Clinical Neurology and Neurosurgery 173 (2018) 20–30

Contents lists available at ScienceDirect

Clinical Neurology and Neurosurgery

journal homepage: www.elsevier.com/locate/clineuro

# From epidemiology and neurodevelopment to antineoplasticity. Medroxyprogesterone reduces human glial tumor growth *in vitro* and C6 glioma in rat brain *in vivo*

Meric A. Altinoz[a,b,*], Josephine Nalbantoglu[c], Aysel Ozpinar[d], M. Emin Ozcan[e], Rolando F. Del Maestro[f], Ilhan Elmaci[a,g]

[a] *Neuroacademy Research Group, Istanbul, Turkey*
[b] *Department of Psychiatry, Maastricht University, Holland, Netherlands*
[c] *Department of Neuroimmunology, Montreal Neurological Institute, McGill University, Montreal, Canada*
[d] *Department of Medical Biochemistry, Acibadem University, Istanbul, Turkey*
[e] *Department of Neurology, Kizilay Hospital, Bakirkoy, Istanbul, Turkey*
[f] *Department of Neurosurgery, McGill University, Montreal, Canada*
[g] *Department of Neurosurgery, Memorial Hospital, Istanbul, Turkey*

ARTICLE INFO

*Keywords:*
Medroxyprogesterone
Progesterone analogues
Dexamethasone
Temozolomide
Glioblastoma

ABSTRACT

*Objective:* Glial tumor growth may accelerate during gestation, but epidemiological studies consistently demonstrated that parousity reduces life long risk of glial tumors. Pregnancy may also accelerate growth of medulloblastoma and meningioma, but parousity does not confer protection against these tumors. We were the first to show that medroxyprogesterone acetate (MPA) reduces rat C6 glioma growth *in vitro*. Now we aimed to determine the effects of MPA on human brain cancers (particularly glioblastoma) *in vitro* and C6 glioma *in vivo*.
*Patients and Methods:* We evaluated the effects of MPA on: i) monolayer growth of human U87 and U251 glioblastoma, ii) 3D-spheroid growth and invasion of C6 rat glioma and human U251 glioma, iii) interactions with PI3-Kinase inhibitors and coxsackie-adenovirus receptor (CAR) in modifying 3D-spheroid invasion of glioma.
*Results:* MPA at low doses (3.25–13 μM) insignificantly stimulated and at high doses (above 52 μM) strongly suppressed the growth of human U87 and U251 cells *in vitro*. MPA also binds to glucocorticoid receptors similar to dexamethasone (Dex) and unexpectedly, PI3-Kinase inhibitors at low doses suppressed anti-invasive efficacies of MPA and Dex. MPA exerted higher invasion-inhibitory effects on CAR-expressing human glioma cells. Lastly, MPA suppressed growth of C6 glioma implanted into rat brain.
*Conclusion:* Progesterone analogues deserve to be studied in future experimental models of high grade glial brain tumors.

## 1. Introduction

High grade glial tumors are among the most frequent neoplasms of the adult brain. Despite the golden standart of care (maximum surgical resection, radiotherapy, and temozolomide), the median survival of patients with glioblastoma multiforme (GBM) is around 15 months. Hence, discovery of novel pathogenesis mechanisms and proposal of novel treatment strategies is an urgent need for GBM treatment. Embryonic differentiation agents/morphogens may be employed as adjunctive agents in treatment of these grave tumors. While pregnancy may accelerate growth of gliomas [1], it was consistently shown that parousity reduces the incidence of high grade glial tumors [2–8]. This effect may occur due to the functioning of progesterone as a proliferative hormone at low doses while suppressing growth and inducing differentiation of glial progenitor cells at higher levels.

In support of this theory, progesterone increases oligodendrocyte number in neonatal rat brain glial cultures [9]. On the other hand, oligodendrocyte pre-progenitors (OPP) and oligodendrocyte progenitors (OP), but not fully differentiated oligodendrocytes (OL) are capable to synthesize progesterone from the immediate precursor pregnenolone, indicating for a differentiation-inducing function of progesterone [10]. Indeed, treatment of cuprizone-demyelinated female mice with

* Corresponding author at: Nurtepe Mh, Guven Sk. No:5, D:6, Kagithane, Istanbul, Turkey.
*E-mail address:* maltinoz@gmail.com (M.A. Altinoz).

https://doi.org/10.1016/j.clineuro.2018.07.012
Received 7 May 2018; Received in revised form 3 July 2018; Accepted 13 July 2018
Available online 19 July 2018
0303-8467/ 

progesterone or nestorone (a 19-nor-progesterone derivative which is 100 times more active than progesterone on reproductive system endpoints) enhances number of differentiated oligodendroglia and the formation of the myelin basic protein (MBP)- and proteolipid protein (PLP)-immunoreactive myelin [11]. In parallel to the physiological functioning, a study group showed that progesterone at a low dose (10 nM) stimulated growth of glioblastoma cells [12–14]; yet another study group showed that higher doses of progesterone (above 10 μM) blocks proliferation human glioblastoma cells via suppressing PI3-Kinase/akt pathway [15,16].

We previously showed that medroxyprogesterone acetate (MPA, employed as a contraceptive and anticancer agents in endometrial cancer) reduced the S-phase and potentiated efficacy of the procarbazine with no significant cell kill in monolayer cultures of C6 rat glioblastoma [9,17]. Procarbazine is a chemotherapeutic employed in the treatment of glioblastoma before the temozolomide era, which has a triazene-type chemical structure similar to temozolomide [9,17]. Procarbazine is still being used in treatment of glial tumors in combination with lomustine and vincristine in the PCV protocol [18]. Lysosomal phospholipidosis, a sign of autophagy accompanied to the chemosensitizing efficacy of MPA in C6 cells. In this study, we aimed to determine the potential of MPA in modifying human brain cancer (glioblastoma) growth and invasion in various cell culture models. Hence, we evaluated effects of MPA on: i) monolayer growth of human U87 and U251 glioblastoma, ii) spheroid growth and invasion of C6 rat glioma (in the absence and presence of 5-Fluorouracil/5-FU) and human U251 glioma, iii) spheroid invasion of glioma in the presence of PI3-Kinase inhibitors in comparison to dexamethasone, iv) spheroid invasion of glioma cells which express coxsackie-adenovirus receptor (CAR), v) *in vivo* growth of C6 glioblastoma.

## 2. Materials and methods

### 2.1. Cell lines, cell culture conditions and drugs investigated in the current study

The rat glioma cell line C6 and human glioma cell lines U87-MG and U251 were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained at 37 °C with 5% $CO_2$ in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS and an antibiotic cocktail (final concentration of 30 μg/ml gentamicin, 100 units of penicillin/ml and 100 μg of streptomycin/ml). Briefly; MPA is medroxyprogesterone acetate, LY294002 and wortmannin are PI3-Kinase inhibitors and 5-Fluorouracil (5-FU) is used as a chemotherapy agent; and details about these agents were provided in the discussion.

### 2.2. CAR transfection

The retrovirus carrying the full-length mCAR1 isoform (CAR-LNCX) was provided by Dr. Nalbantoglu (Montreal Neurological Institute, Canada) and the retroviral structure was described in detail in a previous publication [19]. The transgene was under the control of the cytomegalovirus (CMV) promoter. The retrovirus also expressed a neomycin phosphotransferase gene to allow the selection of stably transduced cells with geneticin (G418 sulfate). A third retrovirus containing only the neomycin resistance gene was used as the control (LNCX). The supernatants from the producer cell lines were used to transduce U87 cells, which were then selected for 10 days with G418 (600 μg/ml). The elimination of the nontransduced cells was ensured by the absence of colonies in a control nontransduced cell plate treated with G418. Clones were pooled together to generate bulk populations stably expressing the full-length mCAR1 (U87CAR) or expressing no CAR (U87LNCX).

