

All-trans retinoic acid influences viability, migration and adhesion of U251 glioblastoma cells

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Abstract: Glioblastoma (GBM) is one of the most aggressive and deadly forms of cancer. Literature data reveals that all-trans retinoic acid (ATRA) has anticancer effects on different types of tumor cells. However, data about the effects of ATRA on glioblastoma cells are contradictory. In this study, we examined whether ATRA treatment affects features of human glioblastoma U251 cells. To that end, the cells were treated with different concentrations of ATRA. Results obtained by MTT and the crystal violet assays imply that ATRA affected the viability of U251 glioblastoma cells in a dose- and time-dependent manner. Fluorescence staining of microtubule cytoskeleton protein α -tubulin revealed that ATRA induced changes in cell morphology. Using semi-quantitative RT-PCR we found that the expression of *SOX3* and *GFAP* genes, as markers of neural differentiation, was not changed upon ATRA treatment. Thus, the observed changes in cell morphology after ATRA treatment are not associated with neural differentiation of U251 glioblastoma cells. The scratch-wound healing assay revealed that ATRA changed the mode of U251 cell migration from collective to single cell motility. The cell-matrix adhesion assay demonstrated that the pharmacologically relevant concentration of ATRA lowered the cell-matrix adhesion capability of U251 cells. In conclusion, our results imply that further studies are needed before ATRA could be considered for the treatment of glioblastoma.

Key words: glioblastoma; ATRA; differentiation; viability; cell migration

INTRODUCTION

Gliomas are the most common type of primary brain tumors in humans [1]. Grade IV of glioma tumors, glioblastoma (GBM), is one of the most aggressive and deadly forms of cancer with a median survival of 15 months despite intensive therapeutic strategies which include surgical resection combined with radiotherapy and temozolomide chemotherapy [2-4]. Therefore, the identification of more effective treatment strategies for patients with GBM is required. Some of the new therapeutic approaches are focused on targeting the main features of human malignant glioblastoma cells,

such as uncontrolled proliferation, migration and invasion, resistance to apoptosis, lack of differentiation and vigorous angiogenesis [5-7].

Retinoids are a group of natural and synthetic compounds that are important modulators of various biological processes such as cell proliferation and differentiation [8]. All-trans retinoic acid (ATRA) is the most potent natural derivative of vitamin A that regulates various cell processes, including proliferation, differentiation and apoptosis of different normal and malignant cell types [9,10]. This compound has been shown to have anticancer effects on several types

of tumors, such as acute promyelocytic leukemia [11], breast [12], prostate [13], colon [14] and pancreatic cancer [15]. On the other hand, it was demonstrated that ATRA can promote the malignant potential of mouse mammary tumor cells [16], suggesting that its effect could be cell-context dependent.

Literature data regarding the effects of ATRA treatment on the malignant characteristics of glioblastoma cells are contradictory [9,17-19]. With this in mind, the aim of this study was to analyze the effects of ATRA treatment on the properties of U251 glioblastoma cells that are widely used as a GBM model system. We demonstrated that ATRA treatment reduced cell viability, induced morphological changes and affected migration and cell-matrix adhesion of U251 cells.

MATERIALS AND METHODS

Cell culture and treatments

The human glioblastoma U251 cell line was maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine and 1% nonessential amino acids (all from Invitrogen, USA) at 37°C in 10% CO₂. ATRA (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution, and the cells were treated with different concentrations of ATRA (1, 5, 10, 20, 40 and 60 µM) for a period of 3 or 5 days. Control cells and all ATRA-treated cells were grown in medium that contained the same volume of DMSO that was used for the treatment with 60 µM ATRA. The DMSO concentration in the assay did not exceed 0.6%.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (indicating the number of metabolically active cells) and crystal violet (CV) (indicating the total number of live adherent cells) colorimetric assays. U251 cells were cultured in 96-well plates at a density of 2x10³ cells/well for 3-day treatments or at a density of 1x10³ cells/well for 5-day treatments, and treated with DMSO (control) or different concentrations of ATRA (1, 5, 10, 20, 40 and 60 µM).

For the MTT assay, at the end of treatment MTT solution was added to cell cultures at a final concentration of 1 mg/mL and the cells were incubated for an additional hour at 37°C. Subsequently, the medium was removed and the cells were lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells was monitored by a microplate reader (Infinite 200 PRO; Tecan, Austria) at a wavelength of 550 nm. The experiment was performed in triplicate and repeated independently three times.

