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Tibolone attenuates inflammatory response by palmitic acid and preserves mitochondrial membrane potential in astrocytic cells through estrogen receptor beta

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ABSTRACT

Palmitic acid (PA) induces several metabolic and molecular changes in astrocytes, and, it is involved in pathological conditions related to neurodegenerative diseases. Previously, we demonstrated that tibolone, a synthetic steroid with estrogenic, progestogenic and androgenic actions, protects cells from mitochondrial damage and morphological changes induced by PA. Here, we have evaluated which estrogen receptor is involved in protective actions of tibolone and analyzed whether tibolone reverses gene expression changes induced by PA. Tibolone actions on astrocytic cells were mimicked by agonists of estrogen receptor α (ERα) and β (ERβ), but the blockade of both ERs suggested a predominance of ERβ on mitochondria membrane potential. Expression analysis showed a significant effect of tibolone on genes associated with inflammation such as IL6, IL1B and miR155-3p. It is noteworthy that tibolone attenuated the increased expression of TERT, TERC and DNMT3B genes induced by palmitic acid. Our results suggest that tibolone has anti-inflammatory effects and can modulate pathways associated with DNA methylation and telomeric complex. However, future studies are needed to elucidate the role of epigenetic mechanisms and telomere-associated proteins on tibolone actions.

1. Introduction

Estrogens play an important role in the brain (Arevalo et al., 2015). During several decades, estrogen withdrawal in women has been associated with cognitive impairment, increased Alzheimer's disease (AD) risk (Pike, 2017) and with psychiatric disorders such as schizophrenia (Kulkarni et al., 2015), depression (Schmidt et al., 2015) and anorexia nervosa (Ramoz et al., 2013). Interestingly, hormone therapy (HT) has demonstrated beneficial effects on cognition and mood (Luine, 2014), and the use of estradiol as an adjuvant in antipsychotic therapy improves symptoms of schizophrenia in women of child-bearing age (Kulkarni et al., 2015). However, HT has some potential side effects in women, including increased risk for heart disease, breast cancer and stroke (Gurney et al., 2014). In this regard, this caveat led to the development and implementation of more specific molecules, such as Selective Estrogen Receptor Modulators (SERMs) and selective Tissue Estrogenic Activity Regulators (STEARs) (Arevalo et al., 2011; Reed and Kloosterboer, 2004).

Tibolone is a synthetic steroid, classified as STEAR, which means that its activation of estrogen receptors in human tissues is dependent on the availability of enzymes in each tissue (Kloosterboer, 2011). Moreover, whereas tibolone actions are mediated by estrogen receptors (ERs) in bone and vagina, it also activates progesterone receptors in endometrial cells (Kloosterboer, 2001). In clinical and preclinical studies, tibolone has shown beneficial effects in the central nervous system (CNS) (Pinto-Almazan et al., 2017). For example, tibolone showed antidepressant effects in women suffering from depressive symptoms during menopause or post menopause (Kulkarni et al., 2018). However, its effect on cognitive abilities is controversial as while it improved the performance in a test of semantic memory it decreased performance in an executive function task (Fluck et al., 2002). Additionally, in *in vitro* studies of neuronal or glial cells, as well as *in vivo* studies using animal

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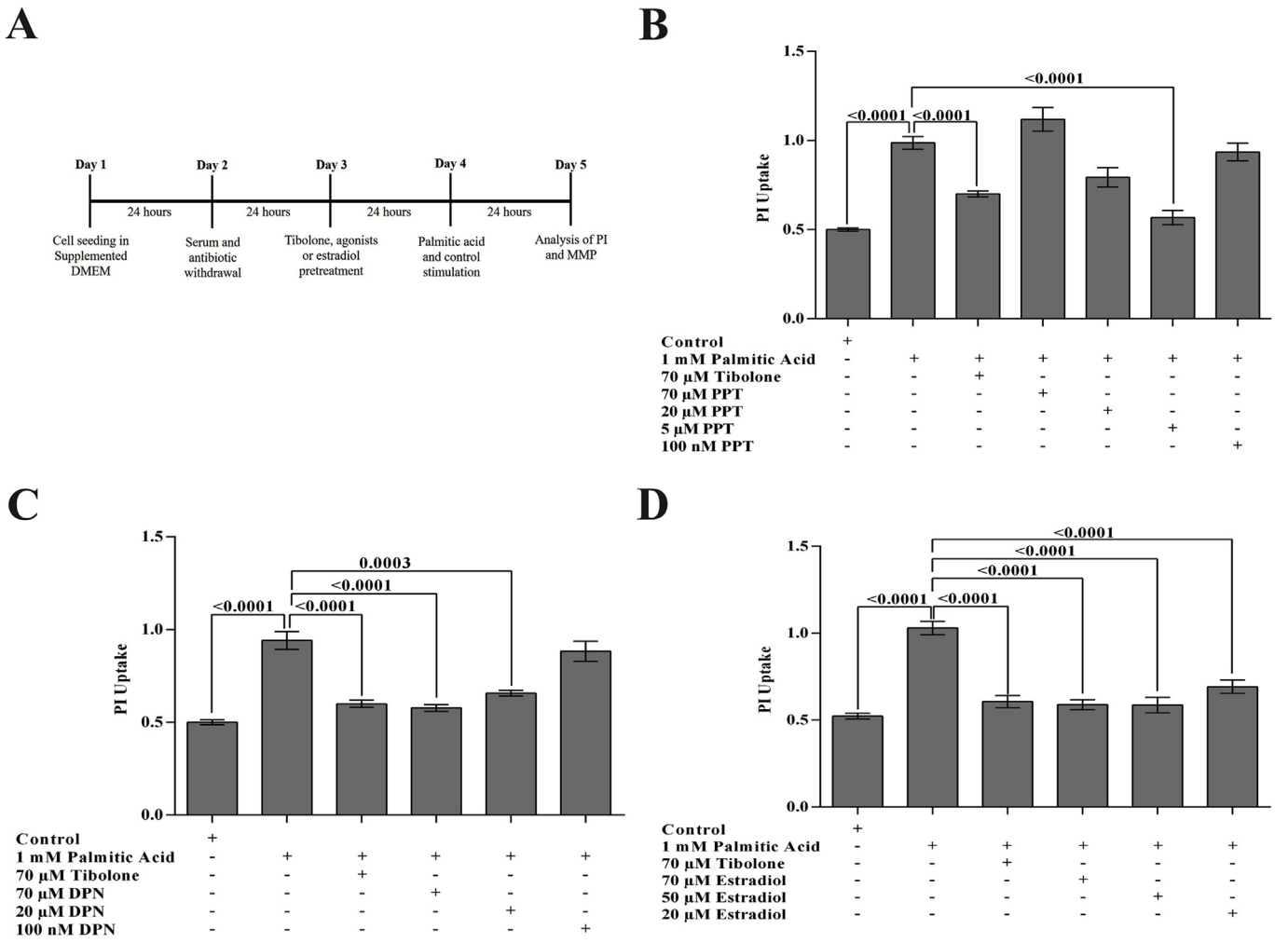


