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Medroxyprogesterone effects on colony growth, autophagy and mitochondria of C6 glioma cells are augmented with tibolone and temozolomide
Cell kinetic and electron microscopical studies with a broad review of the literature

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ABSTRACT

Objective: Risk of high grade gliomas is lower in young females and its incidence enhances after menopause suggesting likely protective roles of female hormones. Hormone replacement therapy (HRT) was widely employed to treat osteoporosis and some epidemiological studies showed that HRT regimes including progesterone analogs such as medroxyprogesterone acetate (MPA) decreased risk of glial tumors. Tibolone is a unique progesterone analog employed in HRT with tissue specific estrogenic effects and stimulates gene expressions very similar to those induced by MPA. Tibolone's pro-estrogenic effects occur particularly in bone and brain and both MPA and tibolone inhibit AKR1C enzymes, which involve in temozolomide chemoresistance. Hence, we aimed to investigate interactions between MPA, tibolone and temozolomide in modification of glioma cell growth and fine structure. Patients and Methods: For our studies, we have particularly chosen C6 rat glioma cell line due to several reasons: i) We previously showed that MPA reduced growth and induced procarbazine-sensitization in C6 cells; ii) temozolomide has a triazene-type molecular structure like procarbazine; iii) other groups previously showed that C6 glioma cell line is more resistant to temozolomide than human glioma cells; hence it may provide a native model of chemoresistance. Monolayer plating efficacy, soft agar colony growth, 3D-spheroid S-phase (as determined by BrdU-labeling) and electron microscopical analyses were performed to assess mutual interactions between MPA, tibolone and temozolomide. Results: MPA inhibited clonogenic growth of C6 glioma and this effect is augmented by both tibolone and temozolomide. MPA and tibolone inhibited DNA synthesis in C6 glioma spheroids to similar levels which can be achieved with temozolomide. Electron microscopical analyses revealed synergisms between MPA, tibolone and temozolomide involved mitochondrial proliferation, condensation, mitophagy and autophagy. Conclusions: MPA and tibolone shall be studied in further experimental models of glioblastoma *in vitro* and *in vivo*.

1. Introduction

Risk of high grade gliomas is lower in young women and its incidence increases after menapause suggesting a likely preventive role of female hormones [1–3]. Despite some controversies, most of the epidemiological studies showed that postmenopausal hormone replacement therapy (HRT) including progesterone analogs associate with lower risk of gliomas [1–3]. Gliomas may exert accelerated growth during pregnancy; yet intriguingly, parousity reduces lifetime risk of gliomas which can be explained by the dichotomous proliferative and antiproliferative influences of progesterone at low versus high levels [4,2,3]. In fact, progesterone levels gradually increase in gestation up to 200-fold and the incidence of highly angiogenic brain tumors decreases in the last trimester [5].

In gliomas, the dominant cytosolic PR is PR-B which enhances cell growth, while PR-A reduces cell growth. Medroxyprogesterone acetate (MPA) is employed as a hormonal agent in treatment of endometrial carcinoma and downregulates PR-B [6]. We previously showed that

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MPA inhibits growth and increased procarbazine sensitivity in C6 rat glioma cell line [7,8]. Procarbazine is a triazene agent with chemical similarity to temozolomide, which is the current chemotherapy for high grade glial tumors. Therefore, one of the aims of our current study is to determine whether MPA and temozolomide synergy in clonogenic assays and in fine structural damage in C6 glioma. MPA exerts partial androgenic activity and in theory, progesterone analogs with partial estrogenic activity – especially in the brain – may block glial tumor growth alone and potentiate MPA efficacy via neutralizing the androgenic effects in the brain.

Tibolone is a progesterone analog, which possess tissue specific estrogenic (bone and brain) effects and which is being used in HRT. The 3α-hydroxytibolone and 3β-hydroxytibolone metabolites bind to the estrogen receptors in the brain, with minimal effects on the endometrium [9]. According to some studies, tibolone may enhance risk of mammary and female reproductive cancers [10,11]; but peculiarly, there exist several basic scientific data that it acts antitumoral at higher concentrations than which can be achieved in HRT [12,9,13,14]. Furthermore, there also exist clinical evidence that tibolone employed as a HRT in older women decreases risk of invasive breast cancer and colon cancer.

These features closely resemble to that of MPA which enhances risk of breast cancer in HRT protocols [15]; but at higher dosages, it can cause regression of far advanced breast, endometrium and renal cancers [16,17,8,18,19]. Moreover, molecular studies have shown that MPA and tibolone induce very similar gene expression patterns [13,14]. Therefore, we hypothesized that high dose tibolone with both progestagenic and estrogenic actions may block glioma growth alone and/or in synergy with MPA or temozolomide. In pilot experiments, we assessed dose dependent effects of tibolone in human glioblastoma cells grown as a primary culture and in C6 rat glioma cells. As we have shown previously [20], tibolone was more effective in reducing growth of human glioblastoma cells.

Nonetheless, we continued our studies with C6 glioma cells to mimicry chemoresistant phenotype of glioblastoma, as human glioblastoma xenografts are more easily inhibited with temozolomide in comparison to orthotopic C6 glioblastoma [21,22]. Moreover, temozolomide at doses between 100 and 1000 μM fail to induce cell death in C6 rat glioma [23]. We used 400 μg/ml (2.06 mM) of temozolomide in our drug combination experiments (continuous exposure to 200 μg/ml of temozolomide was capable to reduce C6 glioma cell counts by 21.3% at 48 h). We determined effects of MPA, tibolone and temozolomide on the S-phase of C6 glioma spheroids as assessed by Bromodeoxyuridine (BrdU)-labeling. At the beginning, we also aimed to determine effects of drug combinations on S-phase. Nonetheless, drug combinations have significantly changed spheroid diameters, caused incoherent results and perturbed correct evaluation of the S-phase fractions. Hence, we only present data on the separate effects of these drugs on DNA synthesis in C6 glioma spheroids.

