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Inhibition of ATP hydrolysis as a key regulator of temozolomide resistance and migratory phenotype of glioblastoma cells

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ABSTRACT

Glioblastoma (GBM) is the most lethal among malignant gliomas. The tumor invasiveness and therapy-resistance are important clinical hallmarks. Growing evidence emphasizes the purinergic signaling contributing to tumor growth. Here we exposed a potential role of extracellular ATPase activity as a key regulator of temozolomide cytotoxicity and the migration process in GBM cells. The inhibition of ATP hydrolysis was able to improve the impact of temozolomide, causing arrest mainly in S and G2 phases of the cell cycle, leading M059J and U251 cells to apoptosis. In addition to eradicating GBM cells, ATP hydrolysis exhibited a potential to modulate the invasive phenotype and the expression of proteins involved in cell migration and epithelial-to-mesenchymal-like transition in a 3D culture model. Finally, we suggest the ATPase activity as a key target to decline temozolomide resistance and the migratory phenotype in GBM cells.

1. Introduction

Malignant gliomas are a challenge in the cancer scenery. The tumor aggressiveness has been attributed to signaling pathways influencing invasive behaviors into the tumor microenvironment (TME) [1]. Glioblastoma (GBM) is the most lethal subtype of malignant glioma and its high invasiveness makes complicated the treatment [2]. The available therapy considering surgical resection followed by radiotherapy and temozolomide is still very limited in confronting the recurrence rates [3,4].

Tumors interact closely with the surrounding microenvironment [5]. Growing evidence provides the importance of nucleotides and nucleosides signaling in the extracellular environment [6–8]. ATP is released to the extracellular space in response to cellular stress and acts in an antitumor manner by binding to P2 receptors. However, its biological activities are limited by the presence of ectoenzymes [9]. The ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) is the main family of ectoenzymes that initiates the cascade of nucleotide hydrolysis. The NTPDase1 (CD39) mostly hydrolyze ATP to adenosine diphosphate (ADP) and subsequently to adenosine monophosphate (AMP), which is finally converted to adenosine, by the action of CD73 [10]. Adenosine exhibits opposite actions in relation to ATP, exerting pro-tumor effects such as restriction of immune cell infiltration, induction of resistance mechanisms and impairment of cell death in the tumor microenvironment (TME) [7].

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The modulation of E-NTPDases, favoring high amount of extracellular ATP (eATP), has been related to decrease tumor-promoting functions [11–13]. The inhibition of ectoenzymes and subsequently adenosine generation showed to prevent invasiveness and migration in breast cancer cell lines [14,15]. Similarly, researchers have reported increased liver metastases in colorectal tumor-bearing mice overexpressing CD39. At the same time, the use of E-NTPDase inhibitor sodium polyoxotungstate (POM-1) showed to reduced metastatic spread in some tumor models [16,17].

Since it is known that the ATPase activity, favoring adenosine generation has been involved in tumor-promoting functions, here we presented a potential role of extracellular ATP hydrolysis as a key regulator of temozolomide cytotoxicity and migration process in GBM cells.

2. Materials and methods

2.1. Chemicals

ATP, Malachite Green, Coomassie Blue, and temozolomide were purchased from Sigma Aldrich. Sodium polyoxometalate (POM-1, $Na_6O_{39}W_{12}\bullet H_2O$) (inhibitor of E-NTPDases CD39, CD39L1, CD39L3) was purchased from Santa Cruz Biotechnology. All culture materials were obtained from Gibco Laboratories (Grand Island, NY, USA).

2.2. Cell culture

Human GBM cell lines M059J and U251 are from American Tissue Culture Collection (ATCC, Rockville, MD). Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml), and amphotericin B (100 g/ml), under humidified conditions (5% CO_2 at 37 °C).