Fig. 1. Pretreatment with estrogen receptor agonists and estradiol protects T98G cells against death induced by palmitic acid (PA), similarly to tibolone. **(A)** Overview of experimental design: T98G cells were pretreated with estrogenic compounds for 24 h followed by 24 h of 1 mM palmitic acid stimulation, and, then, propidium iodide (PI) uptake and mitochondrial membrane potential analysis were performed. Mean fluorescence (a.u.) of PI uptake test for: **(B)** Control (0.5 ± 0.01), 1 mM PA (0.99 ± 0.03), 70 µM tibolone plus 1 mM PA (0.70 ± 0.01); ERα agonist (PPT) at 70 µM (1.1 ± 0.06), 20 µM (0.79 ± 0.05), 5 µM (0.56 ± 0.04), 100 nM (0.93 ± 0.04) plus 1 mM PA. **(C)** ERβ agonist (DPN) at 70 µM (0.57 ± 0.02), 20 µM (0.65 ± 0.01), 100 nM (0.88 ± 0.05) and 70 µM tibolone (0.59 ± 0.02) plus 1 mM PA. **(D)** Estradiol at 70 µM (0.59 ± 0.03), 50 µM (0.058 ± 0.04) and 20 µM (0.69 ± 0.04) and 70 µM tibolone (0.6 ± 0.03) plus 1 mM PA. For all experiments, control sample consisted in 2.5% BSA and 2 mM carnitine. Bar graph represents Mean ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis.

models, tibolone has shown to preserve mitochondrial functions, thus attenuating cell death and oxidative damage (Gonzalez-Giraldo et al., 2017). These findings suggest that tibolone might be a promising neuroactive steroid with therapeutic actions for several conditions affecting the CNS.

Postmenopausal women have increased susceptibility for developing obesity and chronic inflammation. One hypothesis is that, in women, the hormonal withdrawal during this critical period might account for augmented incidence of inflammatory conditions that may increase the risk of developing CNS pathologies. In this regard, these risk factors could contribute to AD onset in women (Christensen and Pike, 2015). Previous studies have shown increased levels of free fatty acids in the brain of AD patients, suggesting a possible implication of these molecules in the pathogenesis of the disease (Fraser et al., 2010). Indeed, obesity has negative effects on memory and cognition (Smith et al., 2011). Using animal models it has been shown that high levels of free fatty acids induce the expression of peripheral pro-inflammatory cytokines, which can cross the blood-brain barrier and induce metabolic and energy deficits in neurons, astrocytes, and other brain cells (Miller and Spencer, 2014). A growing number of studies have used palmitic acid (PA) *in vitro* using cells and *in vivo* in mouse to model the consequences of obesity (Alsabeeh et al., 2018; C. A. Martin-Jimenez et al., 2016). PA is a saturated fatty acid that, in addition to its presence in food, is also present in the cell membranes, where it can be generated by a *de novo* synthesis mechanism (Carta et al., 2017). Changes in cognition induced by PA have been evidenced in animal models (Contreras et al., 2017). In humans, a high diet in PA induced the activation of the basal ganglia regions including the striatum (Dumas et al., 2016). Although in normal conditions PA plays important physiological roles in cells, it is possible that an imbalance in its concentration can be deleterious for brain functions, thus leading to brain dysfunction (Hussain et al., 2013).

Astrocytes play a key role in the brain, as these cells are involved in fluid, ion, pH, and neurotransmitter homeostasis, synapse function, energy and metabolism and blood-brain barrier (BBB) maintenance (Sofroniew and Vinters, 2010). PA is able to activate different damaging responses in astrocytes, such as inflammation (Gupta et al., 2012), *de novo* ceramide synthesis (Patil et al., 2007) and endoplasmic reticulum stress (Ortiz-Rodriguez et al., 2018). Moreover, PA has a significant effect on mitochondrial functionality since this saturated fatty acid is able to dampen mitochondrial membrane potential (Gonzalez-Giraldo et al., 2017; Wong et al., 2014). Additionally, there is evidence that PA

