier method, with P-values determined using the log-rank test.

Results and discussion

Melatonin is toxic specifically in AML cells carrying the FLT3-ITD mutation. Melatonin has exhibited antitumoral properties in various hematological malignancies, including AML (8). However, none of these studies have analyzed in detail the effects of melatonin on AML in relation to prognostic factors. Several gene mutations have been identified as leukemia risk factors, with FLT3 the most frequent site of genetic alterations associated with poor disease outcome (3). Two wt AML cell lines (OCI-AML3 and U-937) and two FLT3-ITD AML cell lines (MV-4-11 and MOLM-13) were incubated with melatonin (0.1-1 mM) for 48 h, and the proportion of viable cells was determined using an MTT assay (Fig. 1A). Melatonin induced a dose-dependent decrease in cell viability in all cell lines, although it exhibited a greater effect in FLT3-ITD mutant cells.

Melatonin can decrease the proportion of viable cells by inhibiting proliferation or inducing cell death, and the MTT assay does not allow to distinguish between these phenomena. PI staining (Fig. 1B), Annexin V staining (Fig. 1C) and Caspase-3 activity (Fig. 1D) assays indicated that melatonin only induces cell death in FLT3-ITD mutant cells, and that it kills these particular AML cells by apoptosis.

The antitumoral activity of melatonin was further evaluated in a MOLM-13 xenograft model *in vivo*. A significant decrease in tumor volume was observed upon treatment with melatonin, starting at day 15 after tumor implantation (Fig. 2A). Moreover, the antitumoral activity of melatonin in MOLM-13 FLT3-ITD cells was associated with a significant increase in survival time (Fig. 2B), with an average survival of 24 ± 0.8 days in the control group and 31 ± 2.4 days in the melatonin-treated group. Altogether, the present findings indicated that melatonin specifically kills FLT3-ITD AML cells.

Melatonin cytotoxicity is associated with reliance on the Warburg effect in AML cells. Tumor cells can obtain their energy from oxidative phosphorylation, in the same manner as physiological cells, or they can exhibit a metabolic adaptation via which they metabolize glucose fundamentally to lactate