### 2.3. Assessment of monolayer growth patterns

#### 2.3.1. XTT and trypan blue assays to assess cell density and cell death

For cell proliferation assays, cell populations were seeded in microtiter plates (tissue culture grade, 96 wells, flat bottom) in a final volume of 200 μl of serum-supplemented medium (standard DMEM with 10% FBS) in a humidified atmosphere at 37 °C and 5% $CO_2$. Proliferation was assessed by a colorimetric assay with the dye (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]2*H*-tetrazolium- 5-carboxanilide) sodium salt (XTT; Sigma, St. Louis, MO). Spectrophotometric absorbance of the samples was measured as optic density (OD) using a microtiter plate reader (EAR400AT, SLTLab Instrument, Austria) at wavelength of 450 nm. In the plotted graphs, values above the x-axis represented growth inhibition/cell kill and under the x-axis represented growth stimulation, since values were calculated according to the increased or decreased OD values as percentage of the control. In conditions when a significant inhibition of cell density was observed, simple trypan blue exclusion tests were applied whether the decrease of cell density was due to inhibition of proliferation or cell death (explained below, detailed quantitative data not given).

#### 2.3.2. Dose-response experiments with MPA on human glioma

For each dose of MPA, U87 and U251 cells in six separate wells of a 24-well plate were used. They were treated with various doses of MPA, however each time same amount of (5 μl) ethanol was used as a solvent in 1 ml DMEM. Control groups always received the same amount of the ethanol solvent. Cells were treated with MPA following 24 h of plating, and XTT assay was performed following 96 h of drug treatment, without replenishing medium or drugs in any of the groups. Drug concentrations were increasing by two-fold stepwise from P1- to P6, which ranged from 3.25 μM to 104 μM (1.25 to 40 μg/ml), respectively.

### 2.4. Establishment of the spheroid model

Spheroids of C6 rat glioma, and human U87, U251 and tranduced U87 gliomas cells were generated by spinner cultures, and spheroids of similar diameter were selected and encased in a collagen type I gel. Briefly, glioma cells in monolayer culture were trypsinized and seeded into spinner culture flasks at approximately $4 \times 10^6$ cells/100 ml DMEM with 10% FBS and spun at 180 rpm for 2 weeks. Thereafter, spheroids of similar diameter were implanted into collagen type I gel (600 μl per well, Vitrogen 100, purchased from Cohesion Technologies, Palo Alto, CA) in 24-well plates. The gel was then overlaid with 500 μl of DMEM supplemented with 10% FBS. The medium was changed every 3 days. Each day for 10 days, cell invasion was assessed with the aid of an inverted microscope. Invasion distance was calculated as the distance in micrometers from the spheroid edge to the most distant population of invasive cells. The experiment was repeated with 3 different preparations of spheroids. Growth values in the right side of Figs. 3–6 represent changes in the spheroid diameter, therefore they begin one day later than the invasion data since they were calculated as percentage changes.

### 2.5. MPA and 5-FU effects on the C6 glioblastoma spheroid growth and invasion

Modulation of C6-glioma spheroid growth and invasion by high (MPAh- 104 μM, 40 μg/ml), medium (MPAm- 52 μM, 20 μg/ml) and low (MPAl- 26 u M, 10 μg/ml) doses of MPA and/or 5-Fluorouracil (5-FU/Flu 200 μM) was determined. Spheroids were incubated throughout 8 days in the same conditions in type I collagen, and both the drugs and feeding media were replenished every 72-h. Con-et represents ethanol-control for 0.5% of ethanol, which was used as a solvent for MPA. This amount of ethanol did not effect neither the growth nor the invasion of C6-spheroids significantly.

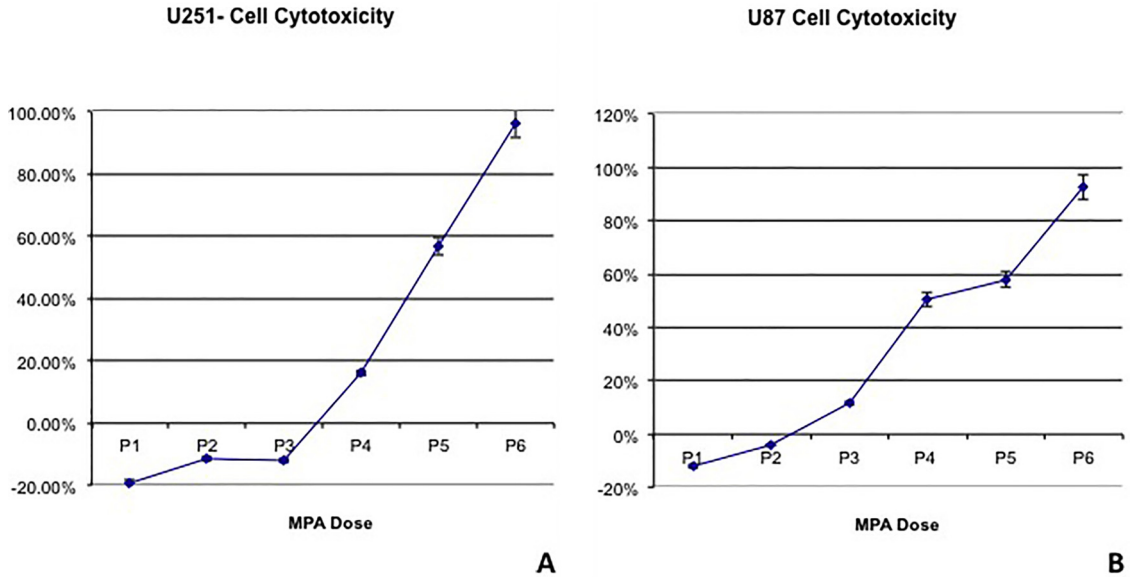


**Fig. 1.** Effects of MPA (stepwise increasing doses two-fold from 3,25 μM (P1) to 104 μM (P6)) on the cytotoxicity in U87MG (a) and U251 (b) human glioblastoma cell lines in monolayer culture *in vitro*. Slight stimulation of cell proliferation in glioma cells treated with 3,25 and 6.5 μM of MPA did not reach significance. Strong reduction of cell density at 52 and 104 μM exerted statistical significance ($p < 0.00001$) and this decrease occurred via cell necrosis as assessed by trypan blue exclusion test (data not shown). For P1-to P3 data from 20, for P4-toP6 and control group data from 24 different samples were pooled and analyzed. Error bars represent standart deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### *2.6. Effects of MPA on the growth and invasion of U251 human glioma spheroids*

MPA treatment at low (26 μM) and high (104 μM) dosages was applied every 48 h, beginning one day after spheroid implantation, which is 'day 1′ as presented on the left side in Fig. 3. Growth values in the right side of Fig. 3 represent changes in spheroid diameter, therefore they begin one day later than the invasion data due to origin function of 'day 1′ data for measuring percentage changes.

### *2.7. Interactions of MPA and dexamethasone with PI3-Kinase inhibitors and coxsackie-adenovirus receptor in modifying glioblastoma growth and invasion*

At first, we determined effects of the high (112 μM) or low (1.4 μM) dose of LY294002 and of the high (400 nM) or low (10 nM) dose of wortmannin on the cell viability of U251 human glioma cells in monolayer culture and we did not witness any cytotoxic effects (data not shown). Modulation of U251 human spheroid growth (Fig. 4a) and invasion (Fig. 4b) were measured following treatment with medium (52 μM) dose of MPA in the presence and absence of high (112 μM) or low (1.4 μM) dose of LY294002. All agents were replenished every two days in fresh medium, and the treatment began at the day of spheroid implantation. The same tests were applied to determine U251 glioma spheroid growth (Fig. 4c) and invasion (Fig. 4d) following treatment with medium (52 μM) dose of MPA in the presence and absence of high (400 nM) or low (10 nM) dose of wortmannin.