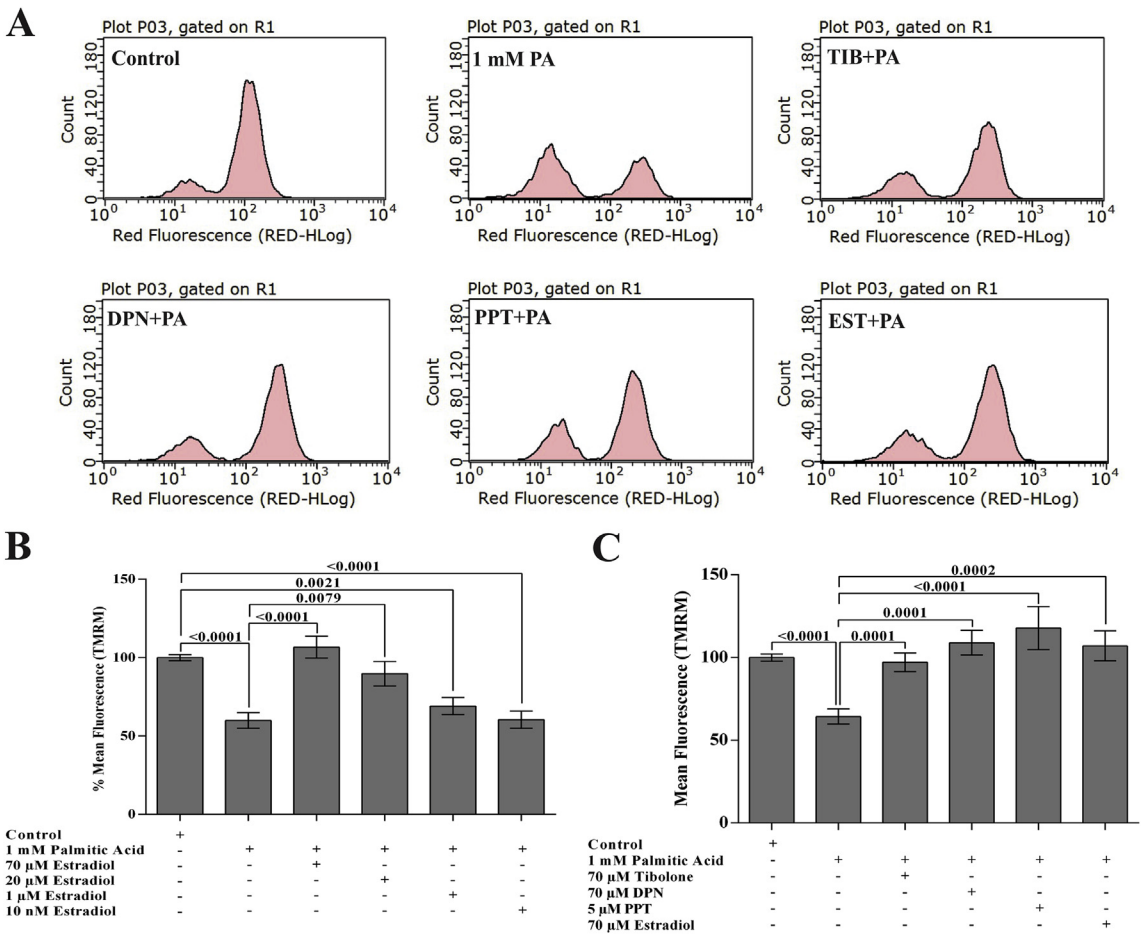


Fig. 2. Mitochondrial membrane potential is preserved by pretreatment with estradiol PPT and DPN. Once finished the treatments, TMRM staining was performed to quantify the Mitochondrial membrane potential (MMP). **(A)** Representative plots for different treatments analyzed by flow cytometry: Control (2.5% BSA and 2 mM carnitine), 1 mM PA, 70 µM tibolone + 1 mM PA, 70 µM DPN + 1 mM PA, 5 µM PPT + 1 mM PA and 70 µM Estradiol + 1 mM PA. **(B)** MMP for cells treated with Control (100 ± 1.9), 1 mM PA (60 ± 5) and estradiol at 70 µM (107 ± 6.9), 20 µM (90 ± 7.8), 1 µM (69 ± 5.4) and 10 nM (60 ± 5.4) before 1 mM PA stimuli. **(F)** Mitochondrial membrane potential analysis for cells stimulated with ERα agonist (PPT) at 5 µM (118 ± 12.9), ERβ agonist (DPN) at 70 µM (109 ± 7.4) and tibolone at 70 µM (97 ± 5.6) plus 1 mM PA. For all experiments, control sample consisted in 2.5% BSA and 2 mM carnitine. Bar graph represents Mean ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis.

actions on astrocytes can involve epigenetic mechanisms, such as DNA methylation (Su et al., 2015) and microRNAs (Geekiyanage and Chan, 2011).

Previous studies from our group showed that tibolone has protective effects against cell damage induced by PA on an astrocytic cell model (Gonzalez-Giraldo et al., 2017). In order to explore the possible mechanisms of actions of tibolone on astrocytic cells, in the present study, we aimed to determine which ERs are involved in the protective effects of this compound and how tibolone can modulate gene expression in cells treated with PA.

2. Materials and methods

2.1. Cell culture and drug treatments

In this study, the T98G cell line (ATCC® CRL-1690™) (Manassas, VA, USA), which expresses genes encoding astrocyte marker proteins (Litovchick et al., 2007), was used as an astrocytic cell model. Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM)-high glucose (Lonza, Walkersville, USA), supplemented with 10% fetal bovine serum (FBS) (Eurobio, Les Ulis, France) and 10 U penicillin/10 mg streptomycin/25 ng amphotericin (Lonza, Walkersville, USA), under 37 °C and 5% CO_2. Prior to experimental treatments, cells were serum deprived for 24 h. Then, cells were treated with 70 µM tibolone (Sigma, St Louis, MO, USA) for 24 h followed by 1 mM PA (Sigma, St Louis, MO, USA) for other 24 h, as previously described (Gonzalez-Giraldo et al., 2017). Control vehicle for PA was 2.5% Bovine Serum Albumin (BSA) and 2 mM Carnitine, and tibolone was dissolved in DMSO at a final concentration of 0.2%, as previously described (Gonzalez-Giraldo et al., 2017).

2.2. Treatment with ER agonists and antagonists

To assess whether tibolone's actions in PA-treated astrocytic cells involve the activation of a specific ER, we treated cells with either the ERβ agonist DPN (2,3-bis(4-Hydroxyphenyl)-propionitrile, Sigma, St Louis, MO, USA) or the ERα agonist PPT (4,4′,4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol, Sigma, St Louis, MO, USA). Treatments with ER agonists were performed at concentrations of 70 µM, 20 µM and 100 nM for 24 h before palmitic acid treatment (Avila-Rodriguez et al., 2016). As a positive control, we used estradiol (Sigma, St Louis, MO, USA) at concentrations of 70 µM, 50 µM, 20 µM and 100 nM, being similar doses to those used for tibolone and ER agonists. To further evaluate which ER is involved in tibolone protective effects, cells were incubated with either the ERβ antagonist PHTTP (4-[2-Phenyl-5,7-bis (trifluoromethyl)pyrazolo[1,5-a] pyrimidin-3-yl] phenol, Sigma, St Louis, MO, USA), or the ERα antagonist MPP (1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole