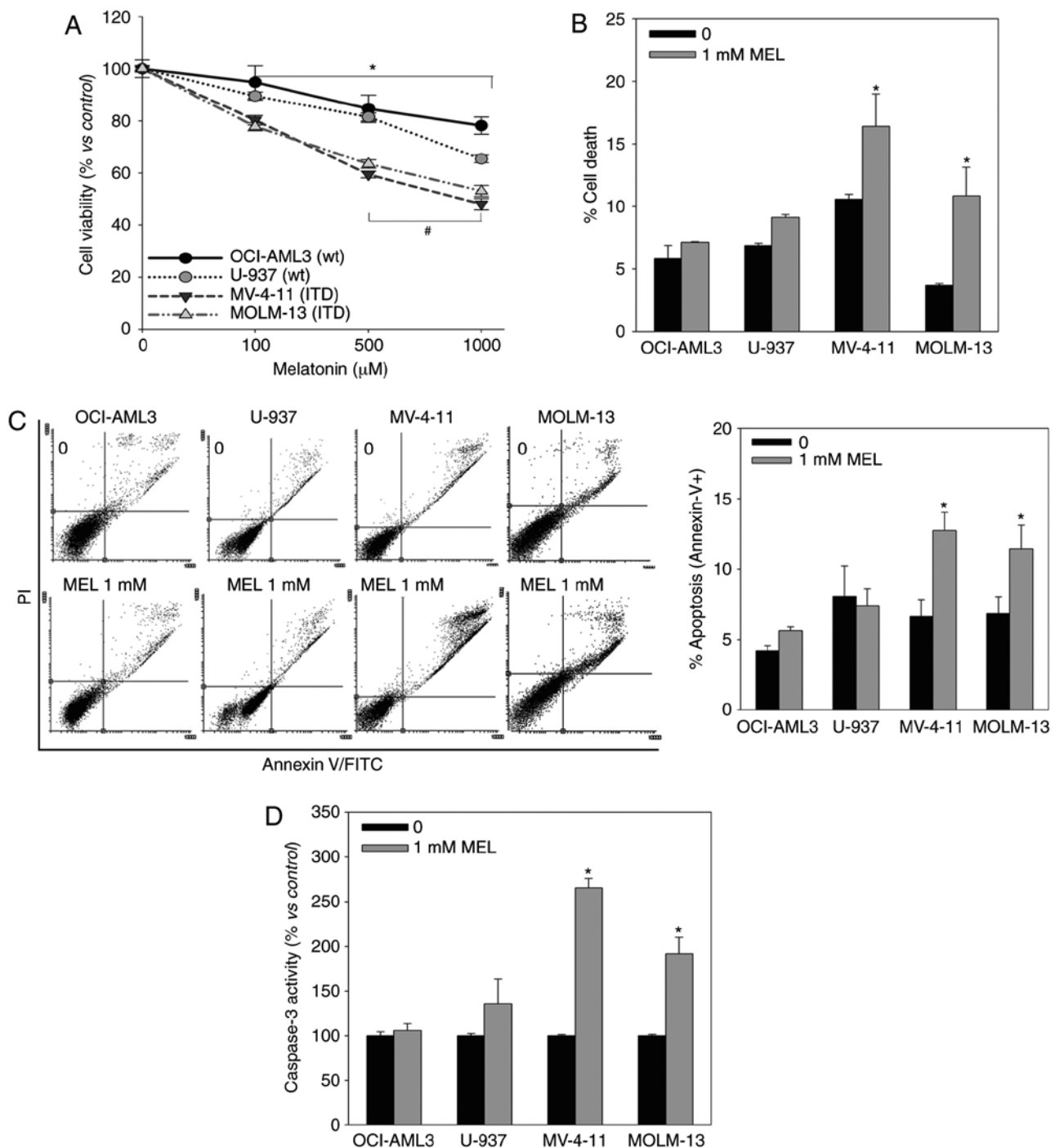


Figure 1. Melatonin is toxic specifically in AML cells carrying the FLT3-ITD mutation. (A) Four AML cell lines (2 wt and 2 FLT3-ITD) were treated with melatonin (100-1,000 μM) for 48 h, and the proportion of viable cells was determined using MTT reduction assays. Data were analyzed using two-way ANOVA followed by Bonferroni test. * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. wt cells. (B) Cell death determined by PI staining after treatment with 1 mM melatonin for 48 h. (C) Effect of 1 mM melatonin (24 h) on the apoptosis of AML cell lines, as indicated by Annexin V staining. A representative flow cytometry plot for each cell line is represented in the left panel; the percentage of apoptosis (late + early apoptosis) is represented in the right panel. (D) Effect of 1 mM melatonin (24 h) on caspase-3 activity in AML cell lines. * $P < 0.05$ vs. vehicle (t-test). AML, acute myeloid leukemia; FLT3-ITD, FMS-like tyrosine kinase 3 internal tandem duplication; MEL, melatonin; wt, wild-type; PI, propidium iodide.

even in the presence of oxygen (the Warburg effect) (13). Our previous study demonstrated that the specificity of melatonin toxicity in certain Ewing's sarcoma cell lines was related to their metabolic profiles, namely whether their glucose metabolism relies on oxidative phosphorylation or the Warburg effect (11).

As previously described for Ewing sarcoma cells, FLT3-ITD AML cells presented a higher dependency on

aerobic glycolysis (Fig. 3A and B). Inhibition of hexokinase II by means of 3-BrPA (5 μM) incubation for 48 h resulted in a higher induction of cell death in MV-4-11 and MOLM-13 mutant cells than U-937 and OCI-AML3 wt cells (Fig. 3A). Conversely, inhibition of mitochondrial complex I by incubation with rotenone (500 nM) for 48 h had less effect on the viability of FLT3-ITD cells compared with wt cells (Fig. 3B). These results indicated that the FLT3-ITD mutation renders