Modulation of U251 human spheroid growth (Fig. 5a) and invasion (Fig. 5b) were measured following treatment with 1 μm of Dexamethasone in the presence and absence of high (112 μM) or low (1.4 μM) dose of LY294002. All agents were replenished every two days in fresh medium, and the treatment began at the day of spheroid implantation. The same tests were applied on U251 glioma spheroids to determine growth (Fig. 5c) and invasion (Fig. 5d left) following treatment with 1 μm of Dexamethasone in the presence and absence of high (400 nM) or low (10 nM) dose of Wortmannin. Lastly, we assessed on the effects of MPA and dexamethasone on the invasion of U87 LNCX and U87CAR cells, which were transduced with empty or CAR-containing retroviral vectors, respectively (Fig. 6).

### *2.8. C6 astrocytoma implantation into female rat brains*

Female Wistar rats (6 weeks old, Charles River Canada, St-Constant, Quebec) were anesthetized by i.p. injection of sodium pentobarbital (25 mg/kg) and placed in a stereotactic apparatus (Kopf). There were 10 rats each in control and MPA-treatment groups. A burr hole was drilled 1 mm anterior and 2 mm lateral to the bregma. C6 glioma cells origined from a methylnitrosourea induced adult rat glioblastoma. The C6 glioma cell suspension ($1 \times 10^5$ cells in 3 μl of Hank's balanced salt solution (HBSS) was injected stereotactically over a 10 min period using a Hamilton syringe at a depth of 3.5 mm. In the treatment group, 20 mg/kg of MPA was injected intraperitoneally. Controls were injected with saline solution at the same volumes of MPA. After 39 days, the animals were euthanized and the tumor volumes analyzed as described below. All animal experimentation was carried out according to the guidelines of the Declaration of Helsinki. After euthanasia, brains were removed and quick frozen in isopentane chilled with liquid nitrogen. Coronal sections (10 μm) were prepared. Tumor volumes were calculated using the formula $a \times b^2 \times 0.4$, in which a represents the longest axis and b represents the width perpendicular to this axis.

### *2.9. Statistical analysis*

Statistical significance was assessed by Student's *t*-test or ANOVA. Statistical significances were defined as $p < 0.05$.

## 3. Results

### *3.1. Dose response effects of MPA on human glioma cell density. Role of cell death versus inhibition of growth*

As depicted in Fig. 1, in U87 cells, only P5 and P6 treatments showed statistical effectiveness, and at high significance values ($p < 0.0001$). Slight proliferations with P1 and P3 doses did not show any statistical difference ($p > 0.05$). In U251 cells, only P5 and P6 treatments showed statistical effectiveness, yet also reflected by high

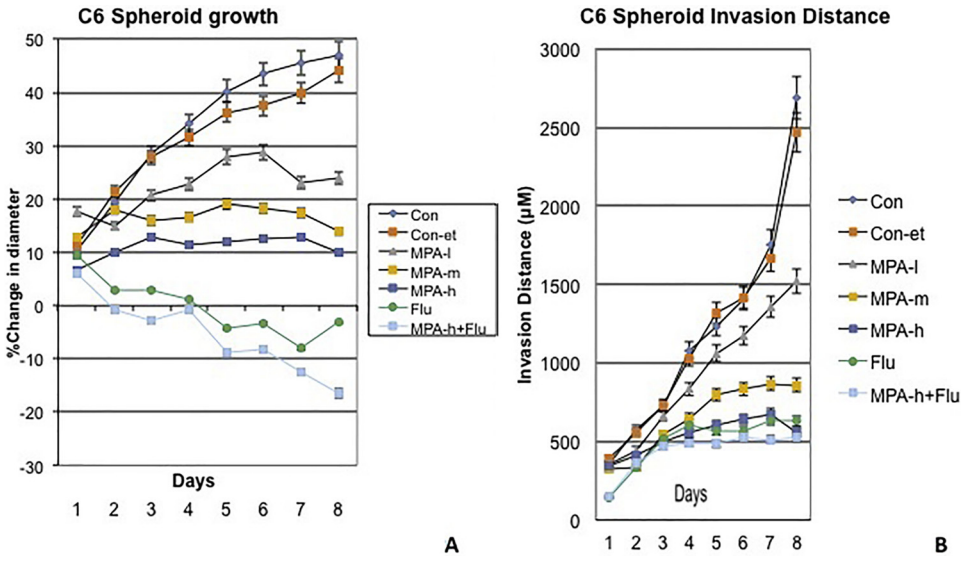


**Fig. 2.** Modulation of C6-glioma spheroid growth (a) and invasion (b) by high (MPAh- 104 μM, 40 μg/ml), medium (MPAm- 52 μM, 20 μg/ml) and low (MPAl- 26 u M, 10 μg/ml) doses of MPA and/or 5-Fluorouracil (Flu- 200 μM). MPA inhibition of growth occurred significantly at both 26 and 104 μM ($p < 0.01$ and $p < 0.005$, respectively). MPA inhibition of invasion was also significant at high dosage ($p < 0.05$). 5-FU inhibition of tumor growth alone and in combination with MPA exerted high significance ($p < 0.0001$ for both effects). 5-FU treatment alone and with MPA also blocked tumor invasion ($p = 0.028$ for single 5-FU, $p = 0.020$ for MPA + 5-FU in comparison to control). Results of triplicate experiments.

significance values ($p < 0.0001$). At the 96th hour, growth inhibitions between 85% to 100% were noted in both glioblastoma cell lines with complete necrosis frequently witnessed at the highest MPA dosage (Fig. 7a, b and c), which was also proven with trypan blue exclusion assay (data not shown). Slight proliferations between P1 and P3 doses did not show any statistical difference ($p > 0.05$).

### 3.2. MPA and 5-FU effects on the C6 glioma spheroid growth and invasion

As depicted in Fig. 2, at the end of the eight day period, control spheroids had a 44% increase in diameter, whereas treatment group spheroids had increased their diameter by only 24%, 14%, and 10% in the presence of 26, 52 and 104 μM MPA, respectively. Differences were statistically significant ($p < 0.01$ for 26 μM and $p < 0.005$ for 104 μM). In 5-FU treated spheroids, there was a reduction of diameter by 3.2%, which was further reduced to 17% with 104 μM MPA (both reductions occurred with significance of $p < 0.0001$ in comparison to control). After eight days, C6 tumor cells had invaded 2700 microns on average, which was reduced to 1520, 860 and 560 microns in the presence of 26, 52 and 104 μM MPA, respectively ($p < 0.05$ for the highest dose). 5-FU treated spheroids were only able to invade 630 microns on average, which was further reduced to 530 microns with 104 μM MPA ($p = 0.028$ for single 5-FU, $p = 0.020$ for MPA + 5-FU in comparison to control).

### 3.3. Effects of MPA on the growth and invasion of U251 human glioma spheroids

As depicted in Fig. 3, in U251 glioma, for the control group, only high dose MPA (104 μM) was statistically effective ($p = 0.022$) in reducing growth, however both low (26 μM) and high (104 μM) doses of MPA were effective in reducing invasion, with p values less than 0.05 and 0.0001, respectively. Fusiform and healthy-appearing cells were noted in the control group, whereas rounded and necrotic cells with cell debris were witnessed at the spheroid periphery in the MPA-treated groups (Fig. 8). The difference between high and low doses of MPA to inhibit invasion was significant ($p < 0.05$).