2. Materials and methods

2.1. General cell culture conditions and assessment of monolayer cell growth with temozolomide treatment

C6 glioma cells obtained from American Type Culture Collection (ATCC) were maintained using RPMI-1640 (Biological Industries, Haemek, Israel) with 15% heat inactivated fetal calf serum (FCS), 0.2 mM glutamine, 50 μg/ml neomycin and 100 μg/ml streptomycin. The flasks were kept in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. Cells prepared in 5 ml of RPMI-1640 were plated into a six-well plate in 1×10^5/ml concentration with 100% vitality. For colony assays, MPA and tibolone were dissolved in ethanol and the ethanol concentration was always 200-fold lower than the incubation medium, which did not influence cell growth. There were separate controls for MPA, tibolone and temozolomide. To determine efficient doses of temozolomide, C6 cells were treated with increasing concentrations of temozolomide (0.1, 1, 10, 100, 200 μg/ml of temozolomide (Sigma-Aldrich, CAS-Number: 85622-93-1) corresponding to 103 μM, 103 μM, 103 μM, 103 mM, 206 mM, respectively. After 48 h of drug treatment, cells were harvested with trypsin-EDTA C (Biological Industries, Haemek, Israel). Vital cells were identified by trypan blue exclusion and counted on a Thoma chamber. To reveal changes of the cell proliferation, only vital cells are compared between different groups.

2.2. Soft agar colony growth assay

Stock agar (Gibco, 152-00391 M) solution of 0.3% concentration was prepared at 45 °C. Four volumes of RPMI-1640 was rapidly mixed with one volume of stock agar solution and 1 ml of this solution was filled into each well of a six-well plate and used as an underlayer. Subsequently, plates were incubated at 4 °C for 15 min. For each well 3×10^3, 100% vital cells were suspended in 1 ml RPMI-1640 media and mixed with 2 ml of (4 vol. RPMI + 1 vol. Stock agar solution mix). One milliliter of this suspension was put onto each under layer of six-well plates. At first, to obtain a dose response curve in regard to tibolone inhibition of colony growth, cells were treated with 10-fold increasing tibolone doses between 0.1–100 μg/ml (equal to 10 fold increasing molar concentrations between 0.3205 and 320.5 μM, respectively; Sigma-Aldrich, CAS-Number: 5630-53-5). In other groups, cells were then treated with MPA (100 μg/ml; 260 μM), tibolone (100 μg/ml; 320.5 μM), tibolone + temozolomide (temozolomide dose was 400 μg/ml) and tibolone + MPA. Six-well plates were then kept in an electronic incubator under a humidified atmosphere of 4.5% CO_2 and air mix. Colonies containing more then 50 cells were counted under an inverted microscope. In dose-response experiments, incubation time was 7 days. In drug combination experiments, when the combination groups exerted no more growth than approximate 20 colonies following 5 days and even begin to degenerate, experiments were stopped and colony growths were compared at that time period. Colony inhibition was calculated with the following formula: Clonogenic Cell Death % = 100 × (1-colony number of drug treated group/colony number of control group).

2.3. Spheroid cultures and S-Phase fractions assessed by BrdU-Labelling index in spheroid cultures

In six-well plates, 1 ml of cell medium (RPMI-1640) was mixed with 1 ml of 3% agar (Biobak) at 42 °C. Following the formation of soft agar and after cooling, 5 ml's of medium was put on to the agar and 1×10^6 vital C6 glioma cells were inoculated in this medium. Cells were cultured at 37 °C with 5% CO_2-air mix in humidified atmosphere (Sanyo Incubator 20 AIC). Every 2nd day after incubation, cells were investigated with inverted microscope to monitor formation of spheroids. Cells formed aggregates in the first two days after inoculation, and they formed spheroids after 3 days. At the 7th day, spheroid diameters were above 120 μm. At that stage, MPA (100 μg/ml), tibolone (100 μg/ml) and temozolomide (400 μg/ml) were added into the cell media. (In fact, there also existed groups consisted of combinations of MPA, tibolone and temozolomide treatments; however, spheroid diameters became smaller and provided incoherent results; hence they were excluded from further experiments and final analyses). After 48 h of drug treatment, plates were shaken on a heated water bath and spheroids were incubated with 20 μM of BrdU (Sigma-Aldrich, CAS Number: 59-14-3) for 1 h. Spheroids were then fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, Missouri, USA) in PBS for 24 h at 4 °C and then washed in phosphate-buffered saline (PBS). Following fixation the spheroids were dehydrated through graded ethanol, cleared in xylene, embedded in paraffin and 5 μm coronal sections were cut on microtome (Leica MR 2145, Heerbrugg, Switzerland). Sections were dewaxed in xylene for 30 min. After soaking in a decreasing series of ethanol,

sections were washed with distilled water and PBS for 10 min and then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37 °C for 15 min and washed with PBS. Sections were incubated in a solution of 3% H2O2 for 15 min, then washed with PBS and incubated with primary mouse anti-BrdU antibody (1:250 dilution, LabVision, UK). Next the sections were incubated with biotinylated IgG followed by streptavidin- peroxidase conjugate (LabVision, UK). The sections were then washed and incubated with the AEC chromogen substrate system and counterstained with Mayer's haematoxylin. The immunostained slides were observed under light microscopy at magnifications of x40. BrdU-labelled cells were observed by the same person. BrdUpositive cell types were determined by observing dark red AEC nuclear staining. Unlabeled nuclei with only blue haematoxylin staining and pale brownish nuclei were considered to be negative. Ten adjacent sections and at last 3000 cells were evaluated for each group.