2.3. Gene expression

The expression of genes listed in Table 1 were determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), utilizing SYBR® Green I (Invitrogen) on the 7500 Real-time PCR System (Applied Biosystems, CA, EUA). The reference gene ribosomal protein lateral stalk subunit P0 (*RPLP0*) or β-2-microglobulin (*B2M*) was used for normalization. Relative mRNA expression levels were revealed using the $2^{\Delta\Delta Ct}$ method [18].

2.4. Cytotoxicity

The compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (Cell Titer 96 AQueous One Solution Reagent, Promega, USA) was applied to measure cell viability of M059J and U251 (3×10^4 per well in 96-well plate) treated with ATP (1, 3, and 5 mM), POM-1 (100 μM), or temozolomide (100 μM) for 24 h, according to the manufacturer's protocol [19]. Cell viability was expressed as a percentage compared to control.

2.5. Cell cycle and apoptosis assay

M059J and U251 cells were treated with temozolomide (100 μM) and/or POM-1 (100 μM) for 24 h. After, the medium was centrifuged at 400×*g* for 6 min and cell pellet was resuspended in staining solution (0.5 mM Tris-HCl at pH 7.6, 3.5 mM trisodium citrate, 0.1% nonidet 40 (v/v), 100 μg ml^{-1} RNase, and 50 μg ml^{-1} PI). The cell cycle and apoptosis analysis were performed in cells suspended in buffer containing Annexin V-APC and 7AAD using a flow cytometer FACSCalibur (BD Bioscience, San Jose, CA, USA). The results were analyzed by the FlowJo® 7.6.5 software.

2.6. Cell migration in a 3D cell culture model

Spheroids were formed spontaneously by depositing drops of U251 cells (5 x 10^3 cells/20 μl) transfected with green fluorescent protein (GFP) onto the bottom of the lid in a 48-wells plate, as previously described [20,21]. Spheroids were treated (n = 4 per group) with temozolomide (100 μM) and/or POM-1 (50 μM). Fluorescent images were captured using a camera coupled in IX-71 Olympus® inverted microscope (4x objective) starting from $t = 0$–72 h. The area covered by the spheroids were measured, normalizing data to the original size of each spheroid recorded at t = 0, using Image-Pro Plus software [formula: (migrated area at t = x/migrated area at t = 0) x 100] [22].

2.7. Morphological analysis

The morphology of single migratory cells (n = 180) was analyzed in images derived from 72 h of cell migration assay. Using Image-Pro Plus, we applied a mask to capture cells in the middle space between the spheroid core and periphery of migrated area. We measured radius ratio (the ratio between Max Radius and Min Radius) of each cell, considering values < 2 as non-migratory cell (epithelial-like phenotype) and values > 2 as migrating cell (mesenchymal-like phenotype). The analysis of roundness and perimeter of spheroid core was performed also using Image-Pro Plus, where circular objects present roundness = 1, indicative of non-deformed core, and other shapes present roundness >1; and more deformed core will present the largest perimeter.

2.8. Statistical analysis

All results were expressed as mean ± standard deviation (SD) from at least three independent experiments. Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by Tukey's post hoc test, using GraphPad Software 5.0 (San Diego, CA, U.S.A.). Statistical significance was deemed if $p < 0.05$.

Table 1
Primer sequences for gene expression analysis.

Gene	Foward primer	Reverse primer
MMP1	5′-GGA AAG ATG GGG TGG CGA C-3′	5′-GGT ACC TGT ACC CCT TGG TC-3′
MMP2	5′-CCC AGC GAC TCT AGA AAC ACA-3′	5′-GGG CCA CTA TTT CTC CGC TT-3′
RACK1	5′-GAG TGT GGC CTT CTC CTC TG-3′	5′-GCT TGC AGT TAG CCA GGT TC-3′
CD274 (PD-L1)	5′-AAA TGG AAC CTG GCG AAA GC-3′	5′-GAT GAG CCC CTC AGG CAT TT-3′
RPLP0	5′-CAG CAA GTG GGA AGG TGT AAT CC-3′	5′-CCC ATT CTA TCA TCA ACG GGT ACA A-3′
B2M	5′-ACT GAA TTC ACC CCC ACT GA-3′	5′-CCT CCA TGA TGC TGC TTA CA-3′

B2M: β-2-microglobulin; MMP1: metalloproteinase 1; MMP2: metalloproteinase 2; PD-L1: programmed cell death ligand-1; RACK1: receptor for activated C kinase 1; RPLP0: ribosomal protein lateral stalk subunit P0.