For the CV assay, at the end of treatment the cells were washed with PBS, fixed for 10 min with 4% paraformaldehyde, stained with 2% crystal violet-PBS for 15 min at room temperature (RT), washed four times in a stream of tap water and air dried. The absorbance of dye dissolved in 33% acetic acid was measured in a microplate reader Infinite 200 PRO at 550 nm. The experiment was performed in triplicate and repeated independently three times.

The results of MTT and CV assays are presented as the percentage of the values for control cells that was arbitrarily set to 100%.

Immunocytochemistry

The morphology of control cells and cells treated with three different concentrations of ATRA (10, 20, 40 µM) for 5 days were analyzed by fluorescence staining of microtubule cytoskeleton protein α -tubulin. For better visualization of cell morphology, 18 h prior to the end of treatment the cells were plated in low numbers on coverslips and grown in medium with the appropriate concentration of ATRA. Following fixation in 4% PFA for 20 min at room temperature (RT) and permeabilization in 0.2% Triton X-100, cells were blocked in 10% normal goat serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 1 h at RT. Mouse monoclonal anti- α -tubulin antibody (Calbiochem, MA, USA, CP06, diluted 1:100) was applied in PBS containing 1% BSA and 0.1% Triton X-100 overnight at 4°C. Coverslips were washed in 0.1% Triton X-100 prepared in PBS and incubated for 1 h at RT with anti-mouse guinea-pig secondary antibody conjugated with Alexa FluorH 594 (Invitrogen, diluted 1:500 in 1% BSA-PBT (phosphate buffered saline with Tween 20)). Nuclei were stained with 0.1 mg/mL diamidino phenylindole (DAPI, Sigma-Aldrich).

Images were taken by a Leica TCS SP8 confocal microscope applying the Leica Microsystems LAS AF-TCS SP8 software (Leica Microsystems, Germany).

Reverse transcriptase (RT) PCR analysis

The total RNA from control and U251 cells treated with different concentrations of ATRA (10, 20, 40 μ M) for 5 days was isolated using the TRI Reagent (Ambion, USA) according to the manufacturer's instructions. Isolated RNA was treated with DNase I using the DNA-free™ kit (Ambion) and subjected to cDNA synthesis. One μ g of the total RNA was reversely transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol. *SOX3* (SRY (sex determining region Y)-box 3) was amplified from the synthesized cDNAs with primers: 5'-CAC GGG TCC TCC GGG TTG CGA GGG GCG GAC C-3' (forward) and 5'-TGG GGA ACA AGG GTG GAC GAG C-3' (reverse); glial fibrillary acidic protein (*GFAP*) with primers: 5' -GCA GAG ATG ATG GAG CTC AAT GAC C- 3' (forward) and 5'-GTT TCA TCC TGG AGC TTC TGC CTC A -3'(reverse) [20] and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) with primers 5'-GCC TCA AGA TCA TCA GCA ATG C-3' (forward) and 5'-CCA CGA TAC CAA AGT TGT CAT GG-3' (reverse) [21]. The expression of *GAPDH* was used to normalize the levels of the total RNA used in the assays. PCR reactions were performed in 20- μ L reactions using the Kapa 2G Fast HotStart Ready Mix (Kapa Biosystems, USA). For *GFAP* and *GAPDH* amplification, samples were denatured for 2 min at 95°C and then cycled at 95°C for 15 s, 60°C for 15 s and 72°C for 15 s for 35 cycles, with reaction aliquots taken at 30 and 35 cycles. For *SOX3* amplification, samples were denatured for 5 min at 95°C and then cycled at 95°C for 30 s, 68°C for 30 s and 72°C for 30 s for 35 cycles, with reaction aliquots taken at 30 and 35 cycles.

Scratch-wound healing assay

U251 cells were treated with DMSO or various concentrations of ATRA (10, 20, 40 μ M) for 5 days. Upon treatment, the cell monolayer was scratched with a 200 μ L tip. Floating cells were washed and cells were incubated in a fresh growing medium containing DMSO or ATRA. Cell migration was monitored with

the DM IL LED Inverted Microscope (Leica) 7 h after the wound was made. The mode of cell migration into the gap was analyzed by counting single cells in 3-4 different parts of the wounded area from three independent experiments.