2.4. Transmission electron microscopy

C6 glioma cell cultures were treated throughout 96 h with ethanol solvent (0.5%) for control, MPA (100 μg/ml), tibolone (100 μg/ml), MPA + tibolone, MPA + temozolomide (400 μg/ml of temozolomide) and tibolone + temozolomide for experimental groups, respectively. Then, the cells were tyripsinized and centrifuged. After harvesting cell pellets were fixed with 2% glutaraldehyde in 0.1 sodium cacodylate for 1 h at 4 °C. They were then washed twice for 10 min with 0.1 M sodium cacodylate (pH 7.4) and fixed with 2% osmium tetroxid in 0.1 M sodium cacodylate for 1 h at room temperature. Then samples were washed three times for 5 min each with same buffer. Cells were exposed 1% uranyl acetate for 1 h and washed three times for 10 min again each with same buffer. Samples were dehydrated via graded ethanol series. After embedding in Epon 812, and sectioning, they were stained with 5% uranyl acetate for 30 min followed by Reynold's lead citrate incubation. Samples were then examined with JEOL 100C transmission electron microscope.

3. Results

3.1. Dose dependent effects of temozolomide on monolayer growth of C6 rat glioma

Following 48 h of drug exposure, only 100 and 200 μg/ml of temozolomide was found to be effective in reducing monolayer growth of C6 glioma (p = 0042 and p = 00,069, respectively) (Fig. 1).

3.2. Soft agar colony growth assay

In dose-response experiments with soft agar clonogenic assay of C6 rat glioma, only 10 and 100 μg/ml of tibolone exerted a significant blockage of colony growth (p = 0032 and p = 000,042; respectively) (Fig. 2). In second set of experiments, both MPA and tibolone alone very efficiently suppressed C6 glioma colony growth (p = 000,017 and p = 000,011; respectively) (Fig. 3). Combination of MPA with tibolone exerted synergism and a more potent colony inhibition was found in comparison to tibolone alone (p = 0,000,036) and MPA alone (p = 000,016). Single temozolomide was also very efficient to block C6 glioma colony growth (p = 0,000,043) (Fig. 3). Both tibolone and MPA very significantly enhanced temozolomide efficacy to block C6 colony growth (p = 0004 and p = 0,000,098; respectively) (Fig. 3).

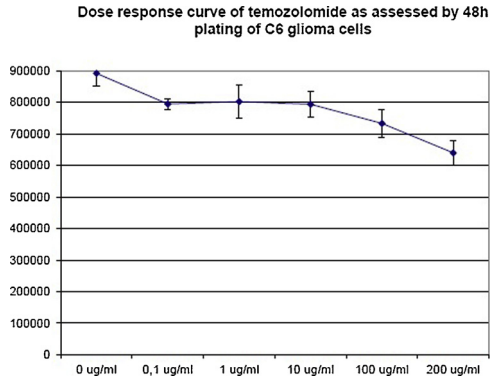

Fig. 1. Effects of various doses of temozolomide on monolayer growth of C6 glioma cells. X axis depicts doses as μg/ml and Y axis depicts total viable cell numbers.

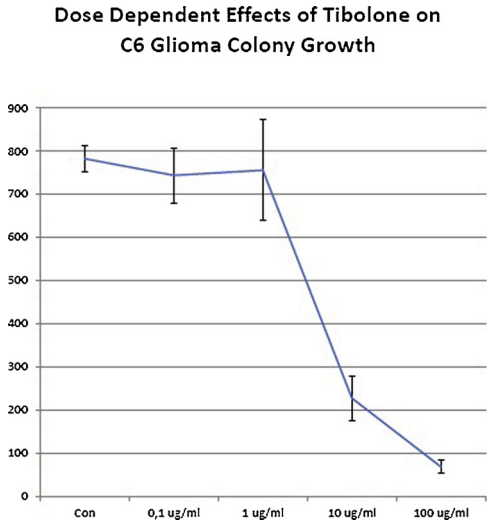

Fig. 2. Effects of various doses of tibolone on soft agar colony growth of C6 glioma cells. X axis depicts doses as μg/ml and Y axis depicts colony numbers.

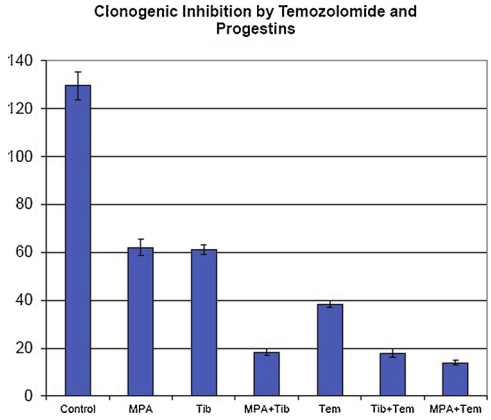

Fig. 3. Effects of progestins and/or temozolomide on soft agar colony growth of C6 glioma. X axis depicts drug names (Tib = tibolone, Tem = Temozolomide). Y axis depicts colony numbers. Dosages: MPA (100 μg/ml; 260 μM), tibolone (100 μg/ml; 320.5 μM), temozolomide (400 μg/ml; 2.06 mM).