### 3.4. Effects of MPA and dexamethasone with PI3-Kinase inhibitors on the growth and invasion of U251 human glioma spheroids

As demonstrated in Figs. 4 and 5, LY294002 efficiently reduced invasion with both low ($p < 0.005$) and high dose ($p < 0.0001$) application, and the dose difference was significant ($p < 0.005$).Wortmannin at both low and high doses also efficiently reduced invasion ($p < 0.0001$) (Figs. 4 and 5). The difference of the potencies between low versus high doses of wortmannin was significant ($p = 0.03$). At the end of 8$^{th}$ day, MPA significantly ($p < 0.05$) reduced spheroid growth (Fig. 4a), but this efficacy was paradoxically blocked by LY294002 ($p > 0.05$ for both LY294002 doses). MPA was also efficient ($p < 0.005$) in reducing spheroid invasion, which was only augmented by high ($p < 0.005$), but not low dose of LY294002 (Fig. 4b). At the end of 8$^{th}$ day incubation in type I collagen, MPA efficacy to block spheroid growth was significantly augmented by high dose wortmannin ($p = 0.023$) (Fig. 4c). However, either low or high dose wortmannin did not significantly alter MPA mediated invasion inhibition (Fig. 4d). As depicted in Figs. 5 and 9, dexamethasone efficiently blocked U251 spheroid growth ($p < 0.0001$) and invasion. LY294002 at low dose reduced dexamethasone efficacy to inhibit spheroid growth ($p < 0.05$) (Fig. 5a) and invasion ($p = 0.0018$) (Figs. 5b, 9). Low dose of wortmannin tended to reduce dexamethasone's effect to inhibit spheroid growth ($p = 0.089$) (Fig. 5c). The lowdose of wortmannin has significantly reduced efficacy of dexamethasone to inhibit invasion ($p = 0.002$) (Fig. 5d).

### 3.5. Effects of MPA and dexamethasone on the spheroid growth and invasion of CAR-Transfected U251 human glioma spheroids

As depicted in Fig. 6, in U87 LNCX cells, neither MPA nor Dex treatment did not significantly influence spheroid invasion, even there was an increase in invasion with Dex treatment. In U87 CAR cells which express Coxsackie-Adenovirus Receptor, both Dex and MPA decreased invasion, which occurred significantly only for the latter ($p = 0.011$).

### 3.6. MPA effects on the intracranial growth of C6 glioblastoma

As depicted in Fig. 10, in the control group, the mean tumor volume was measured as 395.2 mm$^3$, whereas in the MPA-treated group the mean tumor volume was 1947 mm$^3$ (a 49.3% inhibition, $p = 0.0043$). As demonstrated in Fig. 11, histopathological analysis revealed a massive monolymphocytic infiltration around the invasion zones of C6 glioma in the MPA-treated groups (A, D, E, F). Sparce fusiform glioma cells exerted distant infiltration into brain parencyma (B). In glioblastoma, perivascular infiltration by tumor cells is a common feature; however, lymphomonocytes almost formed a barrier between tumor cells and microvascular structures in MPA-treated groups (C). Massive lymphomonocytic around glioma cells (D) also surrounded areas of tumoral necrosis (E). Extracellular punctate staining with β-

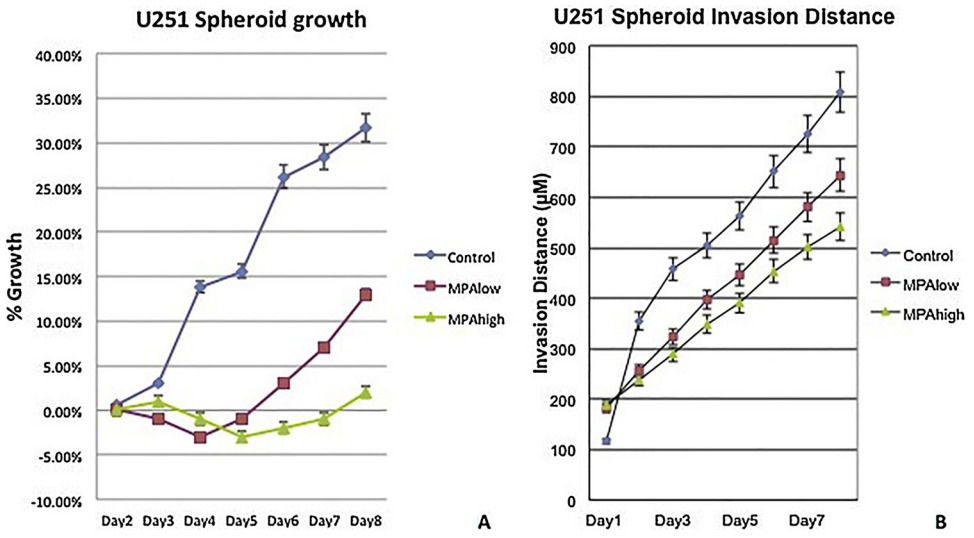


**Fig. 3.** Effects of MPA (MPAhigh- 104 μM, MPA-low – 26 μM) on the percentage growth (a) and invasion distance (b, given as micrometers at the left side) of U251 human glioblastoma. Only high dose MPA (104 μM) was statistically effective ($p = 0.022$) in reducing growth, however both low (26 μM) and high (104 μM) doses of MPA were effective in reducing invasion, with p values less than 0.05 and 0.0001, respectively. Results of triplicate experiments.