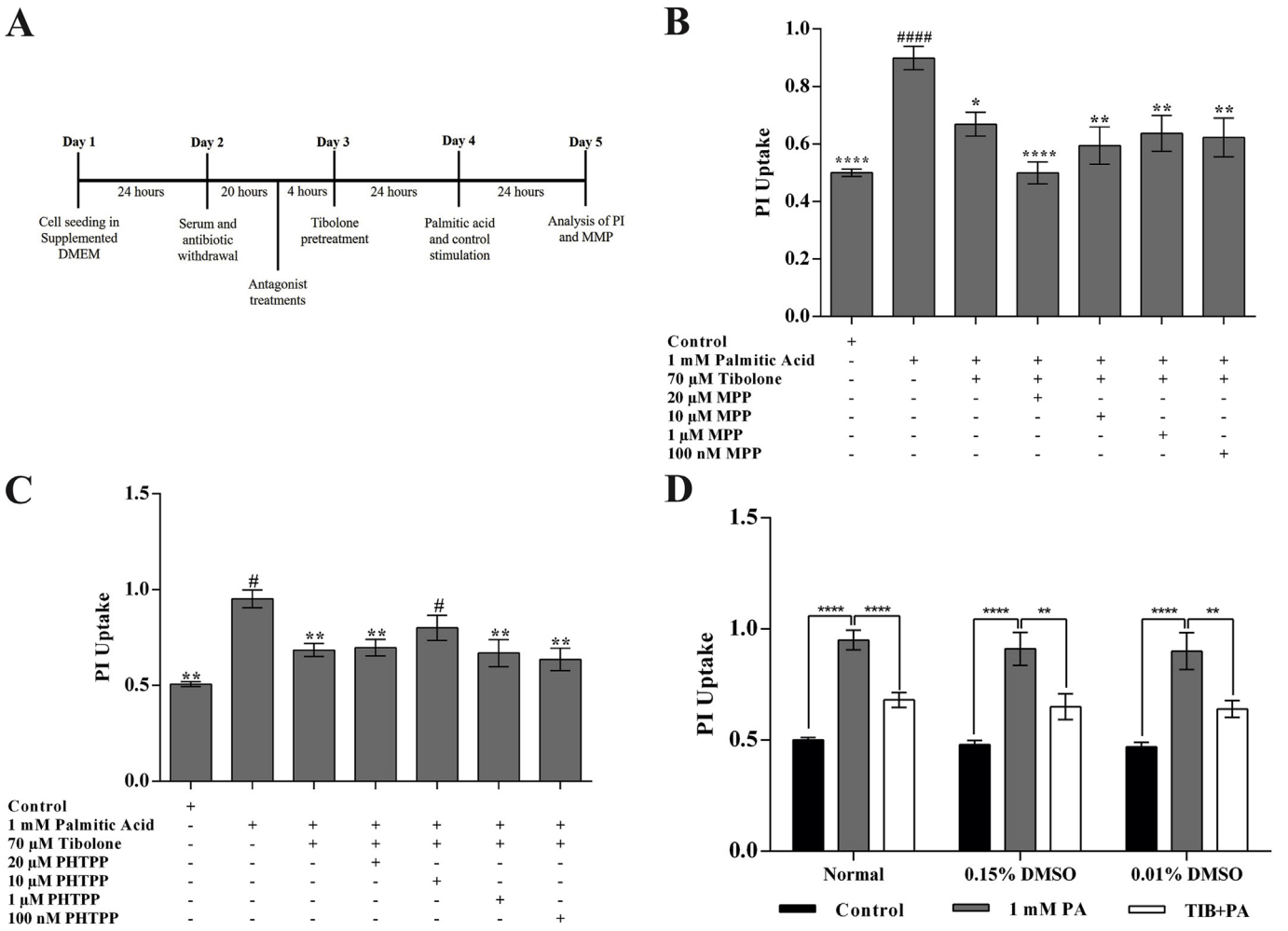


Fig. 3. Effects of ERα and ERβ antagonists on tibolone actions in T98G cells. (A) Overview of experimental design: T98G cells were treated with ER antagonists for 4 h prior to 70 µM tibolone and 1 mM palmitic acid treatments. Once the PA treatment was finished, analysis of propidium iodide uptake test and mitochondrial membrane potential were performed. **(B)** Analysis of PI uptake test for Control (0.5 ± 0.01), 1 mM PA (0.9 ± 0.04), 70 µM Tibolone (0.66 ± 0.04) plus 1 mM PA and ERα antagonist (MPP) treatment for 4-h prior to 70 µM tibolone treatment at 20 µM (0.5 ± 0.04), 10 µM (0.6 ± 0.06), 1 µM (0.63 ± 0.06) and 100 nM (0.62 ± 0.07) plus 1 mM PA. **(C)** Mean fluorescence (a.u.) of PI uptake test for blockade of ERβ by PHTTP at 20 µM (0.69 ± 0.04), 10 µM (0.80 ± 0.06), 1 µM (0.66 ± 0.07) and 100 nM (0.63 ± 0.05). **(D)** 0.15% and 0.01% DMSO were added before PA and TIB + PA treatments in order to evaluate possible effects in cells by DMSO. Bar graph represents Mean ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. In this graph, #### represents < 0.0001 and # < 0.05 respect to control; **** < 0.0001, ** < 0.001 and * < 0.05 respect to palmitic acid.

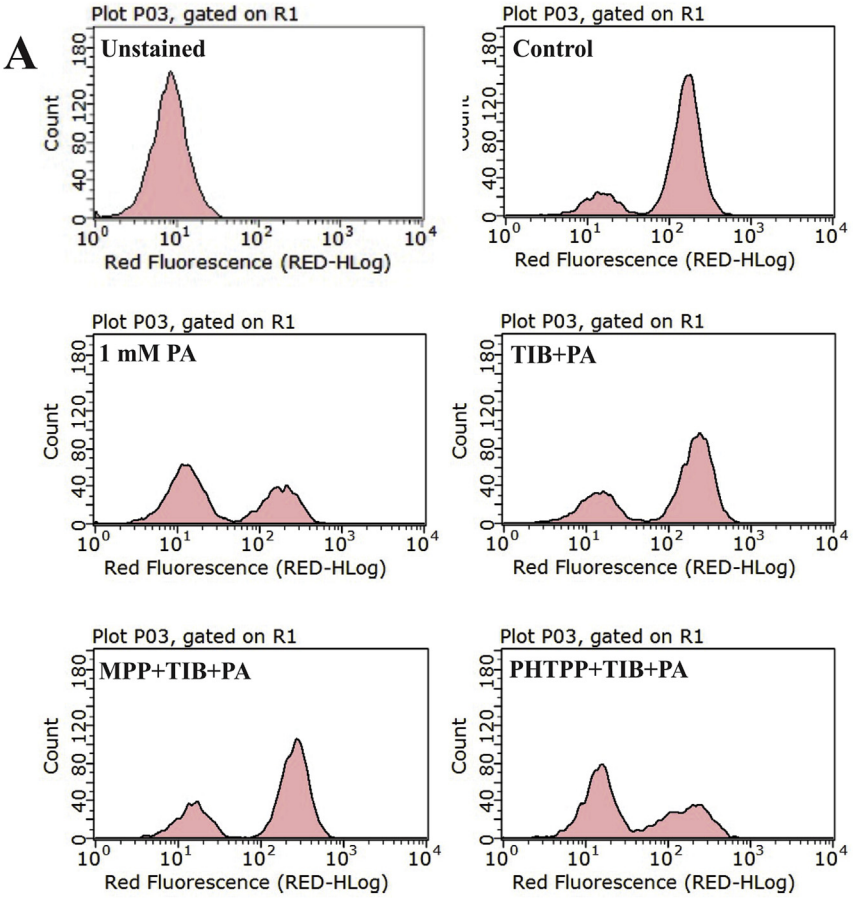
dihydrochloride, Sigma, St Louis, MO, USA) at 10 µM, 1 µM and 100 nM, added for 4 h prior to tibolone treatment (Avila-Rodriguez et al., 2016). Agonists and antagonists were dissolved in DMSO. Final concentrations of DMSO in the treatments for agonists were 0.3%, 0.2%, and < 0.05%; as for antagonists, the final concentrations were 0.15% and < 0.01%. These concentrations did not have any negative impact on cell integrity (Fig. S1).

2.3. Mitochondrial membrane potential and cell death analysis

To evaluate cell death, we used the Propidium Iodide (PI) uptake assay (Crowley et al., 2016). PI (Sigma, St Louis, MO, USA) was used at concentration of 10 µg/ml. Cells were stained for 15 min and fluorescence was detected in a FLUOstar Omega microplate reader (excitation 530 nm/emission 590 nm) (BMG LABTECH, Ortenberg, Germany). We analyzed mitochondrial membrane potential by means of a Guava EasyCyte cytometer (Millipore, MA, USA) using tetramethyl rhodamine methyl (TMRM) dye at 500 nM. Both experiments were performed according to our previous study (Gonzalez-Giraldo et al., 2017). For all experiments, the mean fluorescence is presented in arbitrary units (a.u.).