3.3. Spheroid S-Phase (DNA synthesis) analysis with BrdU-Labeling

In control groups, BrdU-positive cells indicating active S-phase were significantly higher in small (lesser than 50 cells at the counted area) versus (between 50 and 250 cells at the count area) big spheroids. This difference may be due to better oxygenation and higher nutrient availability via diffusion in small spheroids. MPA acted very efficient to suppress S-phase in small spheroids (p = 0.00011); and this efficacy was even more significant in bigger spheroids (p < 0.00,001). Tibolone acted very efficient to block S-phase in C6 rat glioma spheroids, which was even near the same as temozolomide especially in small spheroids. Tibolone suppression of S-phase occurred with very high

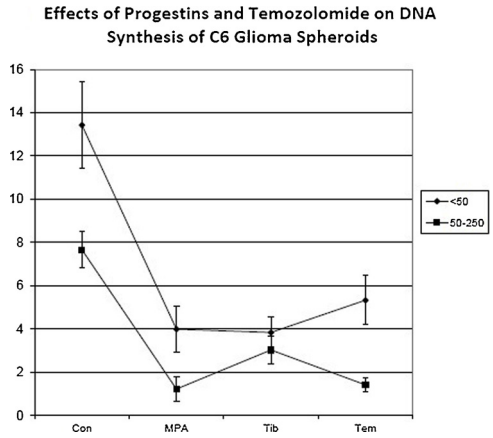

Fig. 4. Effects of progestins or temozolomide on the DNA-Synthesis (S-phase) of C6 glioma. X axis depicts drug names (Tib = tibolone, Tem = Temozolomide). Y axis depicts percentage of cells undergoing DNA synthesis. Dosages: MPA (100 μg/ml; 260 μM), tibolone (100 μg/ml; 320.5 μM), temozolomide (400 μg/ml; 2.06 mM).

significance in both small (lesser than 50 cells at the counted area) and big (between 50 and 250 cells at the count area) spheroids ($p = 0{,}0000018$ and $p = 00{,}069$; respectively). Temozolomide alone was also very efficient in suppression of the S-phase in both small and big spheroids ($p = 00{,}000{,}723$ and $p < 0{,}0000001$; respectively). Temozolomide acted more efficient in blocking S-phase in big versus small spheroids ($p = 0{,}0043$). This effect may be that the outer layer of spheroids underwent apoptosis to a greater extent with temozolomide chemotherapy. Endocrine and chemo-endocrine drug combinations have significantly changed spheroid diameters, caused incoherent results and perturbed correct evaluation of the S-phase fractions. Thus, they were not included into statistical analysis and for S-phase analyses, only the separate actions of MPA, tibolone and temozolomide are reported (Fig. 4).

3.4. Fine structural features as assessed by electron microscopy

In the control groups, (Fig. 5a and c), tubular mitochondria (Mt) and spherical mitochondria (Ms) were witnessed with proper alignment of the matrix. Few desmosomes (D) were seen between adjacent cells. Sparse autophagolysosomes (black arrow) and myelinated bodies (mlb) were observed. Golgi complex (G), centriol (C), and microtubules (Mic and arrows) were discernible (Fig. 5a). Most cell had almost all euchromatic nuclei (N) indicating very active gene transcription, nucleoli (nuc) were discernible in the majority of cells. Few nuclei exerted indentations (black arrow) (Fig. 5a). In the MPA-treated groups (Fig. 5b and d), tubular mitochondria (Mt) were enhanced which juxtaposed to phagolysosomes (Fig. 5b). At relatively lower magnification, mitochondria (Mc) were darker and more distinguishable from the cytoplasm and the majority of the nuclei showed indentations (black arrows) (Fig. 5d). Autophagic vacuoles (av) were also more abundant in comparison to controls (Fig. 5d).

In the MPA + tibolone treated groups (Fig. 6a–d), mitochondrial proliferations occurred to a higher extent which were mostly tubular (Mt) and condensed (mc). Autophagolysosomes (apl) and their coalescence into large residual bodies (apl-rb) were observed. Besides normal nuclei (N) and nucleoli (nuc), peculiar formation of nucleolar channel complex is seen (Ncc), which is normally encountered in endometrial epithelial cells exposed to progesterone (Fig. 6b). Larger and looser myelinated bodies (mlb-l), elongated and dysmorphic mitochondria were discernible (Fig. 6b and d; white arrows). Nuclear indentations (Fig. 6b–d; black arrows) were seen. Myelinated bodies (mlb) were adjacent to autophagolysosomes (apl), proliferated mitochondria were condensed (Mc) (Fig. 6c). A very demonstrative phase of mitophagic process was witnessed (Mpg); a peculiar and perfectly spherical nuclear inclusion body (asterisk sign in the nucleus of the upper cell in the figure) was seen (Fig. 6d).

In the MPA + temozolomide treated groups (Fig. 7a–d); interesting features were seen. While some cells had normal spherical mitochondria with proper cristae (Ms) (Fig. 7a), other cells had mitochondria with peculiar holes (Mh) inside (Fig. 7b; Mh). In some cells, the outer nuclear membrane was separated from the inner nuclear membrane like a blister (Fig. 7a). Enlargement of the endoplasmic reticulum (ER and arrow signs) cisternae was observed (Fig. 7b). Cells with endstage autophagy (Auto) were seen (Fig. 7c) and the nuclear indentations were much more frequent and prominent in comparison to single progestin treatments (Fig. 7d; black arrows).

In the tibolone + temozolomide treated groups (Fig. 8a–d); damaged mitochondria (dM) and lysosomes (Ly) adjacent to myelinated bodies were seen. Demonstrative signs of lysosome-autophagosome fusions were witnessed (Fig. 8b; thick black arrow). Some mitochondria were elongated and dysmorphic (Fig. 8b; white arrows). In higher