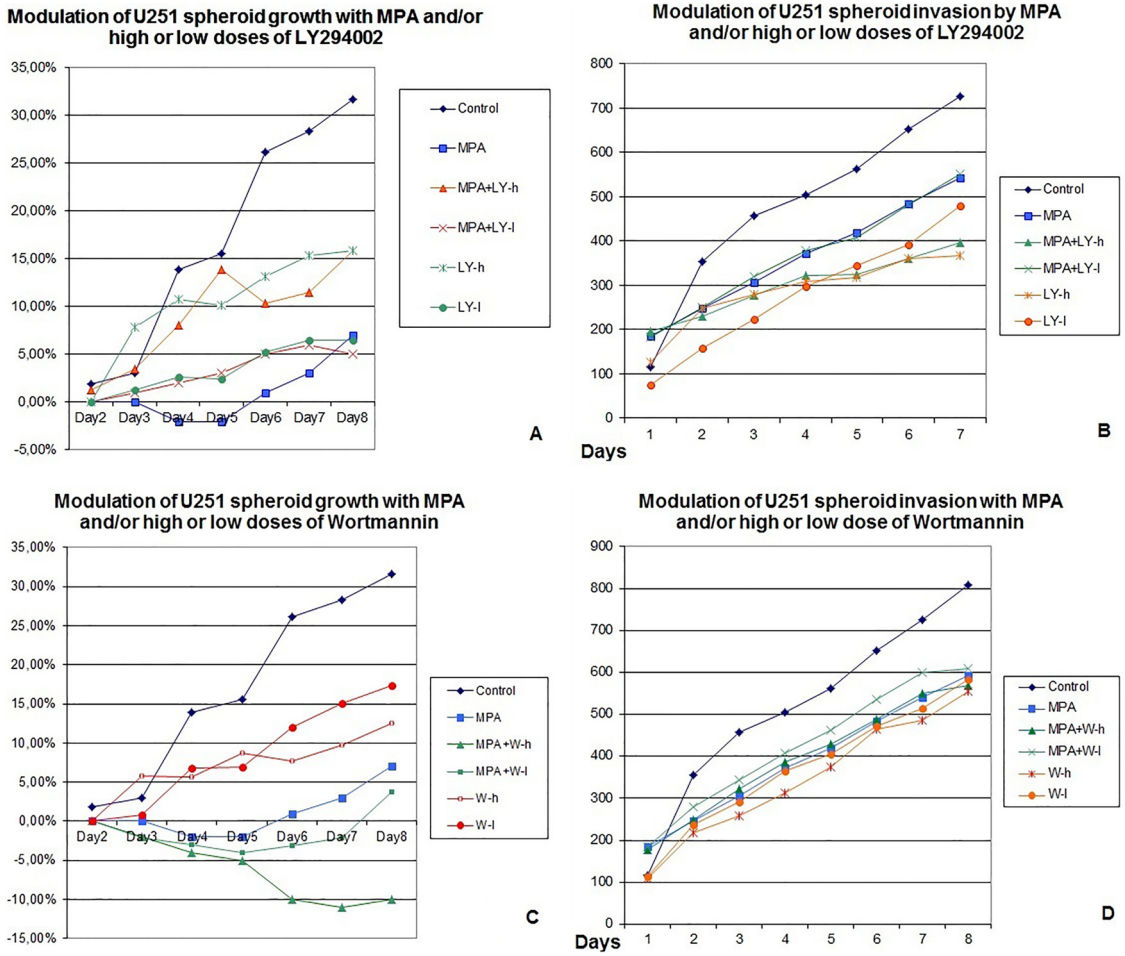


**Fig. 4.** Modulation of U251 human glioblastoma spheroid growth (a) and invasion (b) with medium (52 μM) dose of MPA in the presence and absence of high (112 μM) or low (1.4 μM) dose of LY294002. Modulation of U251 human glioblastoma spheroid growth (c) and invasion (d) with medium (52 μM) dose of MPA in the presence and absence of high (400 nM) or low (10 nM) dose of wortmannin. MPA significantly ($p < 0.05$) reduced spheroid growth (a), but this efficacy was paradoxically blocked by LY294002 ($p > 0.05$ for both LY294002 doses). MPA was also efficient ($p < 0.005$) in reducing spheroid invasion, which was only augmented by high ($p < 0.005$), but not low dose of LY294002 (b). MPA efficacy to block spheroid growth was significantly augmented by high dose wortmannin ($p = 0.023$) (c). However, either low or high dose wortmannin did not significantly alter MPA mediated invasion inhibition (d). In (b) and (d) "x" axis represent days and "y" axis represents the distance of invasion of glioblastoma cells from the edge of the bulk tumor in micrometer values. Results of triplicate experiments.

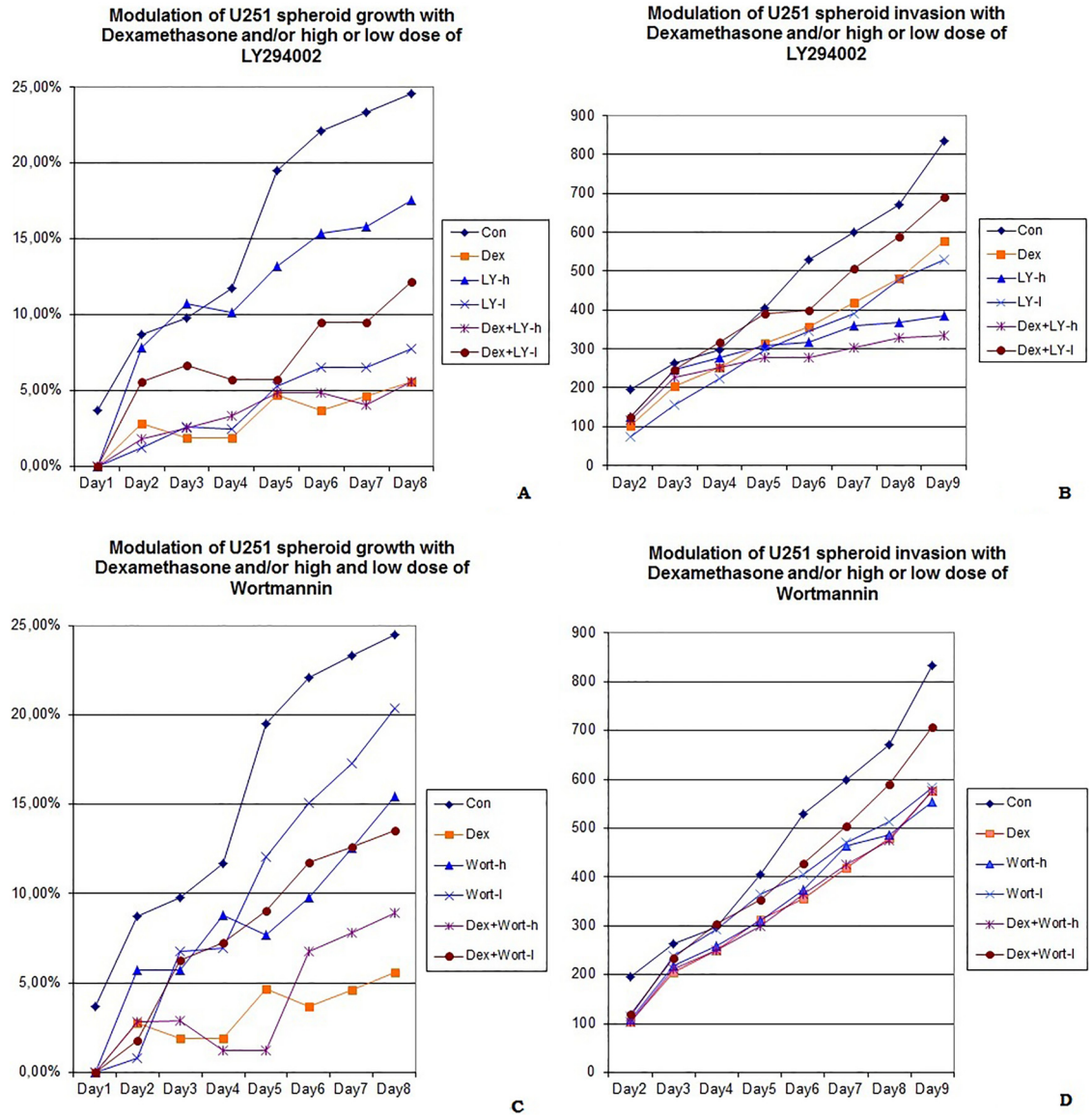


**Fig. 5.** Modulation of U251 human spheroid growth (a) and invasion (b) with dexamethasone (Dex, 1 μM) in the presence and absence of high (112 μM) or low (1.4 μM) dose of LY294002. Modulation of U251 human spheroid growth (c) and invasion (d) with dexamethasone (1 μM) in the presence and absence of high (400 nM) or low (10 nM) dose of wortmannin. dexamethasone efficiently blocked U251 spheroid growth ($p < 0.0001$) and invasion. LY294002 at low dose reduced dexamethasone efficacy to inhibit spheroid growth ($p < 0.05$) and invasion ($p = 0.0018$) (Fig. 9). Low dose of wortmannin tended to reduce dexamethasone's effect to inhibit spheroid growth ($p = 0.089$) (Fig. 7c). The lowdose of wortmannin has significantly reduced efficacy of dexamethasone to inhibit invasion ($p = 0.002$) (Fig. 7d). In (b) and (d) "x" axis represent days and "y" axis represents the distance of invasion of glioblastoma cells from the edge of the bulk tumor in micrometer values. Results of triplicate experiments.

galactosidase-stain surrounded by lymphomonocytes indicates likely immune-mediated cell death of glioma cells (F).

## 4. Discussion

### 4.1. Potential importance of progesterone and MPA research in brain tumors

Among the cytosolic steroid receptors, progesterone receptor (PR) exerts the strongest positive correlation with glial tumor grade [20], where PR-A exerts an inhibitory and PR-B a stimulatory effect on cell proliferation [21]. MPA binds to PR-B with higher affinity than progesterone and acts therapeutic against human endometrium cancer and downregulates PR-B [22]. While low dose MPA enhances risk of breast cancer in hormone replacement treatment (HRT), higher doses of MPA can provide clinical regressions in breast [23–25], renal [26], and endometrium cancer [22,27]. Since MPA is one of the mostly used progestin in the clinic with a high safety, we aimed to define effects of high levels of MPA on the growth of human glioblastoma cells. MPA also binds to the corticosteroid receptors [28] and corticosteroids such as dexamethasone inhibit spheroid invasion of glioblastoma cells [29,30]. Hence, we also aimed to determine effects of MPA and dexamethasone with and without PI3-Kinase inhibitors on the invasion of glioblastoma cells. The reasons that we studied MPA interactions with 5-FU (5-Fluorouracil) and with the expression of CAR are explained below.