Cell-matrix adhesion assay

The adhesion ability of control cells and cells treated with different concentrations of ATRA (10, 20, 40 μ M) for 5 days was studied using 96-well plates coated with Matrigel (Becton Dickerson, NJ, USA). Binding to nonspecific adhesive surfaces was blocked by 2% BSA (Sigma-Aldrich) in DMEM for 1 h at 37°C. Upon treatment, 0.5×10^5 cells/well in DMEM supplemented with 0.1% BSA were seeded in 96-well plates. After 30 min, the cells were gently washed three times with 1xPBS and the number of attached cells was determined using the MTT assay. The tests were done in triplicate and repeated in 3 independent experiments.

Statistical analysis

The results were analyzed using Student's *t* test. Values of $p < 0.05$ were considered significant. This analysis was carried out using IBM SPSS Statistics Version 20 program.

RESULTS

The effect of ATRA on U251 glioblastoma cell viability

U251 human glioblastoma cells were treated with increasing concentrations of ATRA (1, 5, 10, 20, 40 and 60 μ M) for 3 and 5 days. After treatment, the number of metabolically active cells (MTT assay) (Fig. 1A) and the total number of live adherent cells (CV assay) (Fig. 1B) were analyzed. Both MTT and CV assays imply that ATRA affected the viability of U251 glioblastoma cells in a dose- and time-dependent manner as compared to their untreated counterparts. The maximum reduction was reached with 60 μ M ATRA after 5 days of treatment. Interestingly, we detected that 3 days of treatment (Fig. 1B) with 1 μ M ATRA slightly increased (about 10%) the total number of live cells. Since ATRA's effect on cell viability was more

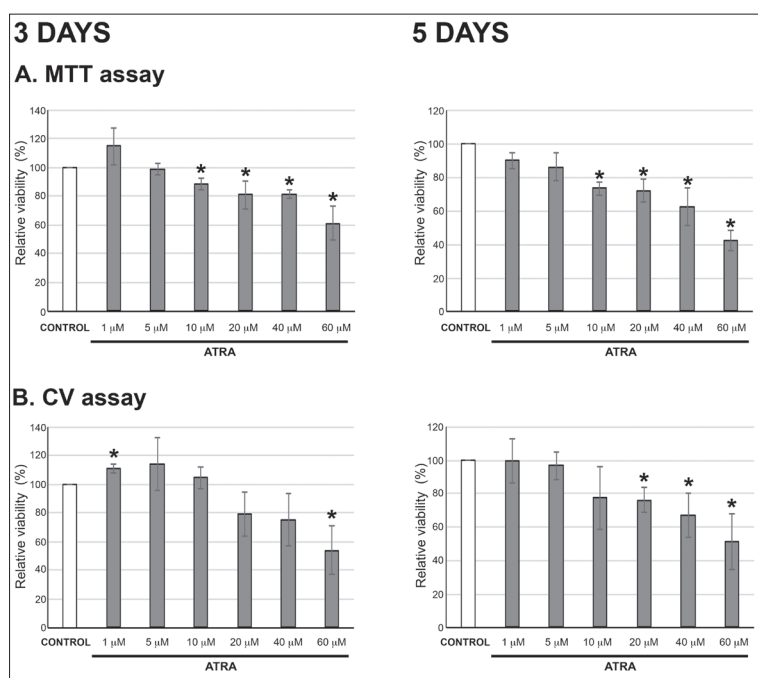


Fig. 1. ATRA treatment affected the viability of U251 glioblastoma cells in a dose- and time-dependent manner. Cell viability was examined by MTT (A) and CV (B) assays. Data are presented as the means \pm SD from three independent experiments; * p <0.05, compared to control cells.

pronounced after longer exposure, all further analyses were performed following 5 days of treatment using 10, 20 and 40 μ M concentrations.

The effect of ATRA on the morphology of U251 glioblastoma cells

The morphology of the control and ATRA-treated U251 glioblastoma cells was analyzed by fluorescence staining of microtubule cytoskeleton protein α -tubulin and nuclear/chromosome counterstaining with DAPI. Changes in cell morphology were induced with all tested ATRA concentrations (Fig. 2A). In cultures exposed to 10, 20 and 40 μ M ATRA most of the cells had long, thin branching processes, while the control U251 cells were mainly round, with small cell bodies and short processes (Fig. 2A).