2.4. Gene expression analysis

Total RNA isolation was carried out using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and it was quantified by means of NanoDrop (Thermo Fisher Scientific, Waltham, Ma, USA). Before cDNA synthesis, RNA was treated with DNase I to eliminate possible DNA contamination, following manufacturer instructions (New England Biolabs, Ipswich, MA, USA). cDNA was generated with 400 ng of RNA using oligo (dt)18 (Bioline, London, United Kingdom) and the M-MLV Reverse Transcriptase kit, following manufacturer instructions (Invitrogen, Carlsbad, Ca, USA). Quantitative PCR for gene expression analysis was performed using 1X SensiFAST™ SYBR® No-ROX Master mix (Bioline, London, United Kingdom), 400 nM of each primer (Forward and Reverse), 1 µl of cDNA containing 10 ng of RNA and water, in a total volume of 10 µl on a CFX96 Touch Real-Time System (BioRad, Hercules, CA). PCR Program consisted in: a cycle of 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s Melting analysis was carried out to verify the amplification specificity (Nolan et al., 2006). PCR efficiency was determined using LinRegPCR program (Ruijter et al., 2009). RPL27 and GADPH genes were used as endogenous controls for normalization, according to previous



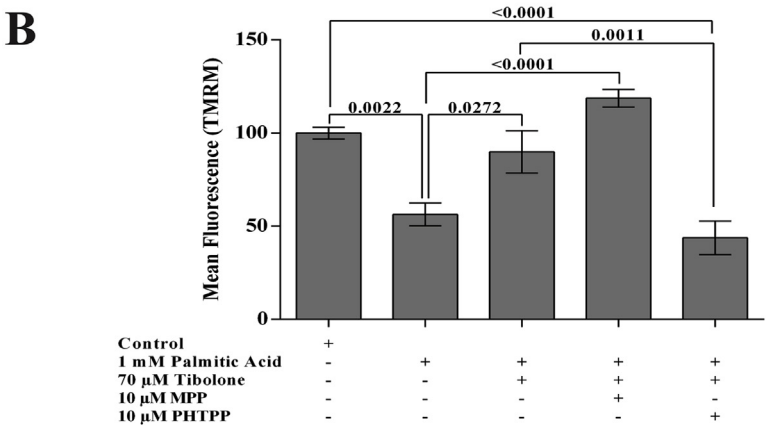


Fig. 4. Protective effects of tibolone are partially mediated by estrogen receptor beta. **(A)** Representative plots for quantification of TMRM fluorescence for mitochondrial membrane analysis in T98G cells by flow cytometry. Control, 1 mM PA, 10 µM MPP +70 µM tibolone +1 mM PA and 10 µM PHTPP +70 µM tibolone +1 mM PA. **(B)** TMRM Mean fluorescence for cells treated with Control (100 ± 3.2), 1 mM PA (56 ± 6.12), 10 µM MPP (118 ± 4.7) and 10 µM PHTPP (44 ± 9.1) before tibolone treatment (90 ± 11.3) and 1 mM PA. For all experiments, control sample consisted in 2.5% BSA and 2 mM carnitine. Bar graph represents Mean ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. In this graph, #### represents < 0.0001 and # < 0.05 respect to control; **** < 0.0001, ** < 0.001 and * < 0.05 respect to palmitic acid.

recommendations (de Jonge et al., 2007). GAPDH was used to normalize the IL6 and TERT expression under agonist (DPN and PPT) treatments. Ct values are shown in supplementary material (Figs. S2a and S2b). Primers used to analyze gene expression are shown in Table S1. Each sample, obtained from biological and technical replicates of cells subjected to tibolone and PA treatments, was run in triplicate for qPCR analysis. Data analysis was performed using the comparative CT method ($2^{-\Delta\Delta CT}$) (Schmittgen and Livak, 2008).

2.5. MicroRNA quantification

First, we used miRTarBase (Chou et al., 2018) and DIANA-TarBase v8 tools (Karagkouni et al., 2018) to find experimentally validated microRNAs targeting candidate genes. cDNA was generated using Verso cDNA kit (Thermo Fisher Scientific, Waltham, Ma, USA), according to manufacturer recommendations. We used small RNA-specific RT primers from TaqMan® MicroRNA Assays (Thermo Fisher Scientific, Waltham, Ma, USA) to quantify the following microRNAs: MIR181a-5p (Assay ID: 000480), MIR124-1-3p (Assay ID: 001182), MIR125a-3p (Assay ID 002199) and MIR155-5p (Assay ID 002623). To normalize the microRNA expression, the MIR191-5p (Assay ID: 002299) was selected due to its stable expression in several tissues and sample types (Peltier and Latham, 2008). Ct values are shown in supplementary material (Fig. S2c). qPCR was performed in a CFX96 Touch Real-Time System (BioRad, Hercules, CA), using: 1x mix (Small RNA-specific forward PCR primer, Specific reverse PCR primer and Small RNA-specific TaqMan® MGB probe), 0,66 µl product from RT reaction, 5 µl TaqMan® Universal PCR Master Mix (2×) and 3,83 µl of water, in a total volume of 10 µl. Data analysis was performed using the comparative CT method ($2^{-\Delta\Delta CT}$) (Schmittgen and Livak, 2008).

Table 1
Protein functions for analyzed genes and their relationship with astrocytes.