### 4.2. Dual effects of MPA on brain Cancer and the relevance of selected doses

We observed slight and insignificant stimulation of glioma cell growth with MPA, but MPA doses above 52 μM were inhibitory and

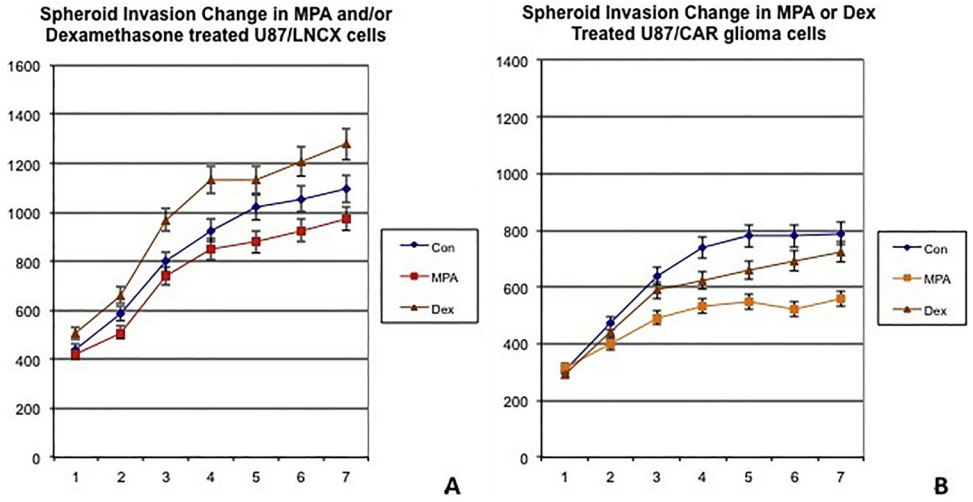


**Fig. 6.** Modulation of invasion in U87 LNCX (a) and U87 CAR (b) glioblastoma cells with MPA and dexamethasone. In U87 LNCX cells, neither MPA nor Dex treatment did not significantly influence spheroid invasion, even there was an insignificant increase in invasion with Dex treatment. In U87 CAR cells which express Coxsackie-Adenovirus Receptor, both Dex and MPA decreased invasion, which occurred significantly only for the latter ($p = 0.011$). "x" axis represent days and "y" axis represents the distance of invasion of glioblastoma cells from the edge of the bulk tumor in micrometer values.

even induced complete cell kill at 104 μM (Fig. 7). Our results are very parallel to the results of Atif et al., which revealed significant cell kill in U87MG, U87dEGFR and U118MG cells after 3 days of progesterone treatment at high concentrations (10, 20, 40, and 80 μM), whereas lower concentrations of progesterone (0.1, 1, and 5 μM) were not cytotoxic even proliferative with prolonged exposure [16]. In breast cancer cells, overlaps in gene regulation by the native progesterone and MPA were determined, but the majority of genes regulated by these hormones differed [28]. 6 out of the 30 genes regulated by MPA were down-regulated (*ITGB5, c-MYB, VEGFR2, ITGA2/CD49B, NPY1R, THBS1*), but progesterone exerted no changes on the expression of these genes [28]. Among these, *c-myb* is a tumor promoting and *VEGFR2* is a proangiogenic gene.

In breast cancer, high dose MPA treatment (1500 mg/day *per os*) yields peak concentrations between 1.5 to 6 μg/ml [31]. Human placental concentrations of progesterone are between 5 to 20 μg/mL corresponding to 15,9–63,7 μM [32]; thus it can be concluded that antitumor concentrations of progesterone may be tolerated in human. In nude mice, progesterone (50 and 100 mg/kg) inhibited neuroblastoma growth by ~50% over 8 d of treatment with serum concentrations reaching to 10–33 μg/mL (corresponding to 31,85–105,1 μM) with no toxicity [33]. Progestins are lipophilic hormones and cross the blood brain barrier and it is also possible that they accumulate in gliomas sufficiently to block cell growth. In human studies, it was shown a transfer from plasma to cerebrospinal fluid of about %10 for progesterone [34]. Synthetic progestins exert very low toxicity such that subcutaneous and oral LD50 doses of MPA are very high (6400 and 8000 mg/kg, respectively) (MPA Product Data Sheet). An alternative approach would be infusing progesterone analogues directly into the tumor cavity via Ommaya reservoirs, as progestins are not toxic like classical chemotherapy agents.

### 4.3. MPA blocks glioma spheroid growth and invasion at high dose

As mentioned, we observed dose dependent dual effects of MPA on monolayer growth of glioblastoma. However, high doses of MPA blocked spheroid growth and invasion of C6 rat glioma and U251 human glioma (Figs. 2 and 3). Our working hypothesis is that high doses of MPA acted via downregulating PR. Indeed, MPA suppressed growth of hormone dependent human breast cancer cells, with simultaneous decrease of the PR content by more than 80% [35]. MPA could downregulate PR expression via heterospecific modulation of the glucocorticoid receptor [36]. In human endometrium cells, progesterone upregulates stromal PR expression but reduces epithelial PR expression [37], whereas MPA downregulates PR expression both in uterine stromal and epithelial cells [38,39] and in endometrial cancer cells [22,27].

The native progesterone hormone also downregulates PR content both in U373 and D54 human glioblastoma cells [40]. High dose progesterone treatment upregulated inhibitory PR-A isoform yet downregulated PR-B isoform in human neuroblastoma xenografts [33]. It was argued that high doses of progesterone acted via non-genomic progesterone receptors in glioma cells as mifepristone was not capable to block progesterone actions and unexpectedly exerted progesterone-mimetic [15] effects as relevant reference. But mifepristone also has PR-agonistic functions [41] and mifepristone and progesterone mutually reinforce their anti-proliferative and apoptotic actions on endometrial cancer cells at high doses. Therefore, we think that high dose progestins and mifepristone acted via similary pathways, mainly via PR downregulation.

Following the downregulation of PR, several mechanisms may account for MPA suppression of invasion in glioma and meningioma spheroids and for the dexamethasone effects in glioma. MPA reduces

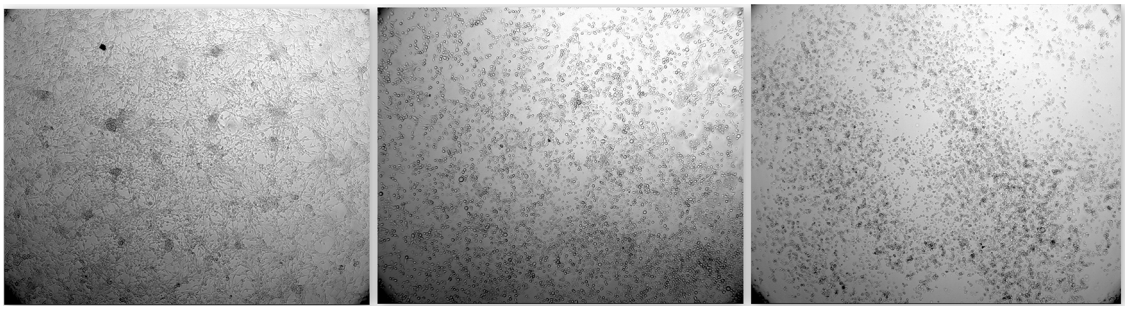

**Fig. 7.** Effects of MPA on the U251 monolayer culture at the 96th hour. a – control, b – 52 μM MPA, c – 104 μM MPA. Note complete necrotic cells at the highest dosage.