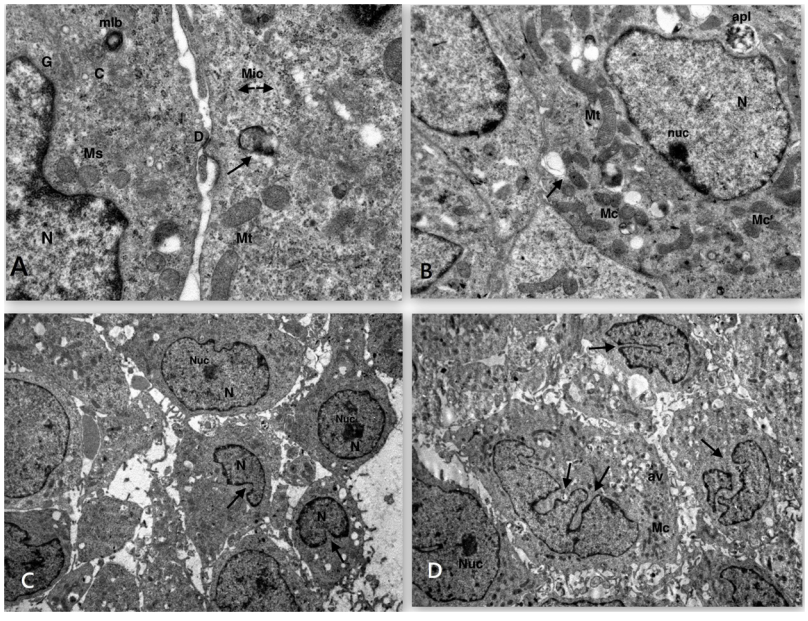

Fig. 5. a) **Control Group:** Healthy cells with heterochromatic nucleus (N), tubular (Mt) and spherical mitochondria (Ts), golgi apparatus (G), centriol (c) and microtubules (mic and "arrow" signs). Sparse myelinated bodies (mlb) and autophagic vacuoles (arrow) are discernible. (magn. × 25,000) **b) MPA Treated Group:** Mitochondrial proliferation and condensed mitochondria (Mc) are predominant, lysosomes juxtapose to mitochondria (arrow); an autophagolysosome (apl) resides besides the nucleus (N); normal heterochromatin and nucleolus (nuc). (magn. × 12,000) **c) Control Group:** Autophagosomes are much lesser, few nuclei showed mild indentation (arrow). (magn. × 5000) Nucleus (N) heterochromatin and nucleolus (nuc) structures are normal. **d) MPA Treated Group:** Proliferation of mitochondria and abundant autophagic vacuoles in the cytoplasm. Nuclei exert widespread indentations (arrows) (magn. × 5000). magn. = magnification.

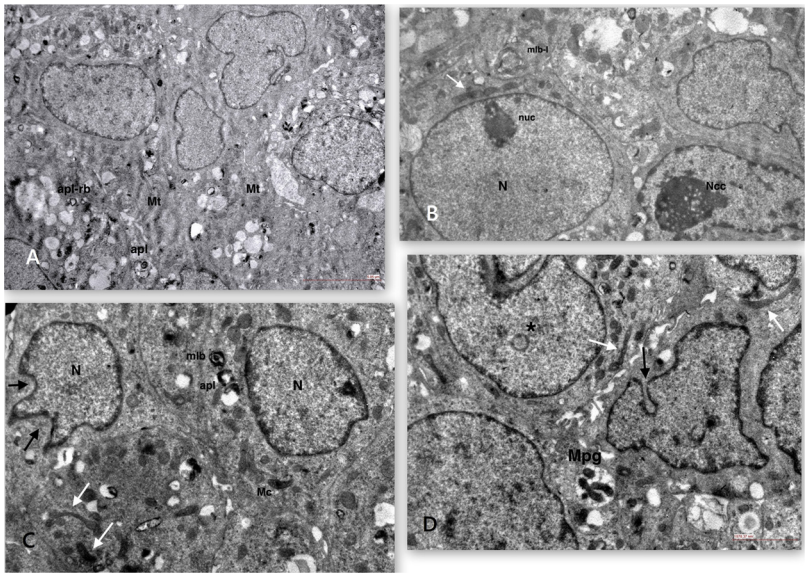

Fig. 6. a) **MPA + Tibolone Treated Group:** Abundant autophagolysosomes (apl) coalesce to residual bodies (abl-rb). Increased numbers of tubular mitochondria (Mt). (magn. × 5000) **b) MPA + Tibolone Treated Group:** Abnormally elongated and dysmorphic mitochondria (white arrow). Nucleolar channel complex formation (ncc) is seen within the nucleus. (magn. × 10,000) **c) MPA + Tibolone Treated Group:** Myelinated body (mlb) and autophagic vacuoles (apl) juxtaposing to the nucleus (N). Nuclear indentations are seen (black arrows). Abnormally elongated and dysmorphic mitochondria are present (white arrows). (magn. × 7500) **d) MPA + Tibolone Treated Group:** A very demonstrative morphological sign of mitophagy (Mpg). Abnormally elongated and dysmorphic mitochondria (white arrow). Nuclear indentations (black arrow). A perfectly spherical nuclear inclusion body (arrow) is discernible within the left upper cell. (magn. × 10,000).

magnification, some mitochondria seemed to be normal yet surrounded with lysosomes (Ly) and autophagic vacuoles (Fig. 8c; av). In completely autophagic cells (Auto), giant lysosomes and intense autophagolysosomes were seen besides the separation of outer nuclear membrane from the inner nuclear membrane like a blister (Fig. 8d).

4. Discussion

4.1. Relevance of research on progestagenic hormones in glioma and their influence on mitochondria in regard to antineoplastic effects

As suggested above, we previously showed that MPA, a progestagenic HRT agent reduced S-phase and increased procarbazine's antitumor efficacy in C6 rat glioma [7,8] and blocked invasion of human glioblastoma cells in spheroid cultures [3]. Another widely used HRT agent, tibolone induces gene expression patterns very similar to MPA [13,14]. Tibolone exerts both estrogenic and progestagenic features [9]; yet hitherto, no study investigated potentials of tibolone in glial tumors except a single study of ours [20]. After oral treatment with tibolone, three metabolites are found in circulation; 3α- and 3β-hydroxytibolone and a 3-keto-Δ4 isomer. The hydroxy metabolites only bind to the estradiol receptor (ER) while the Δ4 isomer and tibolone bind to the progesterone receptor (PR) [24]. Daily 2.5 mg tibolone treatment (equal to the dose used for HRT) yields blood levels of an approximate sum blood level of 2 ng/ml for active tibolone and its metabolites in circulation [25].