3. Results

3.1. ATP hydrolysis inhibition enhances temozolomide effect in non-sensible GBM cells

Initially, we confirmed the capacity of POM-1 to inhibit ATP hydrolysis. POM-1 was able to reduce cell viability and to improve ATP-mediated cytotoxicity in both M059J and U251 cell lines and in a patient-derived GBM culture (LS12) (Supplementary Fig. S1). Next, to gain insight about the effect of ATP hydrolysis on temozolomide cytotoxicity, M059J and U251 cells were treated with POM-1 and/or temozolomide for 24 h (Fig. 1). As expected, the chemotherapy isolated did not induce any effect on viability in both M059J (Fig. 1A) and U251 cells (Fig. 1B). Surprisingly, the POM-1 improved the temozolomide effect reducing the cell viability by 42% ($p < 0.001$) and causing arrest in the cell cycle in the GBM cells (Fig. 1C and D). Temozolomide affected cell cycle in M059J cells more than POM-1 (Fig. 1C); whereas both temozolomide and POM-1, as single agents or in co-treatment, interfere on cell cycle progression of U251, increasing cells in S and G2 phases (Fig. 1D). Specifically, POM-1 improved the percentual of early apoptosis (18.1%, $p < 0.001$), late apoptosis (27%, $p < 0.001$), and necrosis (10% $p < 0.001$) compared to control (1%, 4% and 2%) in M059J (Fig. 1E); the co-treatment showed differences in relation to temozolomide as a single agent causing early apoptosis (17% $p < 0.01$), late apoptosis (17% $p < 0.01$), and necrosis (9% $p < 0.05$), suggesting that the effect on cell apoptosis is probably derived from the POM-1 (Fig. 1E). A similar profile was observed for the U251, 25% ($p < 0.001$) of early apoptosis, 36% ($p < 0.001$) of late apoptosis, much higher than the control group (6% and 2%) (Fig. 1F); however, necrosis does not have been triggered in these cells. Effect of POM-1 on co-treatment was evidenced by the increase in late apoptosis (43% $p < 0.001$) when compared to temozolomide alone (5%) (Fig. 1F).

3.2. Extracellular ATP modulates migration on a 3D cell culture model

As shown, both M059J and U251 are impacted by temozolomide and ATP hydrolysis inhibition in a similar way, hence we choose to follow the next experiments only with U251 cells. The onset of migration in U251 GFP cells was observed before 24 h and a continuing increase in the migrated area, out of the tumor core, was observed in all treatments (Fig. 2A). At 72 h, the migrated area by cells treated with POM-1 was significantly smaller (59% of the migrated area) when compared to the control group. Besides, the co-treatment (temozolomide plus POM-1) showed a significant reduction of 34% in the migrated area when compared to temozolomide alone (Fig. 2B). In addition, cells were distinct in morphology and density. Interestingly, the POM-1 group showed a less deformed tumor core, indicating a reduced number of cells leaving the core (Fig. 2C). The roundness and perimeter of the spheroid core were reduced in POM-1 groups, which are implicated in a less invasive phenotype (Fig. 2D and E).

3.3. Modulation of ATP hydrolysis alters GBM cells phenotype and migration-related genes expression

ATPase activity inhibition can cause changes in the phenotype of GBM cells by reducing the migratory profile, when compared to the control group (Fig. 3A and B). The chemotherapy enhanced the population of GBM cells presenting a mesenchymal-like phenotype (radius ratio 3.2), while POM-1 and the co-treatment with temozolomide were able to minimize migratory profile (radius ratio 2.3) (Fig. 3C and D).