Gene symbol	Gene Name	ERE	Protein function[a]	Evidence related with Astrocytes	Reference
APOE	Apolipoprotein E	x	Lipid transport	It regulates astrocyte activation after exposition to Aβ peptide and it participates in clearance of Aβ plaques	Forero et al. (2018)
BDNF	Brain derived neurotrophic factor		Survival and differentiation of neurons and axonal growth	TNF-α treatment induced BDNF expression by means of NF-kB activation	Saha et al. (2006)
CREB1	cAMP responsive element binding protein 1		Transcription factor that is a member of the leucine zipper family of DNA binding proteins	PA increased CREB phosphorylation to regulate IPAF and IL1B expression	(L. Liu and Chan, 2014)
ALDH1L1	Aldehyde dehydrogenase 1 family member L1		Catalyzes the conversion of 10-formyltetrahydrofolate, nicotinamide adenine dinucleotide phosphate (NADP+), and water to tetrahydrofolate, NADPH, and carbon dioxide	Astrocyte marker downregulated in samples of depressive subjects	Nagy et al. (2015)
GLUL	Glutamate-ammonia ligase	x	It catalyzes the synthesis of glutamine from glutamate and ammonia in an ATP-dependent reaction	Downregulated in samples of depressive subjects; It is increased in temporal cortex of AD patients	(Fluteau et al., 2015; Nagy et al., 2015)
SLC1A2	Solute carrier family 1 member 2 (glutamate receptor)	x	Transporter that clears the excitatory neurotransmitter glutamate	PA increased mRNA levels, it did not affect protein expression, but reduced Glutamate receptor puncta on the cell processes	Huang et al. (2017)
AR	Androgen receptor		Steroid-hormone activated transcription factor	It is expressed in astrocytes and neurons	Acaz-Fonseca et al. (2016)
ESR1	Estrogen receptor 1		Steroid-hormone activated transcription factor	PA reduces expression; ERα is increased in AD patient samples	(Lu et al., 2003; Morselli et al., 2014)
ESR2	Estrogen receptor 2	x	Steroid-hormone activated transcription factor	Involved in tibolone actions in a model of glucose deprivation	Avila-Rodriguez et al. (2016)
PGR	Progesterone receptor		Steroid-hormone activated transcription factor	Activation of PGR induces reduction of TNFα in cells treated with LPS	Kipp et al. (2007)
IL1B	Interleukin 1 beta		Important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.	PA increased its expression	(L. Liu and Chan, 2014)
IL6	Interleukin 6	x	It has pro- and anti-inflammatory properties, and is involved in survival and proliferation, among other functions	PA increased its expression	Gupta et al. (2012)
TLR4	Toll like receptor 4		Acts via MYD88, TIRAP and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response	It is involved in actions of PA, mainly regulating TNF and IL6 expression	Gupta et al. (2012)
TNF	Tumor necrosis factor		Pro-inflammatory cytokine, involved in cell proliferation, differentiation, apoptosis, lipid metabolism, among other	PA increased its expression	Gupta et al. (2012)
BAX	BCL2 associated X, apoptosis regulator		Apoptotic activator	PA increased Bax/Bcl-2 ratio	(Z. Wang, Liu, Wang, Liu, Gao, et al., 2012)
BCL2	BCL2, apoptosis regulator	x	Integral outer mitochondrial membrane protein that blocks the apoptotic death	PA increased Bax/Bcl-2 ratio	(Z. Wang, Liu, Wang, Liu, Gao, et al., 2012)
TERC	Telomerase RNA component		Serves as a template for the telomere repeat	NA	NA
TERF1	Telomeric repeat binding factor 1		Inhibitor of telomerase	NA	NA
TERF2	Telomeric repeat binding factor 2		Negative regulator of telomere length and plays a key role in the protective activity of telomeres	NA	NA
TERT	Telomerase reverse transcriptase	x	Involved in telomere maintenance, aging, anti-apoptotic and reduces ROS	It is increased in a Spinal Cord Injury Model and correlated with GFAP expression	Tao et al. (2013)
TINF2	TERF1 interacting nuclear factor 2	x	Is involved in the regulation of telomere length and protection	NA	NA
DNMT1	DNA methyltransferase 1	x	Transfers methyl groups to cytosine nucleotides of genomic DNA and responsible for maintaining methylation patterns following DNA replication	Protein expression is increased in a model of Ischemia-Hypoxia	Yang et al. (2016)
DNMT3A	DNA methyltransferase 3 alpha	x	Required for genome-wide *de novo* methylation and is essential for the establishment of DNA methylation patterns during development.	Protein expression is increased in a model of Ischemia-Hypoxia	Yang et al. (2016)
DNMT3B	DNA methyltransferase 3 beta	x	Required for genome-wide *de novo* methylation and is essential for the establishment of DNA methylation patterns during development.	NA	NA
MIR181a-5p[b]	microRNA 181a-1		Negative regulation of gene expression: TERT, ESR1, PGR, BCL2	IL1B Treatments reduced expression; LPS reduced expression	(Hutchison et al., 2013; Zumkehr et al., 2018)
MIR124-3p[b]	microRNA 124-1		Negative regulation of gene expression: IL6, TERT, AR, TERF2, BDNF	LPS increased expression	Omran et al. (2013)
MIR125a-3p[b]	microRNA 125a		Negative regulation of gene expression: GLUL, IL6,	NA	NA
MIR155-5p[b]	microRNA 155		Negative regulation of gene expression: IL1B, BCL2, TNF Positive regulation of IL6	LPS increased expression	

(*continued on next page*)

Table 1 (*continued*)

Gene symbol	Gene Name	ERE	Protein function[a]	Evidence related with Astrocytes	Reference
					Mor et al. (2011)

Abbreviations: ERE: (Estrogen response element); PA (Palmitic acid), Aβ (Amyloid β), DNA (deoxyribonucleic acid), IPAF (Ice Protease-Activating Factor), NADP (nicotinamide-adenine-dinucleotide phosphate), NADPH (nicotinamide adenine dinucleotide phosphate), ATP (adenosine triphosphate), AD (Alzheimer's disease), ERα (Estrogen receptor alpha), LPS (lipopolysaccharide), ROS (reactive oxygen species), GFAP (glial fibrillary acidic protein), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), MYD88 (Myeloid differentiation primary response 88), TIRAP (TIR domain containing adaptor protein), TRAF6 (TNF Receptor Associated Factor 6). N/A: No Analyzed in astrocytes.

[a] Information obtained from GeneCards base data (www.genecards.org).

[b] Non-coding RNA.

2.6. Bioinformatics analysis

To investigate the transcription factors stimulating the expression of genes co-regulated in our model, we used the GATHER program (Gene Annotation Tool to Help Explain Relationships) (Chang and Nevins, 2006). The analysis of data was performed through TRANSFAC matrices and the transcription factors were selected considering the Bayes factor and *p* value.

2.7. Statistical analysis

All samples were analyzed in triplicate, with a minimum of three independent experiments. A one-way ANOVA test was carried out on the GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA) and Post hoc analysis were performed using the Tukey's multiple comparisons test. To analyze Pearson correlation data, a correlation matrix analysis was performed. A p value < 0.05 was considered significant. In the graphics, all data are presented as the mean ± SEM.

3. Results

3.1. ER agonists and estradiol imitate the protective effect of tibolone on T98G cells exposed to palmitic acid

Previously, we observed that 70 μM tibolone, when administered before PA, protected astrocytic cells from death, possibly, in part due to the preservation of both mitochondrial membrane potential and mitochondrial integrity (Gonzalez-Giraldo et al., 2017). In order to elucidate the mechanisms involved in the effects of tibolone, we aimed to determine whether its protective actions on T98G cells exposed to PA are also elicited by estradiol and by agonists for ERα and ERβ (Fig. 1A). We found that the ERα agonist PPT reduced cell death at a concentration of 5 μM ($P < 0.0001$), but not at 100 nM, 20 μM or 70 μM (Fig. 1B). ERβ agonist DPN at 70 μM induced a greater reduction of cell death in comparison with 20 μM and 100 nM. Cell death was reduced by 40% ($P < 0.0001$), an effect similar to that of 70 μM tibolone, which was able to reduce cell death by 38% ($P < 0.0001$) (Fig. 1C). Pretreatment with estradiol at 70 μM, 50 μM and 20 μM protected the T98G cells from death, thus reducing the damage induced by PA by 44%, 44% and 33%, respectively ($P < 0.0001$) (Fig. 1D). Physiological concentrations of estradiol did not have protective effects against damage induced by palmitic acid on mitochondrial membrane potential (Fig. 2B).