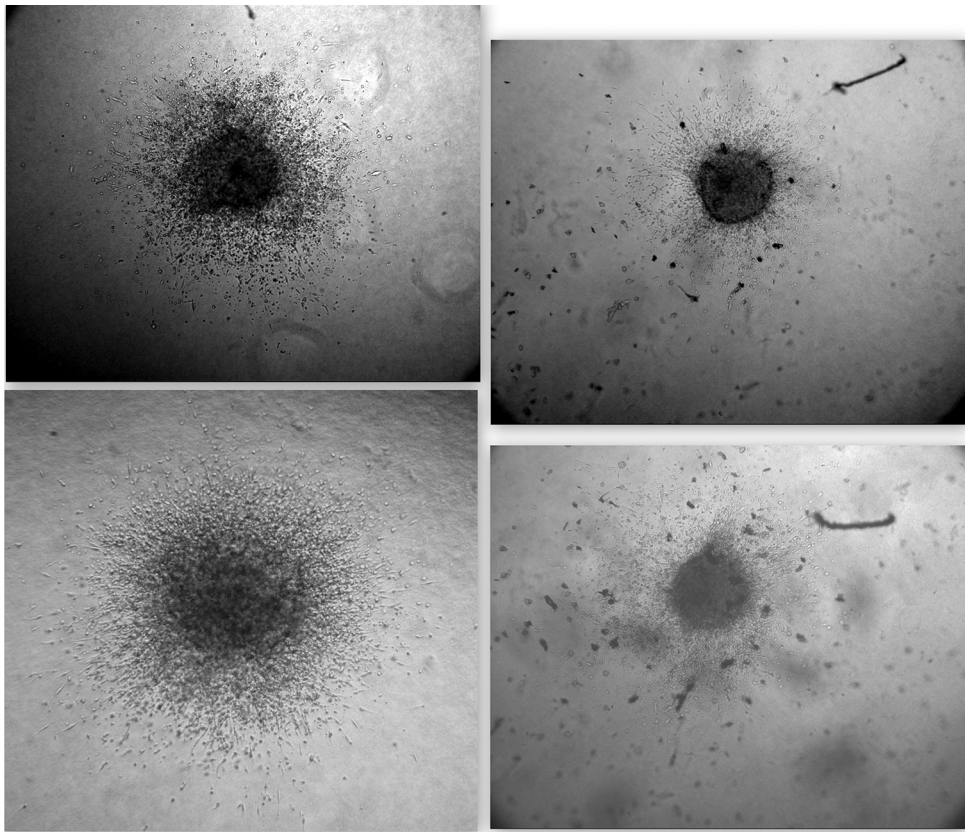

**Fig. 8.** Effects of MPA on the U251 spheroid growth. Upper lane depicts effects of 24$^{th}$ h of treatment, control group (a), 104 μM MPA-treatment (b). Lower lane depicts effects of the 96$^{th}$ hour of treatment, control group (c), 104 μM MPA-treatment (d). Note fusiform invading cells at the controls versus rounded and necrotic cells with cell debris at the spheroid periphery in the MPA-treated groups.

release of metalloproteinases from endometrial cancer cells including proMMP-9, proMMP-2, and MMP-2, which degrade extracellular matrix and facilitate tumor invasion [42]. MPA attenuates breast cancer metastasis and enhances the anti-invasive and anti-metastatic Nm23-H1 protein expression within the metastatic nodules similar to dexamethasone [43]. Lastly, MPA is capable to block RANK/ RANKL induced endometrial cancer cell invasion [44]. Overall, MPA exerts versatile inhibitory effects on cancer invasion, which is parallel to our findings in glioma spheroids and in a primary spheroid culture of meningioma.

### *4.4. Additive actions of 5-FU and MPA in blocking C6 glioma spheroid growth and invasion*

5-FU is a potent anticancer chemotherapeutic, which is used in the treatment of a number of malignancies including cancers of the colon, stomach, pancreas and breast; it principally acts via inhibition of thymidilate synthase and subsequently the production of thymine essential for DNA replication and cell proliferation. Recent data from a clinical study revealed that delivering high levels of 5-FU directly to gliomas was well tolerated with responses lasting a median of 3 years [45]. In breast cancer, adding adjuvant MPA to a chemotherapy regimen including 5-FU enhanced survival in postmenapasual patients [46,47]. In animal breast cancer, MPA augmented antitumor activity and reduced systemic toxicity of 5-FU [48]. 5-fluoro-2′-deoxyuridine (FdUrd) acts tumoricidal after converting to 5-FU by thymidine phosphorylase (which is also a growth factor, platelet derived-endothelial cell growth factor PD-ECGF); and murine glioma cells are less sensitive to FdUrd [49]. 17α-hydroxyprogesterone enhances thymidine phosphorylase [50]; however, it is unknown how MPA and 5-FU interact in glioblastoma. As murine glioma cells are less sensitive to the tumoricidal efficay of 5-FU, we determined MPA and 5-FU interactions in C6 rat glioma spheroids, to mimicry a relatively resistant tumor model. In our study, MPA did not hinder anti-tumor efficacy rather slightly augmented antitumor efficacy.

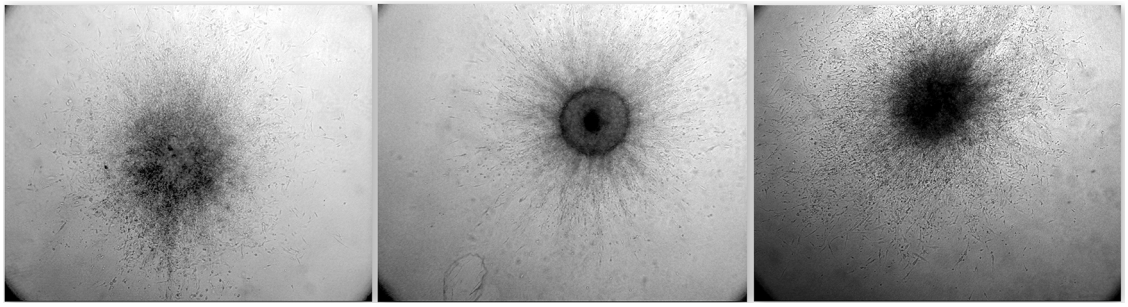

**Fig. 9.** Reduction of U251 glioma spheroid invasion with dexamethasone and alleviation of dexamethasone efficacy with low dose (10 nM) of wortmannin. a) Control, b) Dexamethasone, c) Dexamethasone + Wortmannin.

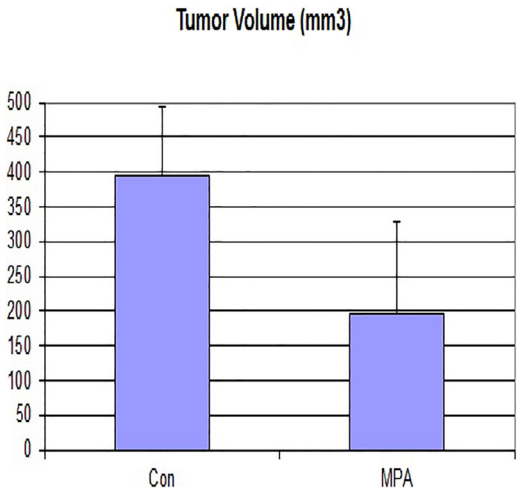


**Fig. 10.** Tumor volumes in the control group and MPA-treated rats. MPA-treated reduced the mean tumor volume by 49.3% inhibition ($p = 0.0043$). Error bars represent standart deviations.

### *4.5. Lesser known antitumor functions of the PI3-Kinase pathway. Emphasis to glial tumors and relevance to actions of MPA and dexamethasone*

PI3-Kinase pathway is mostly known for its tumorigenic effects, and as expected, LY294002 and wortmannin blocked growth and invasion of human glioma spheroids in our study. However, low doses of PI3K-inhibitors reduced anti-growth and anti-invasive functions of MPA and dexamethasone, which was more prominent for the latter. These results were surprising for us at the first glance, yet a detailed literature analysis provided clues to understand these findings. Corticosteroids are used in the clinic in alleviation of glial tumor edema [51], yet corticosteroids may also compromise survival in glioblastoma [52]; indicating that corticosteroids may induce both beneficial and detrimental pathways on glioblastoma cells. Several studies exist which demonstrated autophagic, anti-tumor and anti-invasive roles of the PI3K pathway, which may associate with the dual actions of glucocorticoids on the biological behavior of brain tumors. Autophagic death of liver cells, colorectal cancer cells, 3-deoxyadenosine/cordycepin-induced human glioma cell autophagy and panaxydol-induced glioma cell differentiation are blocked by PI3K-inhibitors [53–56]. An unexpected crosstalk was discovered between the PI3K/TOR pathway and PINS (*partner of inscuteable*) to suppress tumor formation in neural stem cells in Drosophila [57].