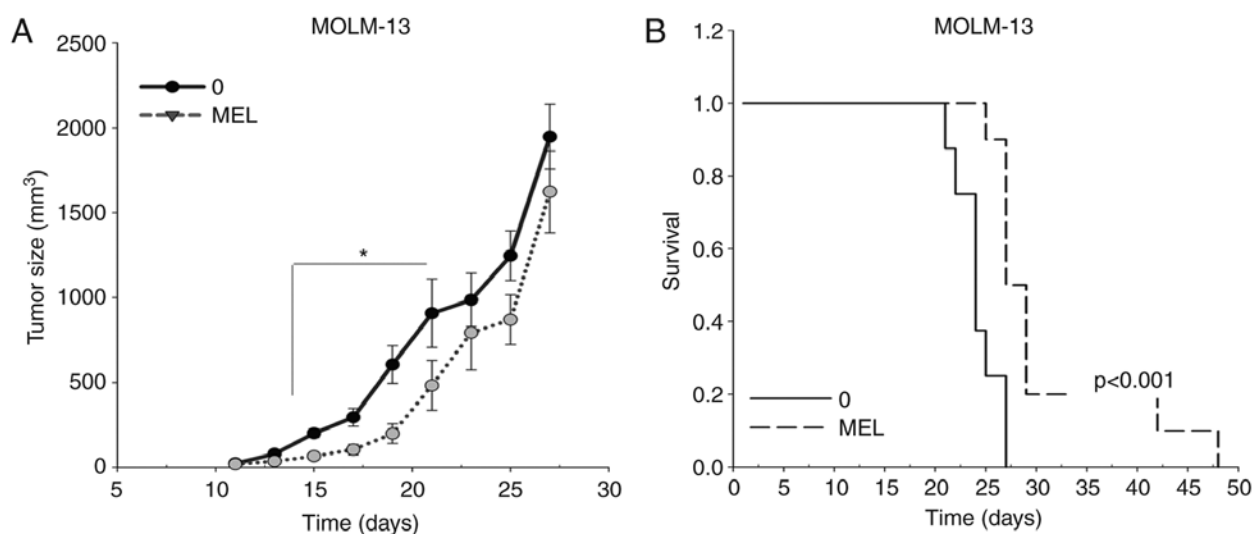


Figure 2. Melatonin decreases tumor growth and increases animal survival in FLT3-ITD animal models. (A) Tumor size following subcutaneous injection and (B) animal survival following tail vein injection with MOLM-13 mutant cells were evaluated over time in CB-17 SCID mice. Melatonin administration via the drinking water (10 mM) was initiated 10 days after tumor cell injection. * $P < 0.05$ vs. vehicle (t-test) at the highlighted time points. MEL, melatonin.

AML cells more dependent on the Warburg effect than on oxidative phosphorylation as their energy supply, consistent with a previous independent study (5).

Melatonin inhibits key signaling and metabolic pathways controlling aerobic glycolysis only in FLT-ITD AML cells. The Warburg effect is characterized by an increased uptake of glucose, which in turn is rapidly metabolized to lactate instead of entering the Krebs cycle and undergoing oxidative phosphorylation (13). Melatonin induced a significant decrease in glucose uptake (measured using the fluorescent D-glucose analogue 2-NBDG) only in FLT3-ITD cells after 8 h of incubation (Fig. 3C). Additionally, LDH activity (Fig. 3D) and intracellular lactate levels (Fig. 3E) were significantly reduced in mutant cells after treatment with melatonin for 24 h, while no changes in any of these two parameters were observed in U-937 and OCI-AML3 wt cells. These results indicated that FLT3-ITD cells relied more on aerobic glycolysis to obtain energy than non-mutant AML cells, suggesting that melatonin partially blocks aerobic glycolysis in these cells.

Differences found in the effects of melatonin on wt cells compared with mutant cells suggested the possibility that melatonin regulated receptor-mediated intracellular pathways. This signaling includes activation of downstream prosurvival effectors such as JAK/STAT5 and PI3K/AKT/mTOR (14); previous studies have shown that melatonin regulates this pathway in other types of tumor (15,16). Western blot analysis demonstrated that melatonin inhibits these pathways as determined by decreased levels of p-STAT5, p-AKT and p-mTOR/p-S6 only in FLT3-ITD cells (Fig. 4A). As a role for FLT3-ITD signaling in the shift of glucose metabolism from oxidative phosphorylation towards aerobic glycolysis has been recently described (5), the regulation of this intracellular signaling by melatonin could be responsible for its effects on glucose metabolism in FLT3-ITD cells. Supporting this hypothesis, treatment of mutant cells with the FLT3-ITD inhibitor EC-70124 (10 nM) (17) resulted in a decrease in

glucose uptake after 8 h of treatment, and LDH activity and lactate production after 24 h of treatment (Fig. 4B), similar to the effects of melatonin.