Since mitochondria play an important role in neuroprotection by estradiol (Simpkins et al., 2005) and are main targets of hormonal compounds (Baez et al., 2017; Giatti et al., 2018; C. Martin-Jimenez et al., 2018; C. A. Martin-Jimenez et al., 2016; C. A. Martin-Jimenez et al., 2017), the mitochondrial membrane potential was assessed by quantification of TMRM dye in cells incubated with the concentrations of DPN, PPT and estradiol that significantly reduced cell death induced by PA (Fig. 2A). The results show that, similarly to tibolone, both ER agonists and estradiol attenuate the loss of mitochondrial membrane potential induced by PA (Fig. 2B and C). Mitochondrial membrane potential was preserved by 33% when cells were treated with tibolone ($P = 0.0001$), 44% with DPN ($P = 0.0001$), 53% with PPT ($P < 0.0001$) and 43% with estradiol ($P = 0.0002$).

3.2. Protective effects of tibolone on cell death and mitochondrial membrane potential are in part mediated by ERβ

As protective effects of tibolone were mimicked by agonists of ERα and ERβ, we assessed whether antagonists of these receptors blocked the action of tibolone. Different concentrations of ER antagonists were added 4 h before 70 μM tibolone pretreatment (Fig. 3A). We observed that the ERα antagonist MPP at concentrations of 20 μM, 10 μM, 1 μM

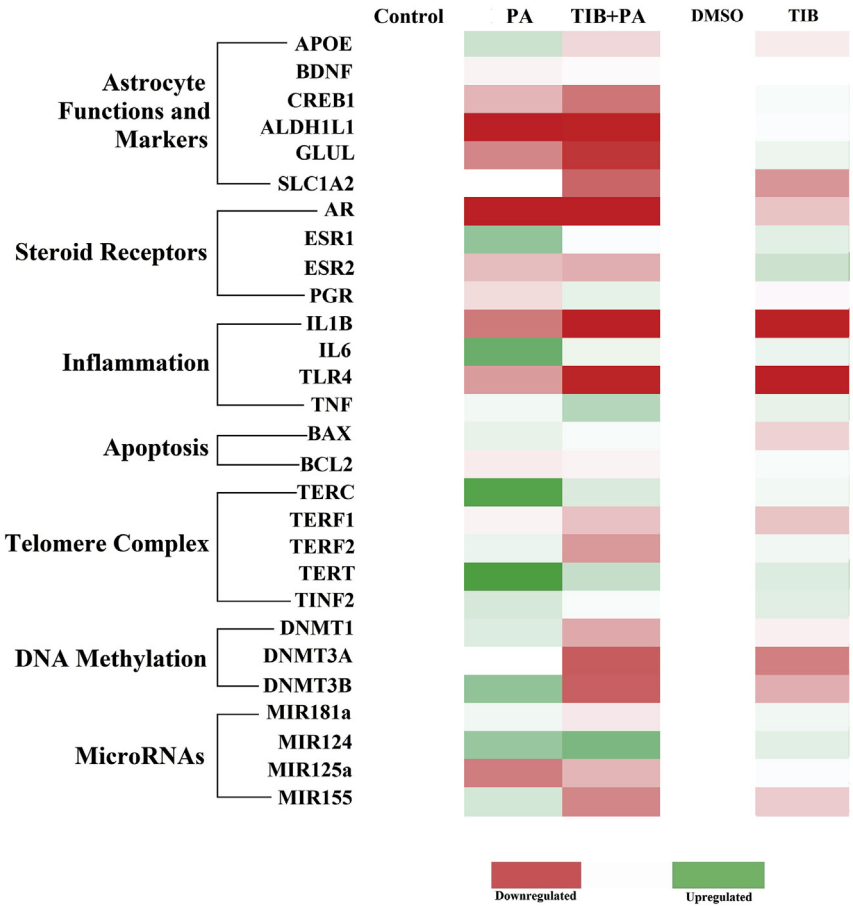


Fig. 5. Tibolone reduces inflammatory gene expression and affects epigenetic and telomere pathways, but does not ameliorate the negative effect of PA on astrocyte genes. Heat map of relative normalized expression for 28 genes in three conditions: Control, 1 mM PA and 70 μM TIB +1 mM PA, DMSO and tibolone. Total RNA was isolated from T98G cells treated with 70 μM tibolone for 24 h followed by 1 mM palmitic acid stimulation for 24 h. Gene expression analysis was normalized using the constitutive gene RPL27 for genes encoding proteins; for microRNAs, MIR191-5p was used to normalize the expression. Control sample consisted in 2.5% BSA and 2 mM carnitine for PA treatment and 0.2% DMSO for Tibolone. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis.

Table 2
Mean ± SEM for gene expression data (Fold change).