PI3K-pathway may also involve in blockage of invasion with dexamethasone and MPA. Loss of adhesiveness correlates with enhanced tumor cell motility and LY294002 blocked breast cancer cell adhesiveness induced by dexamethasone [58]. Dexamethasone also induced pro-adhesion and anti-migration in MG-63 osteosarcoma cells by up-regulating RhoB via the PI3K/Akt pathway [59]. These results are parallel to our findings that low doses of PI3K-inhibitors attenuated antigrowth and antiinvasive actions of dexamethasone on glioma spheroids. On the other hand, while both low and high doses of LY294002 reduced anti-growth efficacy of MPA in spheroids, wortmannin at both low and high doses potentiated the anti-growth efficacy of MPA. Here, it shall be noted that there exist substantial differences between the fungal metabolite wortmannin and a flavonoid quercetin derivative, LY294002 to modify cell signaling [60]. These inhibitors block a variety of kinases and lack specificity within the PI3K family [61]. Wortmannin, but not LY294002 blocks myosin light chain kinase – MLCK [62], autophosphorylation of Platelet-Derived Growth Factor (PDGF) [60] and PI4-Kinase [63], which may account for the differences between these two pharmacological agents to modify steroid inhibition of invasion.

### *4.6. Higher antigrowth and antiinvasive actions of MPA in coxsackie-adenovirus receptor expressing glioma cells*

Coxsackie-adenovirus receptor (CAR) is a membrane glycoprotein [64] which involves in embryonic development, cell adhesion and tumor cell growth [65]. CAR expression decreases with enhanced aggressiveness and grade of human glial tumors and transfection of U87MG glioma cells with CAR reduces its 3D-spheroid growth and invasion *in vitro* and its intracranial growth *in vivo* [66]. CAR and JAML (Junctional Adhesion Molecule-Like Protein) interact, in which CAR-mediated clustering of JAML recruits PI3-K to a JAML intracellular sequence motif [65]. Anti-invasive actions of both CAR and steroid hormones may involve PI3K-associated pathways and *CAR* gene has a promoter site for estrogen receptor and stimulated with estrogen in breast cancer cells [67]. Hitherto, no study questioned interactions of progestins and glucocorticoids with CAR signaling in modifying tumor growth and invasion. Therefore, we also compared effects of MPA and dexamethasone on 3D-spheroid cultures of the native and CAR-

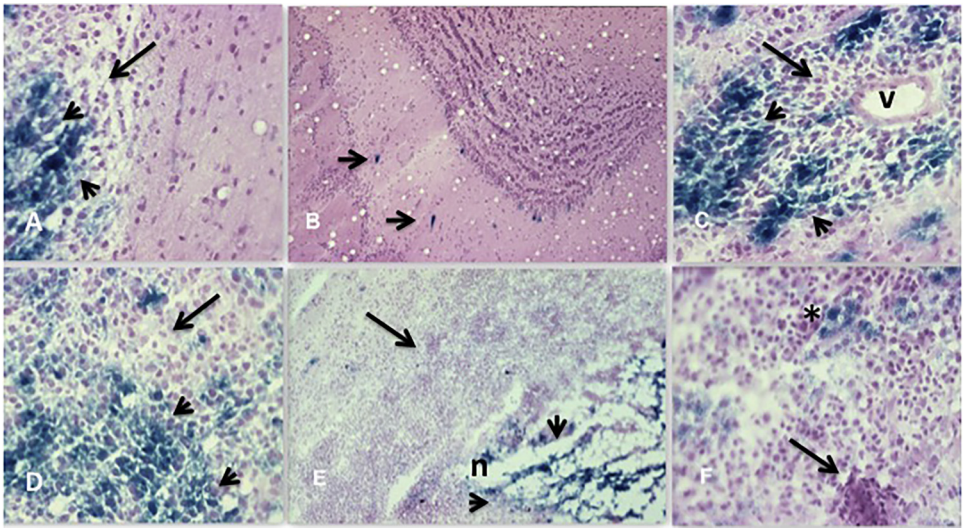


**Fig. 11.** Morphological effects of MPA treatment on the intracranially implanted C6 glioblastoma. Blue-stained cells are β-galactosidase transfected C6 glioblastoma tumor cells. A) Transition zone between glial tumor cells and healthy parenchyma, lymphomonocytic infiltration (large arrow) is seen between the dark blue-stained tumor cells (short arrows) and healthy brain parenchyma. B) Distant infiltration of fusiform glioma cells (short arrows) into the brain parenchyma. C) Perivascular (v) lymphomonocytic infiltration (large arrow) surrounded by dark blue-stained tumor cells (short arrows). D) Intense lymphomonocytic entrapment (large arrow) of dark blue-stained tumor cells (short arrows). E) Tumoral necrosis area (n) surrounded by lymphomonocytic cells (large arrow). Necrotic clusters of tumor cells are witnessed (short arrow). F) Extracellular punctate staining with β-galactosidase-stain (*) surrounded by lymphomonocytes indicating likely immune-mediated cell death of glioma cells. In some areas lymphomonocytes exerted follicle-like clusters (large arrow). Magnifications: A), C), D) and F): 40 × 0.25, B) and F): 10 × 0.25. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transfected glioblastoma cells. In our study, anti-growth and anti-invasive effects of MPA were more prominent in glioblastoma cells enforced to express CAR. In these experiments, basal anti-growth and anti-invasive effects of MPA and Dexamethasone were lower in glioblastoma cells transfected with an empty vector (even dexamethasone slightly promoted invasion). To provide stable gene expression, transfection experiments involve application of geneticin, a lipophilic antibiotic and analogue of neomycin, which is shown to block glioblastoma growth [68]. Hence, transfected glioblastoma cells – even when they were transfected with empty vectors – may be more resistant to lipophilic steroids.

### 4.7. MPA effects on Rat Glioma Model in vivo

Lastly and most importantly, MPA treatment (40 mg/kg) reduced C6 glioma by around 50%. One important feature what we witnessed is the prominent lymphomonocytic infiltration around and into glioma cells in groups treated with MPA. Considering that the MPA also binds glucocorticoid receptors, it may be assumed that MPA would act immunosuppressive. However, pregnancy and progesterone analogues exert peculiar dual inhibitory and stimulating effects on immunity (likely to avoid immune rejection of the semi-allogeneic fetus without compromising general immune defence of the mother). Indeed, addition of MPA to IL-2 immunotherapy in renal cancer enhances free-from progression period [69] and stimulates natural killer cell infiltration into endometrial cancer cells [70]. It would be tempting to study the interactions of MPA with immune stimulating agents in future animal models of glioma.

## 5. Conclusions

Considering the grave prognosis of gliomas, development of new therapeutic protocols is an essential need. Chemo-endocrine protocols may be novel and promising strategies in treatment of glioblastomas. Progestins may not just block growth of gliomas but also protect against marrow toxicity of chemotherapeutic drugs [71]; and hence, there is also the chance that progestins may allow to apply dose-intense chemotherapy (e.g. temozolomide) protocols in treatment of glioblastomas. Since progestins interact with other sex steroid pathways, gender specific effects of these agents shall also be studied in animal models. Future studies shall also measure intracerebral and intratumoral levels of progestins following high dose systemic progestin application. Considering that dosages such as 40 mg/kg and above are high for human application, animal studies with microinfusion pumps may also be logical for modelling human Ommaya reservoir applications. Progestins are not toxic like cytotoxic chemotherapy agents; hence, high doses of progestins can be easily applied via intratumoral route without causing significant cerebral toxicity. In conclusion, we think that progestins strongly deserve to be studied in future cell culture and animal models as likely candidates for novel treatments in high grade glial tumors.

## Conflict of interest

None.

## Acknowledgement

The authors thank to Dr Kuo-Chen Huang for providing CAR-transfected glioma cells and Ms Zafiro Koty for intracranial implantation of C6 glioblastoma cells.

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