The effect of ATRA on neural differentiation of U251 glioblastoma cells

Bearing in mind that alternations in cell morphology could be associated with differentiation and that

ATRA could induce astrocytic differentiation of glioblastoma cells, our next aim was to analyze whether the treatment with ATRA induces neural differentiation of U251 glioblastoma cells. To that end we analyzed the expression of markers of neural differentiation after 5-day treatments with 10, 20 and 40 μ M ATRA (Fig. 2B). Using semi-quantitative RT-PCR analysis we did not detect statistically significant changes in the level of *SOX3* and *GFAP* expression after 5 days of ATRA treatment (Fig. 2B). The presented results suggest that the observed changes in cell morphology after ATRA treatment were not associated with neural differentiation of U251 glioblastoma cells.

The effect of ATRA on cell migration of U251 glioblastoma cells

To analyze whether ATRA treatment influences the migration capability of U251 glioblastoma cells we performed the scratch wound healing assay. At the end of the treatment, the migration of cells into the wounded area was monitored (Fig. 3A). The ATRA treatment affected the mode of cell migration. Namely, we detected about two times more single cells in the wounded area of treated cells compared to the control (Fig. 3B). Additionally, we did not detect differences in the number of single cells in the wounded area between cells treated with different concentrations of ATRA.

The effect of ATRA on cell-matrix adhesion of U251 glioblastoma cells

Considering that the ability of cancer cells to form metastasis is influenced by their capability to adhere to and degrade the extracellular matrix (ECM), our next goal was to analyze if ATRA treatment changes the cell-matrix adhesion capacity of U251 glioblastoma cells. The 5-day treatment of U251 cells with 10 μ M ATRA decreased the cell-matrix adhesion ability of U251 cells by approximately 25% compared to control cells (Fig. 4). On the other hand, treatment with 20 and 40 μ M ATRA did not affect the cell-matrix adhesion ability of U251 cells compared to their DMSO-treated counterparts.

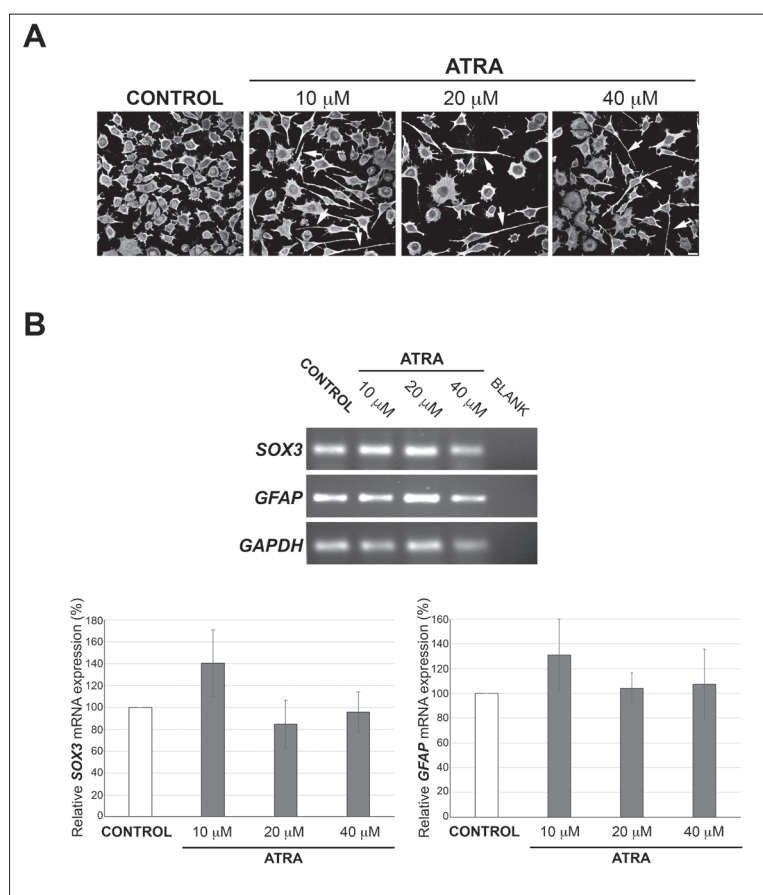


Fig. 2. ATRA treatment changed the morphology, but did not induce neural differentiation of U251 glioblastoma cells. **A** – Changes in cell morphology were analyzed by fluorescence staining of microtubule cytoskeleton protein α -tubulin. Arrows indicate thin branching processes; scale bar=20 μ m. **B** – Expression of *SOX3* and *GFAP* was examined by semi-quantitative RT-PCR analysis. Three independent experiments were performed and representative images are shown. The bands were digitalized, quantified with ImageJ software and normalized for GAPDH values. The relative gene expression was calculated as the fold expression compared to control cells which were set as 100%. Data from three independent experiments are presented in a histogram as the means \pm SD; * p <0.05, compared to control cells.