Although several intracellular pathways can regulate aerobic glycolysis, transcription factor HIF1 α has been described as the master regulator of the Warburg effect (18). In normal cells, this transcription factor is regulated by oxygen levels and activated under hypoxia; however, in tumor cells, HIF1 α can be activated by oncogenic signals independently of oxygen levels, such as the PI3K/AKT/mTOR signaling pathway (19). As shown in Fig. 5A, melatonin induced a significant increase in the hydroxylated (inactive) form of HIF1 α in MV-4-11 and MOLM-13 FLT3-ITD, cells while no changes were found in OCI-AML3 wt cells. The inhibition of the PI3K/AKT/mTOR pathway observed in FLT3-ITD cells is consistent with the inactivation of HIF1 α .

HIF1 α regulates a large number of enzymes involved in aerobic glycolysis (18). However, evaluation of the expression levels of various genes involved in glucose metabolism using a reverse transcription-PCR array showed that melatonin only induced a significant decrease in DLD mRNA levels, and an increase in H6PD and PCK2 mRNA levels in FLT3-ITD AML cells (Fig. 5B). These results were confirmed by western blotting with antibodies against these proteins in the presence or absence of melatonin (Fig. 5C). These results indicated that melatonin altered tumor glucose metabolism in AML mutant cells, potentially in search of new routes to obtain energy and intermediate metabolites necessary for survival and proliferation. Consistent with this, it has been reported that, at least in tumor cells, the activity of H6PD could contribute to the complete conversion of glucose into water and CO₂ at the endoplasmic reticulum (ER) (20). Moreover, H6PD produces NADPH in the ER lumen, which constitutes a cofactor for various reducing enzymes and serves as the ultimate donor of reductive power for most reactive oxygen species (ROS)-detoxifying enzymes (21). As melatonin induction of cell death in numerous cancer cells, including AML cells, has been associated with an increase