Experimental data suggest that tibolone at higher levels than used for HRT significantly blocks cancer growth. In benign human epithelial breast (HBE) cells, tibolone and its Δ4 isomer inhibited proliferation at 1 μM and induced apoptosis [26]. In the breast cancer cell line ZR75-1 containing progesterone and androgen receptors, tibolone increased apoptotic cells by 2 and 2.4 fold at 100 nM and 1 μM concentrations, respectively [12]. Moreover, in both ZR75-1 and T47-D cancer cell lines, tibolone significantly decreased expression of the anti-apoptotic bcl-2 protein [12]. When endometrial cancer cells were incubated with tibolone, total conversion into the Δ4 isomer was observed within 6 d [27]. When cells were treated with tibolone or its Δ4 isomer or MPA, prominent antiproliferative effects were observed with each of these three agents [13]. Upon measuring the expression of two progesterone-regulated genes (fibronectin and IGF-binding protein-3), tibolone, its

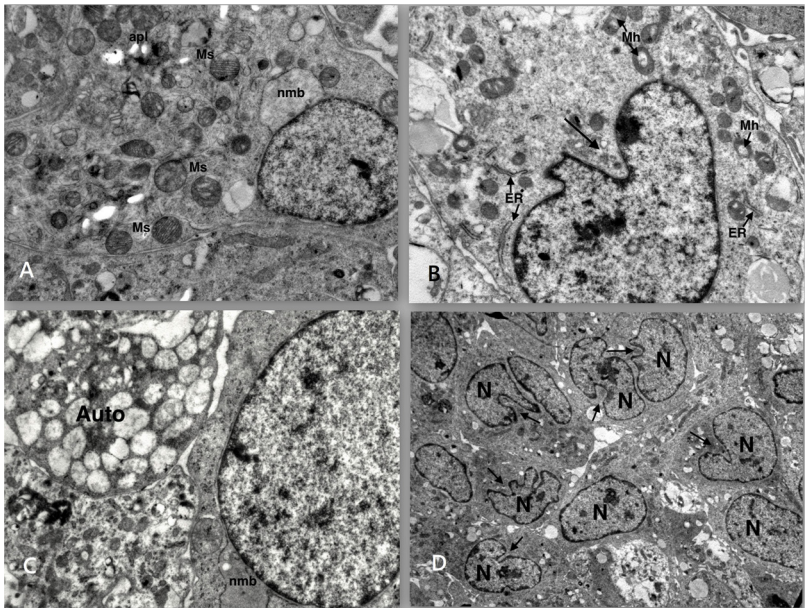

Fig. 7. a) **MPA + Temozolomide Treated Group:** Blister-like separation of the outer nuclear membrane (nmb). Abundant spherical mitochondria (Ms), autophagolysosomes (apl) are discernible. (magn. × 12,000) **b) MPA + Temozolomide Treated Group:** Nuclear indentation (black arrow), prominent enlargement of endoplasmic reticulum cisternae (ER) and peculiar mitochondrial holes (Mh). (magn × 12,000) **c) MPA + Temozolomide Treated Group:** Blister-like separation of the outer nuclear membrane (nmb) below the nucleus. The cell on the left is completely autophagic (auto). (magn. × 12,000) **d) MPA + Temozolomide Treated Group:** Cell nuclei exerted widespread indentations (black arrows) (magn × 4000).

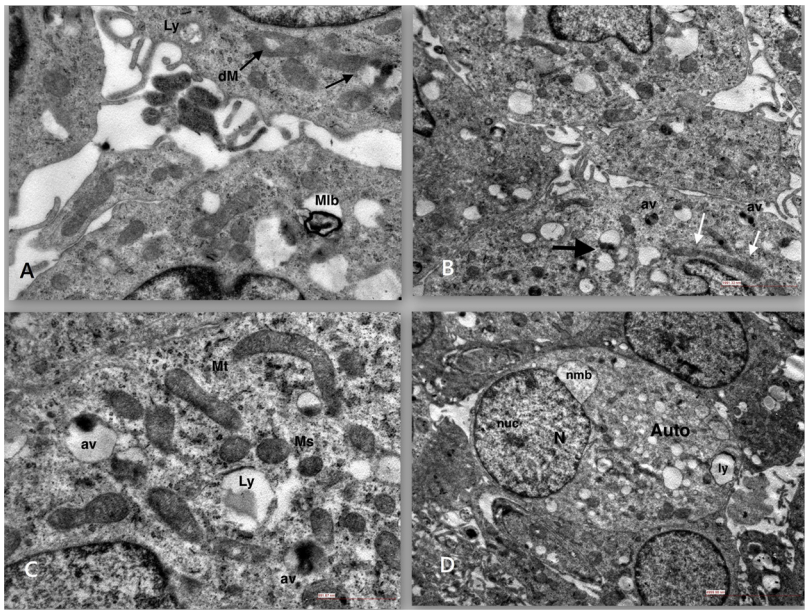

Fig. 8. a) Tibolone + Temozolomide Treated Group: Damaged mitochondria (dM), Lysosomes (Ly) and myelinated bodies (mlb) are discernible. Black arrows indicate wholes in the mitochondria and fusion of mitochondria with the autophagic vacuole. (magn. × 12,000) **b) Tibolone + Temozolomide Treated Group:** Autophagic vacuoles (av) are discernible, abnormally elongated dysmorphic mitochondria (white arrows), a demonstrative morphological feature of autophagosome-lysosome fusion (thick black arrow). (magn. × 12,000) **c) Tibolone + Temozolomide Treated Group:** Autophagic vacuoles (av) and lysosomes are witnessed, tubular (Mt) and spherical (Ms) mitochondria are seen. (magn. × 25,000) **d) Tibolone + Temozolomide Treated Group:** The cell in the center of the picture exerts blister-like separation of the outer nuclear membrane (nmb). The cytoplasm is completely autophagic (auto) with large lysosomes (Lys). (magn. × 6000).