DISCUSSION

Glioblastoma is one of the most common, highly malignant and most therapy-resistant human tumors, with high morbidity and mortality [7]. One of the promising strategies for cancer therapy is treatment with ATRA [22]. The effect of ATRA on glioblastomas and heterogeneous responses depending on the

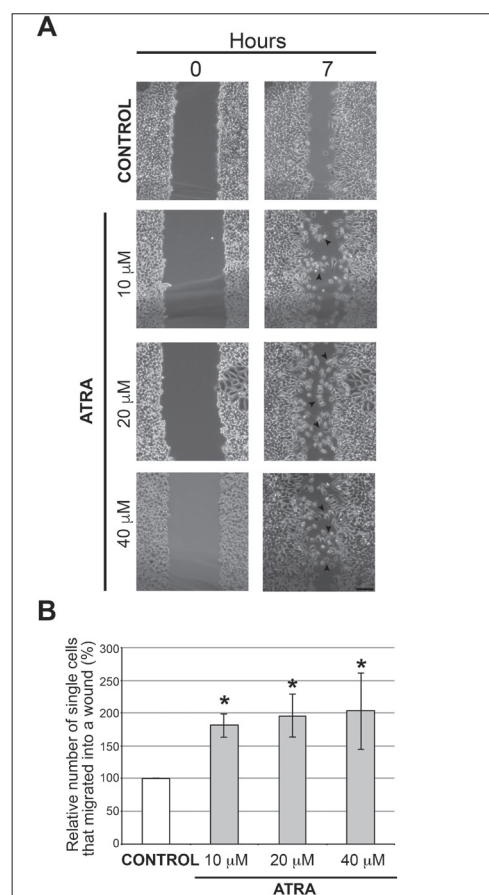


Fig. 3. ATRA changed the mode of U251 cell migration from collective to single cell motility. **A** – Migration capability was analyzed by the scratch wound healing assay. Confluent cell monolayers of U251 cells treated with DMSO or different concentrations of ATRA were scratched, and wound recovery was monitored 7 h after the wound was made. Representative phase contrast images of 3 independent wound closure experiments are shown; scale bar=50 μ m. **B** – The number of single cells migrating into the wounded area of U251 cells treated with DMSO or different concentrations of ATRA was counted. Data of three independent experiments are presented in a histogram as the means \pm SD; * p <0.05, compared to control cells.

concentration of ATRA, exposure times and types of glioblastoma cells have been noted [9,17-19,23]. Some studies have demonstrated that ATRA inhibits proliferation, migration and invasion and induces differentiation and apoptosis of glioblastoma cells [9,19,23]. On the other hand, differences in response to ATRA between long-term glioblastoma cell lines and primary cultures isolated from glioblastoma tumors were

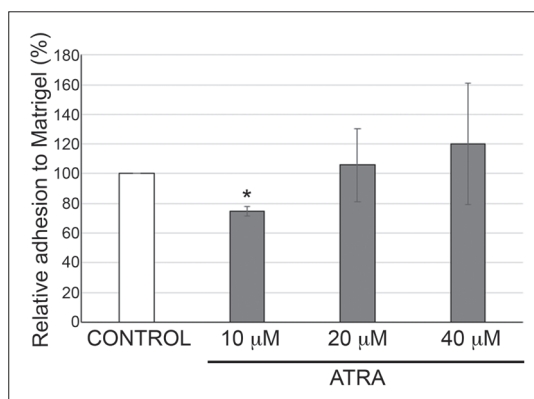


Fig. 4. Pharmacologically relevant concentration of ATRA decreased the adhesion capability of U251 cells. Adhesion capability was examined by the cell-matrix adhesion assay. Results are expressed as the fold of adhesion of control cells, which was set as 100%. Experiments were performed in triplicate and repeated three times. Data of three independent experiments are presented in a histogram as the means \pm SD; * p <0.05, compared to control cells.

reported [17]. In addition, it was shown that ATRA treatment elevated the transcription of a group of cancer-associated genes in glioblastoma cells [18].