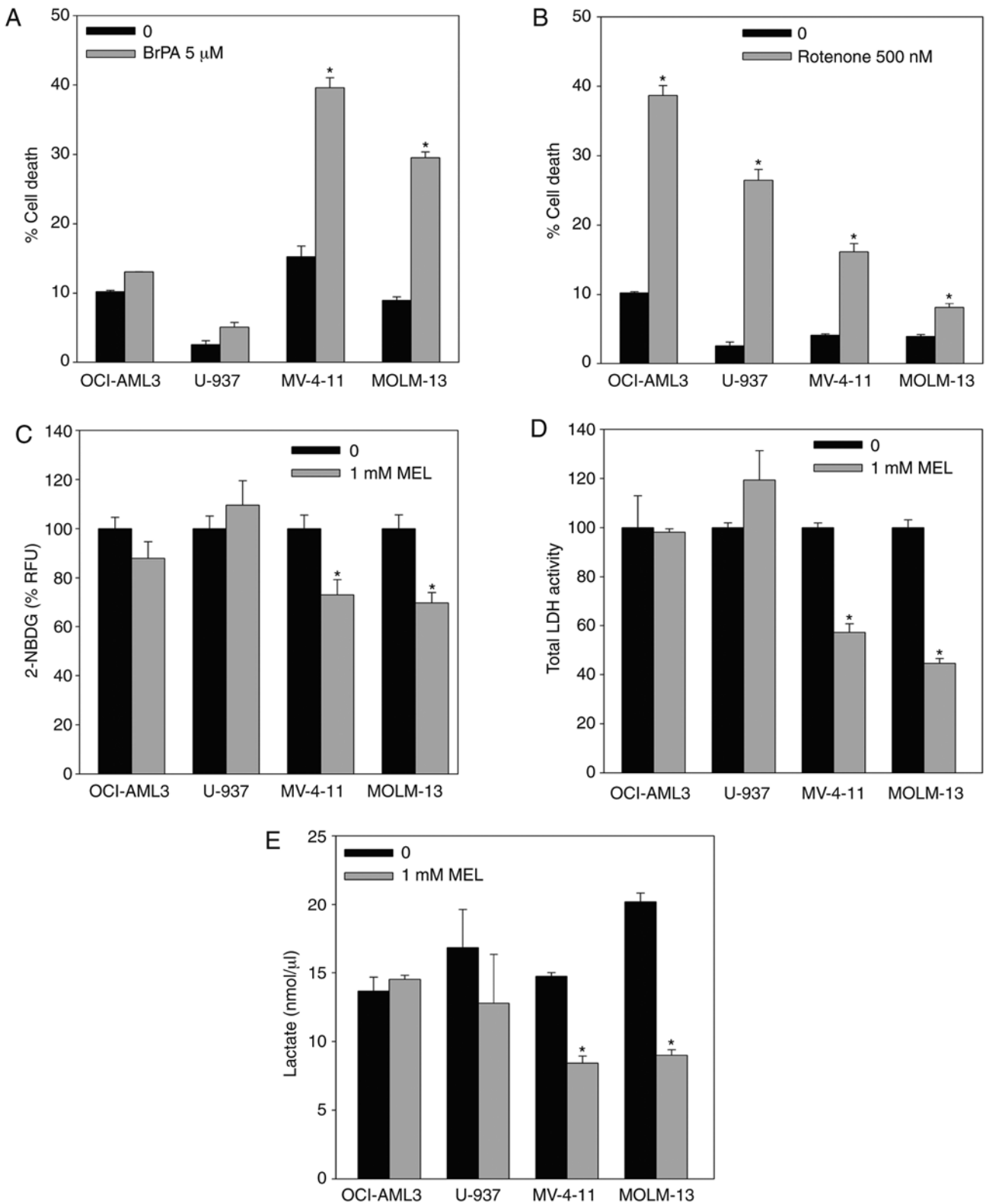


Figure 3. Melatonin inhibits aerobic glycolysis in FLT3-ITD AML cells. (A) Cell viability was evaluated by PI staining after the incubation of FLT3-ITD and wild-type AML cells with 3-BrPA (5 μ M) for 48 h. (B) Cell viability evaluated by PI staining after the incubation of AML cells with rotenone (500 nM) for 48 h. (C) Glucose uptake was measured in AML cells treated with or without 1 mM melatonin for 8 h. Data are expressed as RFU. (D) LDH activity was evaluated in AML cells incubated with 1 mM melatonin for 24 h, and results were normalized to the protein content (μ g) in each sample. (E) Lactate levels (nmol/ μ l) were quantified in AML cells treated with 1 mM melatonin for 24 h. *P<0.05 vs. vehicle (t-test). AML, acute myeloid leukemia; FLT3-ITD, FMS-like tyrosine kinase 3 internal tandem duplication; MEL, melatonin; 3-BrPA, 3-bromopyruvate; PI, propidium iodide; LDH, lactate dehydrogenase; RFU, relative fluorescence units.

in intracellular ROS (22), the increased expression of this enzyme could be related to an attempt to maintain controlled levels of oxidative stress.

Conversely, DLD functions as the E3 component of the pyruvate dehydrogenase and α -ketoglutarate dehydrogenase complexes, both of which form part of the tricarboxylic acid