Differences in cell migration are also related to the expression of proteases that degrade the extracellular matrix or even enzymes related to epithelial-to-mesenchymal-like transition- (EMT-like) and other migratory pathways [23]. Here we evaluated the expression of MMP1 and 2 (Fig. 3E and F), as well as the expression of RACK1 (Fig. 3G) and PD-L1 (Fig. 3H). Temozolomide significantly augmented MMP2 expression, while POM-1 reduced the metalloproteinases expression to undetectable levels. In addition to acting in collagen degradation, POM-1 was demonstrated to inhibit RACK1 and PD-L1 expression, impacting in the EMT-like.

4. Discussion

The adenosine pathway is a signaling route used by the glioma to increase immune evasion, migration process and therapy resistance [11,24,25]. In tumors, ATP is released to the extracellular space in response to cell damage and also after surgical tumor resection, chemo and radiotherapy [26,27]. Tumor cell killing by eATP has been described [28,29]. This outcome is mediated mainly by the P2X7 receptor (P2X7R) expressed by cancer cells. When activated, the P2X7R can be converted to a large nonselective transmembrane pore, leading to cell death [30].

Gliomas are proficient in adenosine generation, which is implicated in the quickly hydrolysis of eATP by ectoenzymes [9]. Once is relevant to inhibit this process and prevent protumor effects, the aim of this study was to modulate the ATPase activity, favoring high amount of eATP, to improve the temozolomide effect and reduce the migratory characteristics of GBM cells.

In fact, the chemotherapy has revealed to impact on nucleotide release [31]. Temozolomide is an alkylating agent used for GBM patients. The mechanism of action is described by arresting the G2 phase of the cell cycle, eventually leading to apoptosis of cancer cells [32]. However, intense temozolomide resistance has been described [33]. In this study, temozolomide exhibited no effect on glioma cells. The inhibition of ATPase activity was able to interfere in S and G2 phases of the cell cycle, expanding the temozolomide effect and leading GBM cells to apoptosis. Late apoptosis was related to POM-1 in this study. A study has already shown that a type of polyoxometalate was able to induce apoptosis in U251 cells more effectively than temozolomide [34]. Also, late apoptosis induced by polyoxometalates has been already described [35]. POM-1 acts generating oxygen reactive species [34] and in our study also showed to stop ATP hydrolysis probably increasing concentrations of this nucleotide in the extracellular space. In fact, researchers suggest that eATP is able to causing arrest of cell proliferation [30] and to stimulate the P2X7R transmembrane pore opening, which is implicated in cell membrane permeability [36]. Early apoptosis can turn into late apoptosis when the cell membrane becomes permeabilized [37].

In addition to eradicating tumor cells, an important part of GBM treatment is to minimize the invasiveness. Studies showed that even standard therapy can increase cell migration and invasion [33,38]. In our study, the inhibition of ATP hydrolysis showed to be a potent tool against GBM cell migration. Surprisingly, POM-1 reduced in 41% the migrated area in a 3D cell culture model. Furthermore, we observed differences in the spheroid core among groups of treatment. Control and temozolomide showed spheroid cores presenting a higher perimeter and roundness index when compared to POM-1, which are related to an invasive nature of GBM cells. In a very interesting manner, ATPase activity inhibition significantly reduced the movement of cells out of the spheroid. In fact, POM-1 had previously been described to have potent anti-metastatic activity. Yan et al. (2020) shown the significant effect of E-NTPDases modulation on reducing lung metastases in a renal carcinoma model [39]. Similarly, CD39-inhibited mice demonstrate