Δ4 isomer and MPA showed similar gene expression regulation [13]. The human endometrial cancer cell line ECC1 was transfected to stably express PR-A and PR-B and then treated with E2, MPA, E2 + MPA or tibolone and their gene expression profiling was evaluated [14]. Growth and gene expression patterns revealed that tibolone acted prominently like MPA. Robust blockage of proliferation inhibition by both tibolone and MPA seemed to involve prereplication complex genes, such as the minichromosome maintenance genes [14]. In our study, both MPA and tibolone suppressed the S-phase (DNA Synthesis Phase) of 3D-spheroids of C6 glioma, synergized in suppression of clonogenic growth and mutually increased their mitochondrial and proautophagic effects.

It is generally accepted that cancer cells rely on glycolysis to cope with their energy demands, which is called as Warburg effect [28], yet it is more and more appreciated that cancer cells also use mitochondrial oxidative metabolism to produce energy [29]. Since many years of our experience in our electron microscopical studies, we observe that cancer cells increase their mitochondria following noxious stimuli mostly independent of the type of stressor. Our observations are supported with recent molecular studies. In endometrial carcinoma but not in the healthy endometrium, mitochondrial fission protein Drp1 is elevated and acts for adaptive response against respiratory complex I deficiency [30]. In stress conditions, not only the Drp1 but also the mitophagy protein BNIP3 is elevated in endometrial carcinoma [30], which is also parallel to our morphological observations demonstrating simultaneous mitochondrial proliferation and autophagy under endocrino-chemotherapy. In breast cancer cells, mitochondrial fission contributes to stem cell phenotype [31], and it is well-established that cancer stem cell phenotype increases in malignant cells surviving chemotherapy and radiotherapy. Here, intricate associations may also exist. Mitochondrial proliferations may be favoured in comparison to mitochondrial fusion events to cope with energy demands; yet it is not just quantity also the quality of mitochondria which is important for cancer cell survival. Damaged mitochondria may produce reactive oxygen species (ROS) more than the healthy mitochondria; hence their removal with autophagy may help cancer cells to survive [32]. On the other hand, excessive removal of mitochondria may also be detrimental for cancer cells. Therefore, the balance between mitochondrial fission/proliferation and also the extent of mitochondrial autophagy may determine the net outcome of the mitochondrial influence on cancer cell survival.

Effects of progestins on mitochondrial metabolism are complex. In carbontetrachloride-induced hepatic injury, MPA reduces mitochondrial swelling and liver damage [33]. On the other hand, MPA also increases the number of liver mitochondria suggesting that it may act as a stressor on liver cells [34]. Indeed, both progesterone and MPA enhance mitochondrial permeability transition pore opening in liver cells [35]. Progesterone induces the formation of superoxide anion and hydrogen peroxide in mitochondria during oxidation of complex I respiratory chain substrates but does not affect ROS production during oxidation of complex II respiratory chain substrates [35]. By their impact on ROS production in mitochondria, progesterone analogs were classified in the descending order as follows: progesterone > buterol > or = acetomepregenol > MPA > megestrol acetate indicating that MPA induces lesser ROS production in mitochondria than the native hormone [36]. On the other hand, MPA may diminish beneficial effects of estrogen on the cerebral mitochondrial metabolism by reducing mitochondrial levels of pyruvate dehydrogenase, cytochrome oxidase, ATP synthase, manganese-superoxide dismutase, and peroxiredoxin V [37]. It is also possible that MPA may uncouple detrimental effects on tumoral versus benign tissue mitochondria; for instance in endometrial carcinoma, high dose progestins deplete succinate dehydrogenase complex which resides in the inner mitochondrial membrane and which is the only enzyme that participates in both the citric acid cyle and the electron transport chain [38]. Moreover, recent studies showed that the antineoplastic activity of MPA in B-Cell Lymphoma cells correlated with mitochondrial superoxide radical formation [39].

Tibolone suppresses complex I of the mitochondrial respiratory chain by the inhibition of the NADH-oxidase activity in liver [40]. Tibolone also induces oxidative stress in perfused livers and isolated mitochondria, as indicated by the increased production of thiobarbituric acid reactive substances [40]. On the other hand, tibolone preserves mitochondrial membrane potential in T98 G glioblastoma cells under glucose deprivation [41] and palmitate stress [42]. Such actions may be detrimental during treatment of glioblastoma. Nonetheless, we think that *in vivo* studies would determine effects of tibolone and tibolone + chemotherapy effects on glioblastoma growth; since our ultrastructural findings on mitochondria and colony assays did not demonstrate glioblastoma-protective effects of tibolone. In the MPA and MPA + tibolone treated groups, mitochondria were condensed. Condensed mitochondria exert a smaller size with a darker matrix and packaging crista, which correspond to state 3 (enhanced) respiration and ATP formation. In T-lymphoblastic leukemia cells, TNF-α induced apoptosis is related to ultracondensed rather than swollen

mitochondria, favoring the theory that cytochrome C is released from state 3-respirating mitochondria in a reduced state.

4.2. Autophagy and mitophagy in MPA + Tibolone and MPA + Temozolomide groups

In the MPA + tibolone treated group there was enhanced mitophagy in comparison to their single effects. The first mechanism to cope with mitochondrial damage is the elimination of damaged mitochondrial proteins via chaperones and proteases; the second is the cytosolic ubiquitin-proteasome system (UPS) which removes proteins embedded in the outer mitochondrial membrane and the last step is the removal of the entire mitochondria through mitophagy, in the case of intensive organelle injury and dysfunction [43]. Hence, both tibolone and temozolomide seemed to shift MPA-induced mitochondrial stress to irreversible mitochondrial injury. As mentioned above, we also observed prominent increase in autophagy, enhancement of tubular mitochondria, and formation of bizzarre mitochondria with “holes” in MPA + temozolomide groups. The roles of autophagy and mitochondrial changes in the antineoplasticity of temozolomide are complex.