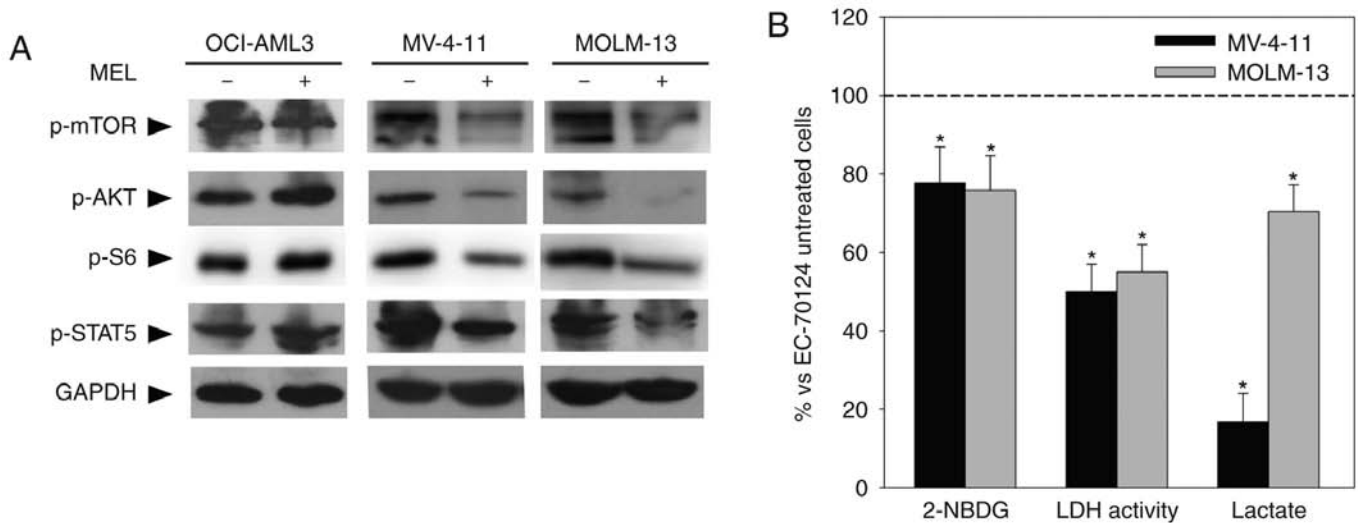


Figure 4. Melatonin inhibits key signaling pathways only in FLT3-ITD-positive AML cells. (A) Western blot analyses were conducted to identify the effect of melatonin (1 mM, 8 h) on the levels of p-mTOR, p-AKT, p-S6 and p-STAT5 in AML cells; GAPDH was used as a loading control. (B) Effects of FLT3-ITD inhibitor EC-70124 (10 nM) on glucose uptake after 8 h treatment, and on LDH activity and lactate levels after 24 h treatment in mutant cells. * $P < 0.05$ vs. vehicle (t-test). AML, acute myeloid leukemia; FLT3-ITD, FMS-like tyrosine kinase 3 internal tandem duplication; MEL, melatonin; p, phosphorylated; LDH, lactate dehydrogenase.

(TCA) cycle (23). Of note, low levels of α -ketoglutarate can activate HIF1 α (19). Thus, a decrease in DLD levels and subsequent inhibition of the α -ketoglutarate dehydrogenase complex could account for α -ketoglutarate accumulation and be related to the observed inactivation of HIF1 α . Moreover, both enzymes are essential not only for TCA cycle progression but also for the incorporation of TCA intermediates that come from other sources of carbon, particularly glutamine and gluconeogenic amino acids (24). In this way, TCA flux is tightly regulated by the process of replenishing (anaplerosis) and removal (cataplerosis) of TCA intermediates (24). Thus, decreased DLD expression induced by melatonin could result not only in an alteration of progression through the TCA cycle, but also in an imbalance between anaplerosis and cataplerosis. PCK2 catalyzes the first step and limiting step of gluconeogenesis, the main process responsible for cataplerosis that is required to shuttle glycolytic intermediates into biosynthetic pathways (25). Although studies of this metabolic pathway in tumors are not numerous, PCK2 appears to have a dichotomous role depending on the tumor type, so that it favors survival in tumors that express high levels of the enzyme, while it is capable of promoting cell death in those that express low or undetectable levels of the enzyme (26). Thus, in hepatocarcinoma or renal cell carcinoma, which express low levels of PCK, metabolic reprogramming with activation of this enzyme has been reported to induce tumor death by creating a 'futile' cycle between glycolysis and gluconeogenesis that induces metabolic stress in the cells leading to the induction of apoptotic cell death (27,28). Of note, melatonin has been described to be deleterious for both cancer types (29,30).