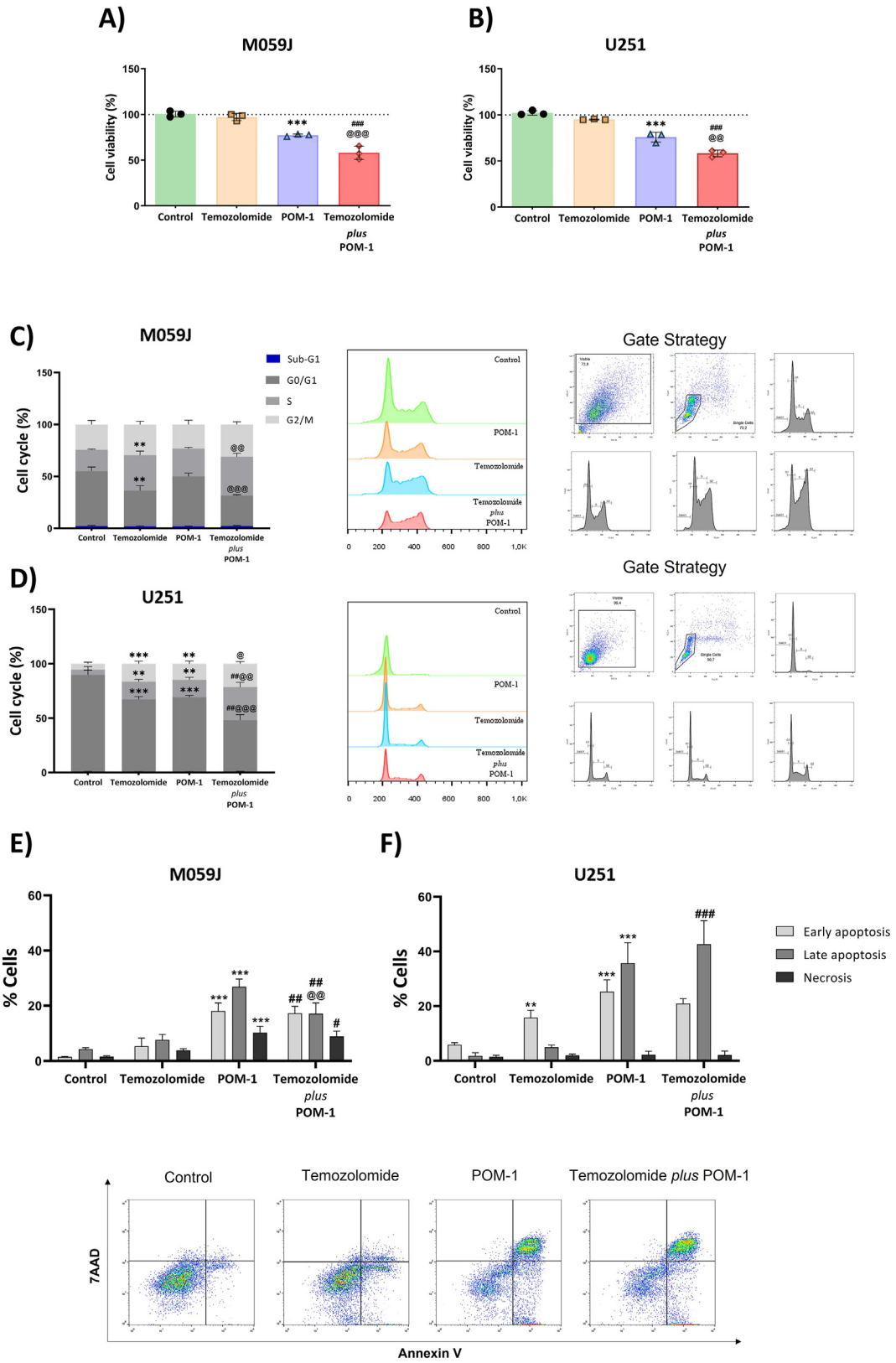

Fig. 1. Effects of ATPase activity in M059J and U251 cell lines. GBM cells were treated with temozolomide (100 μM) and/or POM-1 (100 μM) for 24 h. DMSO 0,1% (v/v) was used as a vehicle control. (A and B) Cell viability measured by MTS assay. (C and D) Histograms and quantitative cell cycle analysis by flow cytometry after staining with propidium iodide. (E and F) Cell death analyzed by flow cytometry after staining with Annexin V-APC and 7AAD. The graph shows the mean ± SD; * means difference from the control, # means difference from the temozolomide, and @ means difference from the POM-1 group. ***p < 0.001, **p < 0.01, *p < 0.05.