In this study, we demonstrated that ATRA affected the viability of U251 cells. Additionally, our results strongly suggest that only high concentrations of ATRA (10, 20, 40 and 60 μ M) might significantly inhibit the viability of U251 glioblastoma cells. Contrary to our results, Lu et al. [23] showed that treatment with a lower dose of ATRA (2.5 μ M) reduced the growth of U251 cells by approximately 60%. A possible explanation for the observed differences between our results and theirs could be variations in genotype, phenotype and growth characteristics of different subclones of U251 cells, as was observed recently by Torsvik et al. [24]. Furthermore, we found that among the pharmacologic doses of retinoic acid (1-10 μ M) [25], only 10 μ M ATRA affected the viability of U251 cells, which is in accordance with the results of Pijuan-Thompson et al. [25], who showed that treatment with 10 μ M ATRA decreased the proliferation of U251 cells.

Similar to the results obtained on U87 glioblastoma cells [26], we observed that ATRA affected U251 cell viability in a dose- and time-dependent manner. Interestingly, we noted that after 3 days of treatment,

1 μ M ATRA slightly increased the total number of live cells (by about 10%). This is in accordance with the results obtained on GL-15 glioblastoma cells where low concentrations of retinoic acid (0.1-1 μ M) increased the proliferation of cells, while higher concentrations (5-10 μ M) reduced the cell proliferation rate [27].

Induction of differentiation in cancer cells in order to eliminate tumor phenotypes is the main goal of differentiation therapy of cancer [22]. It was shown that ATRA could induce astrocytic differentiation of T98G and U87MG glioblastoma cells [7]. Our results showed that U251 cells undergo morphological changes after ATRA treatment. Although association between morphological changes and the induction of neural differentiation has been demonstrated in several glioma cell lines [7,28-30], we did not detect that U251 cells undergo neural differentiation. A possible explanation is that neural differentiation of U251 cells could be induced only with concentrations of ATRA that are smaller than 10 μ M, as previously described for glioma stem/progenitor cells [31].

Metastasis is a complex multistep process that includes the ability of malignant cells to migrate and adhere [32,33]. Data about the effect of ATRA on the migration ability of glioblastoma cells are sparse. We observed that ATRA changed the mode of U251 cell migration from collective to single cell motility. Since it has been shown that glioma cells migrate into distant parts of normal brain tissue as single cells [34], we postulated that ATRA treatment might enhance the infiltration of tumor cells into normal brain tissue.

Adhesion of cancer cells to the ECM represents another important step in the metastasis process and is critical for enabling metastatic spread [35,36]. It has been demonstrated that impaired cell-cell adhesion results in an increased metastatic potential of cancer cells (reviewed in [37,38]). However, data regarding the impact of cell-matrix adhesion on the metastatic potential of cancer cells are different. Some authors have indicated that impaired cell-matrix adhesion decreases the metastatic potential of cancer cells [38,39]. On the other side, it was suggested that a reduction in cell-matrix adhesion is accompanied by an increased metastatic potential of cells [37,40,41]. Our results revealed that only the pharmacologically relevant concentration of ATRA (10 μ M) after a 5-day treatment significantly

decreased the cell-matrix adhesion capability of U251 cells. The anti-adhesive influence of ATRA was also observed in cervical SiHa cells [39] and A375 human melanoma cells [42]. Further investigations, including the determination of the strength of cell-matrix adhesion, the ability of matrix-remodelling, the invasion potential and the rate of cell spreading, should clarify whether the impaired cell-matrix adhesion capability of ATRA-treated U251 cells is accompanied by a decreased or an increased metastatic potential.

In summary, here we studied the effects of ATRA treatment on features of human glioblastoma U251 cells. Our results demonstrate that treatment with ATRA might reduce the viability and cell-matrix adhesion capability of U251 glioblastoma cells and increase the number of single cells in the wounded area. Moreover, despite visible changes in cell morphology, the expression of neural-specific markers was not changed. Based on these results, we conclude that further studies are warranted before ATRA could be considered as a therapy for glioblastoma.

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