Evaluation of basal levels of expression of these proteins revealed that AML wt cells presented low expression of DLD and high expression of PCK2, contrary to FLT3-ITD cells that exhibited high expression of DLD and low levels of PCK2 (Fig. 5D). No changes in basal expression of H6PD were reported. Differences in the basal levels of

expression of DLD and PCK2 appear to indicate that wt and mutant cells exhibit distinct anaplerosis/cataplerosis balances. Melatonin treatment in mutant cells modified the equilibrium, increasing PCK2 expression. The induction of the enzyme by melatonin could be responsible for cell death, as occurs in hepatocarcinoma, where the induction of PCK expression in the absence of glucose leads to the death of tumor cells (27). In fact, in the present study, treatment with melatonin induced both reduced glucose uptake and PCK2 induction. To evaluate this hypothesis, cells were treated with the PCK2 inhibitor 3-MP (250 μ M) along with a glucose supplement (10 mM). As shown in Fig. 5E, the combination of 3-MP and glucose was able to prevent, at least partially, the cytotoxic effect of melatonin on the FLT3-ITD mutant MV-4-11 cells after 48 h of treatment, without any effect on the wt OCI-AML3 cells, which indicated that the overexpression of PCK2 would be involved in the effects of melatonin in these cells.

Blockage of glycolytic metabolism currently constitutes a major target to prevent cancer growth (31,32), and glycolysis inhibitors exhibited antitumoral effects on numerous acute leukemia subtypes, including AML (33). The present findings indicated a differential effect of melatonin dependent on the presence or absence of the FLT3-ITD mutation in AML cells. Thus, melatonin induced cell death in relation to the inhibition of aerobic glycolysis in FLT3-ITD AML cells that are more dependent on this metabolic pathway than wt cells. A recent study suggested that FLT3/ITD promotes aerobic glycolysis in an AKT-dependent manner (5). The present study indicated that melatonin is able to inhibit not only AKT but also other FLT3/ITD downstream targets such as STAT5 only in mutant cells. Collectively, these findings suggested that melatonin selectivity may be related to the ability to inhibit FLT3/ITD downstream signaling. Moreover, treatment of cells with an FLT3/ITD inhibitor induced similar effects on glucose metabolism to melatonin, reinforcing the idea of FLT3/ITD