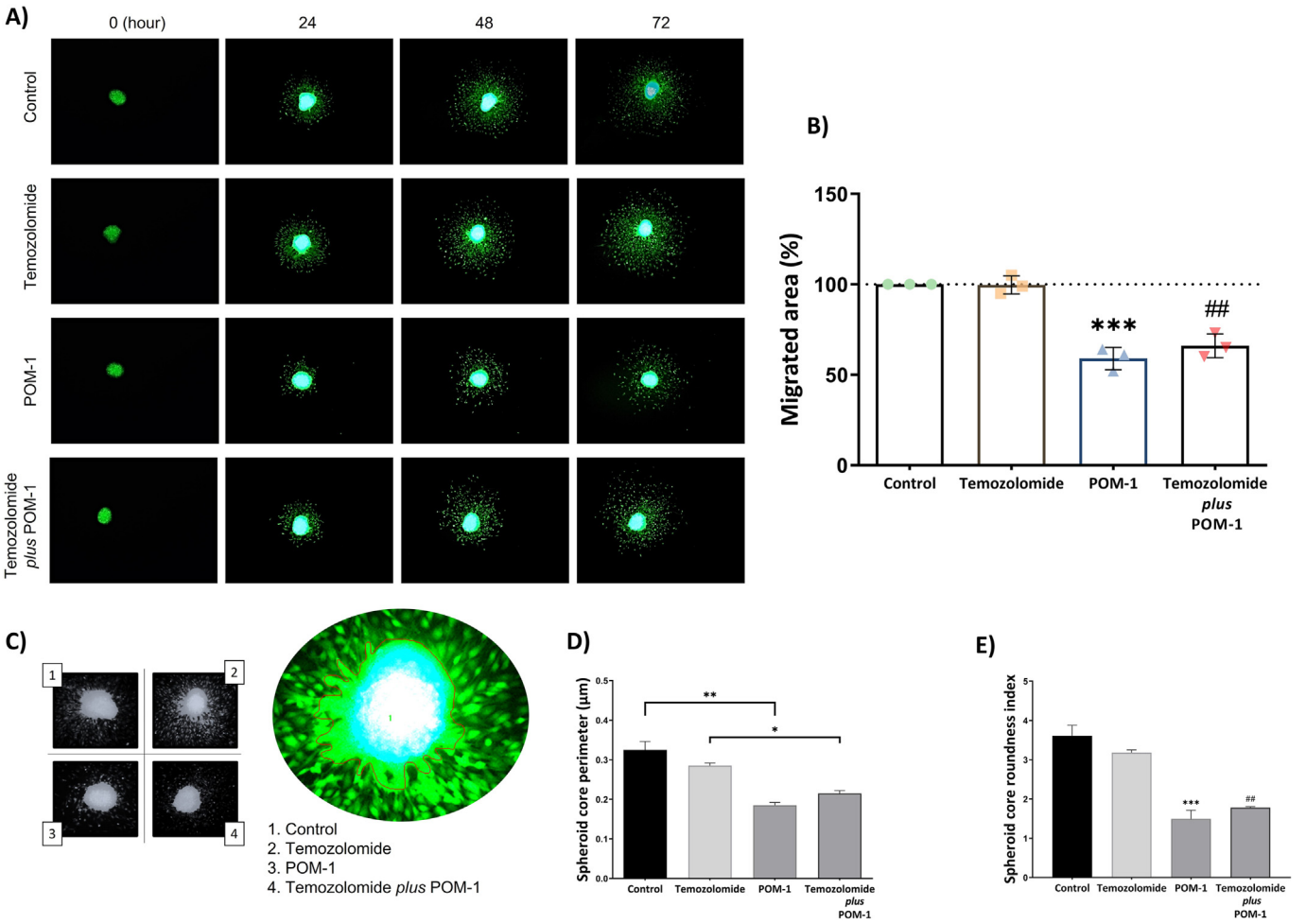

Fig. 2. Analysis of migratory profile of U251 GFP cells by Image Pro Plus. Spheroids (n = 4 per group) were treated with temozolomide (100 μM) and/or POM-1 (50 μM). DMSO 0,1% (v/v) was used as a vehicle control. (A) Representative images of cell migration at intervals starting from $t = 0–72$ h. (B) Percentual of migrated area for cells leaving the spheroid at 72 h. (C) Schematic representation of spheroid cores according to the treatment 1. Control; 2. Temozolomide; 3. POM-1; 4. Temozolomide plus POM-1. Analyses were performed by the automatic delimitation of spheroid core and measuring (D) perimeter (μm) and (E) index of roundness. The graph shows the mean ± SD; * means difference from the control, # means difference from the temozolomide, and @ means difference from the POM-1 group. ***p < 0.001, **p < 0.01, *p < 0.05.

to suppress spontaneous and experimental metastasis formation in different tumor models [13,40,41].

It is known that the cell migration is closely related to the EMT. In GBM, the induction of EMT-like is marked by changes in cell morphology, involving up-regulation of proteases that degrade the ECM and mesenchymal proteins (i.e. RACK1, MMPs, fibronectin, and vimentin) [42,43]. Evidence is mounting that EMT-like affects GBM growth and also confer GBM resistance to both chemo and radiotherapy [44]. The movement of GBM cells through the space in the brain needs to be arranged by disintegration and remodeling the ECM. Matrix metalloproteinases are responsible for the degradation of the majority of ECM proteins [45]. GBM cells express MMPs [46], which could be associated to improved GBM invasion and poor prognosis [47,48]. In our study, the mesenchymal morphology of cells treated with temozolomide seems to be in part related to MMPs expression. While temozolomide increased the expression of MMP2, POM-1 was able to reduce drastically the presence of MMP1 and 2 in GBM cells.

The ATP hydrolysis inhibition decreased RACK1 and PD-L1 expression. Several studies have demonstrated that RACK1 expression is associated with the EMT-like in cancer, including gliomas [49,50]. POM-1 also showed to influence in PD-L1 expression. PD-L1 was recently related to promoting U251 cells migration via EMT-like in a MEK/Erk dependent way [51]. In our study, temozolomide increased PD-L1 expression, while POM-1 drastically decreased it. Corroborating with us, Wang et al. (2019) pointed the improved PD-L1 expression in U87 and U251 GBM cells treated with temozolomide [52]. The close relationship between ATPase activity and PD-L1 needs to be better understood, however, putting together, these data suggest POM-1 is changing the migratory phenotype of GBM cells on different fronts, which could be an interesting strategy to reduce the invasiveness of this type of tumor.

In this study, the inhibition of ATP hydrolysis was able to induce cell death and improve the temozolomide effect, besides to minimize tumor movement by down-regulation of different genes related to the invasiveness and EMT-like process in a new way for GBM. More experiments are needed to define the underlying intracellular mechanisms triggered by E-NTPDases modulation. The reported findings presented insights about E-NTPDases as key ectoenzymes capable to modify GBM cells to a less invasive nature, then contributing against tumor resistance.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