Kanzawa et al. showed that at a clinically achievable dose (100 μM), temozolomide induced autophagy, but not apoptosis in glioma cells [44]. The role of this autophagy on cellular death is intricate. When autophagy was inhibited at an early phase by 3-methyladenine, antineoplasticity of temozolomide was blocked [44]. It is not easily explicable why early events of autophagy are more related to the anticancer potency of temozolomide. Mitochondrial depolarization and mitochondrial permeability transition pore (MPTP) opening occured prior to temozolomide-induced autophagy, which were followed by the loss of mitochondrial mass. Electron transport chain (ETC) inhibitors reduced mitochondrial damage-induced autophagy, and enhanced temozolomide-induced apoptosis [45]. In parallel, chloroquine which inhibits autophagy increases temozolomide-induced apoptosis in glioma cells [23]. It may be likely that glioma cells sense the intensity of ROS and induces mitophagy to remove injured and potentially “dangerous” mitochondria. If there exists robust levels of ROS damage, the extent of mitophagy may not balance with formation of new mitochondria and the fate of cells are shifted to death.

Inside eukaryotic cells, mitochondria harbor their own extranuclear DNA (mtDNA), which is a major target for mutagens due to the lack of protective histones associated with mtDNA and the fact that mtDNA polymerase-γ replicates the DNA with poor fidelity [46]. In glioma cells, temozolomide decreases mtDNA copy number, enhance mitochondrial heteroplasmy, and induce prominent changes in the mitochondrial electron transport chain and cellular bioenergetics [46]. Several studies indicate that while inducing mitochondrial autophagy in response to temozolomide, glioma cells also try to increase the number of their mitochondria via fission events; which is parallel to our observations. For instance, Tbx2 protein increases in correlation with the grade of glial tumors and confers temozolomide-resistance which is an inducer of DRP1, a mitochondrial fission protein [47]. In parallel, melatonin inhibits mitochondrial transcription factors TFB1M and TFB2M and thereby the mitochondrial DNA transcription and enhanced temozolomide sensitivity of glioblastoma cells [48]. Further corroborating these findings, doxycycline – an antibacterial agent which can block mitochondrial proliferation – reverses temozolomide-induced glioblastoma cell aggressiveness and stem cell phenotype [49]. Both chloroquine and doxycycline are cheap –and relatively safe medicines and in future; they can be added to endocrino-chemotherapy protocols to further enhance anticancer efficacy.

Ceramide, a central intermediate of sphingolipid metabolism triggers lethal mitophagy in cancer cells [50] and in glioblastoma [51]. Ceramide synthesis is stimulated by progesterone in oocytes [52], and by temozolomide in glioblastoma cells [53,54]. Thus, enhancement of ceramide may explain synergistic increase of mitophagy, “mitochondrial holes” and reduction of tumor clonogenicity by progestins and temozolomide. Mutual synergisms between MPA, tibolone and temozolomide may also involve AKR1C enzymes, members of the aldoketo reductase (AKR) superfamily. AKR1C subfamily involves in cancer progression and agents which block these enzymes may act as novel antitumor agents [55]. For instance, jasmonates, plant stress hormones with molecular resemblance to prostaglandins exert anticancer activities [55]. AKR1C is a selective mediator of temozolomide resistance in glioblastoma [56] and MPA is a pan-AKR1C inhibitor [57]. Tibolone is also metabolized by AKR1C enzymes to its estrogenic metabolites and exerts substrate inhibitory efficacy on AKR1C2 [58]; thus MPA and tibolone augmentation of temozolomide efficacy in reducing C6 glioblastoma colony growth may also occur via AKR1C pathway.

We previously showed also that MPA-suppression of growth and increase of procarbazine sensitivity associated with lysosomal phospholipidosis/myelin body formation [8]. MPA and tibolone combination caused noteworthy amount of myelinated bodies suggesting increased lysosomal phospholipidosis. As lysosomal phospholipidosis is a sign of early autophagy, we conclude that progestin’s physiological autophagic actions may associate with its autophagic efficacy in tumor cells. For instance, in blastocyst implantation, the uterine luminal epithelia undergo programmed cell death which associates with progesterone stimulated Cathepsin-D and autophagy. Progesterone blocks the re-esterification of cholesterol in the endoplasmic reticulum and P-glycoprotein activity, which mediates glioma chemoresistance [8]. Among glial tumors, drug-sensitive types exert enhanced lamellated myelin bodies as signs of lesser tumor aggressiveness [8]. Hence, progesterone analogs may also affect glioma’s phospholipid and cholesterol metabolism, the permeability of cell membranes and the entrance of chemotherapeutic agents into the malignant cell cytoplasm.

5. Conclusions

Worldwide, huge numbers of experimental studies are conducted to find cures for malignancies with very poor prognosis such as pancreatic cancers and glioblastoma. Unfortunately, very limited progress is obtained until now. For instance, temozolomide - the golden standart of glioblastoma chemotherapy - yields only few months of additional survival. It is without doubt that discovery of novel molecules exerts tremendous importance in cancer research. Nonetheless, we think that repurposal studies are also important which investigate whether drugs approved for other indications than cancer treatment or for other types of cancers are efficient as novel antitumor agents for grave types of malignancies (eg. MPA is employed for the treatment of endometrial carcinoma and our experimental studies were the first to demonstrate its activity in glioblastoma models). We do not presume that progesterone analogues alone would provide a cure for glioblastoma, yet they may be suitable adjuvants for future chemo-endocrine protocols. In the future, we will conduct studies in primary human glioblastoma cell cultures and in animal models whether progestins exert this potential. We believe that progesterone analogues deserve to be studied further in this aspect.

Conflict of interest

None to declare.

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