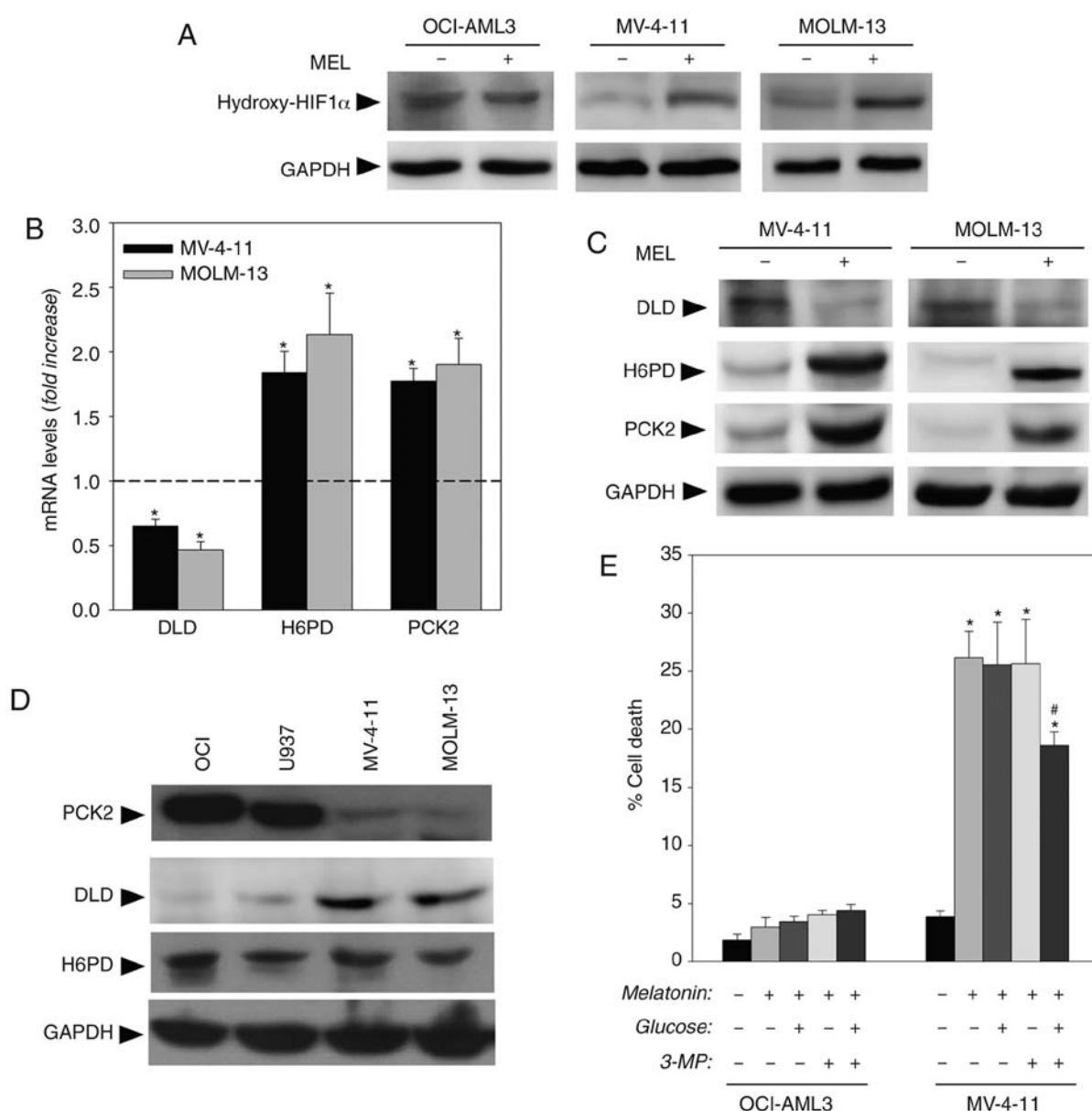


Figure 5. Melatonin regulates the expression of glucose-related genes and proteins. (A) Western blot analyses were conducted to identify the effect of melatonin (1 mM, 8 h) on the activation of HIF1 α using a specific antibody against the inactive hydroxylated form. (B) mRNA levels of glucose metabolism-related genes in AML cell lines after melatonin treatment (1 mM, 8 h) as determined by reverse transcription-quantitative PCR. The dashed line represents basal levels of expression. Data were analyzed using one-way ANOVA followed by Tukey test. * $P < 0.05$ vs. vehicle. (C) mRNA changes in AML cells were validated at the protein level via western blot analysis. (D) Basal levels of protein expression in wild-type and mutant cells. GAPDH was used as a loading control for all assays. (E) Effect of the PCK2 inhibitor 3-MP (250 μ M) and glucose supplementation (10 mM) on the melatonin-induced cell death as determined by propidium iodide staining 48 h after treatments. Data were analyzed using one-way ANOVA followed by Tukey test. * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. melatonin only. AML, acute myeloid leukemia; MEL, melatonin; HIF1 α , hypoxia-inducible factor-1 α ; PCK2, phosphoenolpyruvate carboxykinase 2; DLD, dihydroliipoamide dehydrogenase; H6PD, hexose-6-phosphate dehydrogenase; 3-MP, 3-mercaptopicolinic acid.

downstream signaling inhibition as responsible for melatonin selectivity. However, as melatonin has not been described to exhibit kinase inhibitor activity directly, further studies are required needed to clarify the exact mechanism by which melatonin is able to regulate these kinases. Nevertheless, the present findings in AML cells are consistent with our previous observations in sarcoma cell lines, where melatonin only killed those dependent on the Warburg effect (11), as well as the already described regulation of glucose metabolism in prostate cancer cells (34). Data suggested that glucose uptake and metabolism could be a major target of the indole in cancer cells. Collectively, the results suggested that melatonin not only

limited the aerobic glycolysis of FLT3-ITD AML cells but also altered the anaplerosis/cataplerosis equilibrium, inducing metabolic stress in FLT3-ITD AML cells that can induce apoptotic cell death. Melatonin should be further evaluated as a possible therapeutic tool for cancer types relying on the Warburg effect in general, particularly those that present low levels of gluconeogenesis, and for those patients with AML carrying the deleterious FLT3-ITD mutation in particular.

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Availability of data and materials

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

Authors' contribution

NPM, AMSS, CDO and MTC performed laboratory experiments. IA, FH and JRB conceived and designed the study, and interpreted the data. VM and CR contributed to the conception and design of the study, interpretation of the data and writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal research protocols were approved by the Animal Research Ethical Committee of the University of Oviedo.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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