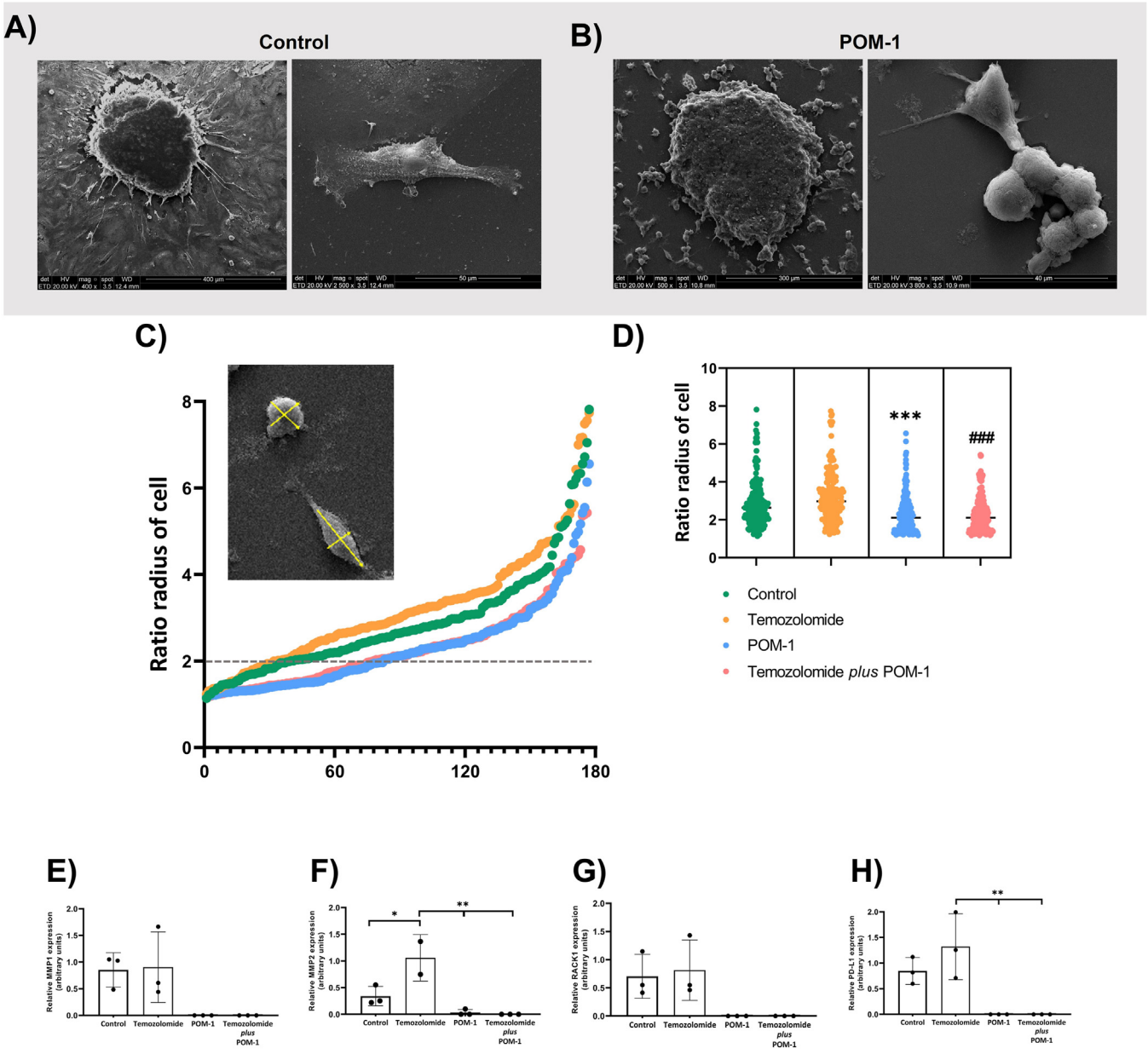

Fig. 3. Morphological analysis and gene expression of migratory U251 GFP cells. Representation of cell phenotypes by scanning electron microscopy in (A) control group and (B) POM-1 group. (C and D) Distribution graph of the ratio radius of cells. Radius ratio <2 indicates an epithelial-like phenotype and radius ratio >2 a mesenchymal-like phenotype. The relative expression of (E) metalloproteinase 1, (F) metalloproteinase 2, (G) the receptor for activated C kinase 1 (RACK1) and (H) programmed cell death ligand-1 (PD-L1) were assessed by real-time PCR. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.02.062.

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