Gene	Control	PA	TIB-PA	DMSO	TIB
APOE	1.00 ± 0.05	1.65 ± 0.25*	0.89 ± 0.18	1.00 ± 0.51	0.95 ± 0.36
BDNF	0.99 ± 0.05	0.97 ± 0.18	0.99 ± 0.34	1.00 ± 0.33	1.01 ± 0.25
CREB1	1.00 ± 0.03	$^{+}$0.81 ± 0.05	$^{+++}$0.63 ± 0.08	1.00 ± 0.39	1.10 ± 0.35
ALDH1L1	1.01 ± 0.14	$^{++++}$0.18 ± 0.04	$^{+++}$0.33 ± 0.05	1.00 ± 0.43	1.08 ± 0.38
GLUL	1.00 ± 0.17	0.68 ± 0.12	$^{++}$0.42 ± 0.08	1.00 ± 0.64	1.22 ± 0.68
SLC1A2	1.01 ± 0.04	1.00 ± 0.23	0.58 ± 0.10	1.00 ± 0.67	0.72 ± 0.35
AR	1.00 ± 0.03	$^{++++}$0.22 ± 0.02	$^{++++}$0.28 ± 0.06	1.00 ± 0.40	0.85 ± 0.26
ESR1	1.01 ± 0.05	$^{++}$0.83 ± 0.12*	0.80 ± 0.10	1.00 ± 0.45	1.36 ± 0.47
ESR2	1.01 ± 0.12	2.45 ± 0.48	1.07 ± 0.24	1.00 ± 0.53	1.64 ± 0.78
PGR	0.97 ± 0.15	0.91 ± 0.21	1.32 ± 0.31	1.00 ± 0.28	0.98 ± 0.28
IL1B	1.00 ± 0.04	$^{++}$0.64 ± 0.13**	$^{++++}$0.23 ± 0.04	1.00 ± 0.42	0.32 ± 0.1$^{\#\#}$
IL6	1.01 ± 0.10	$^{+}$3.20 ± 0.75*	1.25 ± 0.42	1.00 ± 0.21	1.26 ± 0.23
TLR4	1.00 ± 0.11	0.74 ± 0.21	$^{+}$0.34 ± 0.08	1.00 ± 0.64	0.21 ± 0.10$^{\#\#}$
TNF	1.01 ± 0.09	1.17 ± 0.15	$^{+}$1.92 ± 0.39	1.00 ± 0.35	1.30 ± 0.34
BAX	1.01 ± 0.09	1.31 ± 0.29	1.13 ± 0.35	1.00 ± 0.24	0.89 ± 0.17
BCL2	1.01 ± 0.09	0.95 ± 0.21	0.98 ± 0.29	1.00 ± 0.49	1.12 ± 0.38
TERC	1.01 ± 0.14	$^{++}$3.80 ± 0.87*	1.46 ± 0.53	1.00 ± 0.30	1.17 ± 0.30
TERF1	1.01 ± 0.08	0.97 ± 0.15	0.84 ± 0.09	1.00 ± 0.21	0.85 ± 0.13
TERF2	0.95 ± 0.12	1.26 ± 0.23	0.73 ± 0.10	1.00 ± 0.36	1.17 ± 0.31
TERT	1.01 ± 0.09	$^{++++}$4.10 ± 0.5****	1.72 ± 0.15	1.00 ± 0.14	1.42 ± 0.21$^{\#}$
TINF2	1.00 ± 0.08	1.52 ± 0.30	1.11 ± 0.42	1.00 ± 0.58	1.38 ± 0.56
DNMT1	1.01 ± 0.08	1.42 ± 0.24*	0.79 ± 0.12	1.00 ± 0.59	0.96 ± 0.40
DNMT3A	1.00 ± 0.10	1.01 ± 0.21	0.56 ± 0.11	1.00 ± 0.76	0.65 ± 0.40
DNMT3B	1.01 ± 0.22	$^{+}$2.47 ± 0.64**	0.57 ± 0.17	1.00 ± 0.66	0.79 ± 0.38
MIR181a	1.01 ± 0.09	1.19 ± 0.32	0.94 ± 0.36	1.00 ± 0.10	1.17 ± 0.12
MIR124	1.01 ± 0.26	2.36 ± 0.67	2.87 ± 0.85	1.00 ± 0.14	1.36 ± 0.19$^{\#}$
MIR125a	1.01 ± 0.09	$^{+}$0.66 ± 0.05	0.81 ± 0.11	1.00 ± 0.12	1.079 ± 1.20
MIR155	1.01 ± 0.11	$^{+}$1.53 ± 0.13**	0.68 ± 0.18	1.00 ± 0.17	0.87 ± 0.17

$^{++++}$ < 0.0001; $^{+++}$ < 0.001; $^{++}$ < 0.01 and $^{+}$ < 0.05 versus control; **** < 0.0001, *** < 0.001; ** < 0.01 and * < 0.05 versus TIB + PA. TIB vs DMSO: $^{\#\#\#\#}$ < 0.0001; $^{\#\#\#}$ < 0.001; $^{\#\#}$ < 0.01 and $^{\#}$ < 0.05.

Table 3
Pearson's correlation matrix of gene expression.

	IL6	TERT	ESR1	DNMT3B	TERC	IL1B	GLUL	TLR4	APOE	DNMT1	MIR125a
TERT	0,993										
ESR1	0,998*	0,985									
DNMT3B	0,949	0,905	0,966								
TERC	0,999*	0,998*	0,994	0,931							
IL1B	−0,062	−0,178	−0,003	0,257	−0,114						
GLUL	−0,156	−0,270	−0,097	0,164	−0,207	0,996					
TLR4	0,024	−0,093	0,082	0,338	−0,029	0,996	0,984				
APOE	0,974	0,941	0,986	0,996	0,961	0,166	0,072	0,250			
DNMT1	0,901	0,844	0,925	0,992	0,877	0,377	0,288	0,455	0,976		
MIR125a	−0,886	−0,934	−0,858	−0,695	−0,909	0,517	0,595	0,442	−0,758	−0,598	
MIR155	0,884	0,823	0,910	0,986	0,858	0,413	0,325	0,489	0,967	0,999*	−0,567

* Indicates statistically significant correlation at *P value* < 0.05.

Table 4
Transcription factors identified by bioinformatics analysis of genes upregulated by palmitic acid in comparison to control and tibolone pretreatment.

TRANSFAC matrix	Transcription Factor	Genes	P Value	Bayes Factor
V$EBF_Q6	EBF (Early B Cell Factor)	DNMT3B, ESR1, IL6, TERT	0.001	3
V$NFKB_Q6: NF-kappaB	NF-kB (Nuclear Factor Kappa B)	DNMT3B, IL6, TERT	0.002	3
V$ZID_01: zinc finger with interaction domain	ZID (zinc finger with interaction domain)	DNMT3B, IL6, TERT	0.002	3
V$PAX6_Q2	PAX6 (Paired Box 6)	DNMT3B, TERT	0.002	2
V$PTF1BETA_Q6	PTF1A (Pancreas Specific Transcription Factor, 1a)	ESR1, IL6	0.006	1
V$MYCMAX_B: c-Myc:Max binding sites	MYC (MYC proto-oncogene, bHLH transcription factor)	DNMT3B, ESR1, IL6, TERT	0.008	1

Table 5
Transcription factors identified by bioinformatics analysis of genes downregulated by tibolone pretreatment in comparison to control or palmitic acid.

TRANSFAC matrix	Transcription Factor	Genes	P Value	Bayes Factor
V$IRF_Q6_01	IRF (interferon-regulatory factor)	APOE DNMT1 GLUL IL1B TLR4	0,003	2
V$PBX_Q3	PBX1 (PBX homeobox 1)	DNMT1 GLUL IL1B	0,005	2
V$TITF1_Q3: TTF-1, TITF1 (thyroid transcription factor 1)	TITF1 (thyroid transcription factor 1)	APOE DNMT1 IL1B TLR4	0,005	2
V$CAAT_01: cellular and viral CCAAT box	Cellular and viral CCAAT box	APOE DNMT1 GLUL IL1B TLR4	0,008	1
V$FOXJ2_02: fork head box J 2	FOXJ2 (fork head box J 2)	APOE GLUL IL1B TLR4	0,01	1

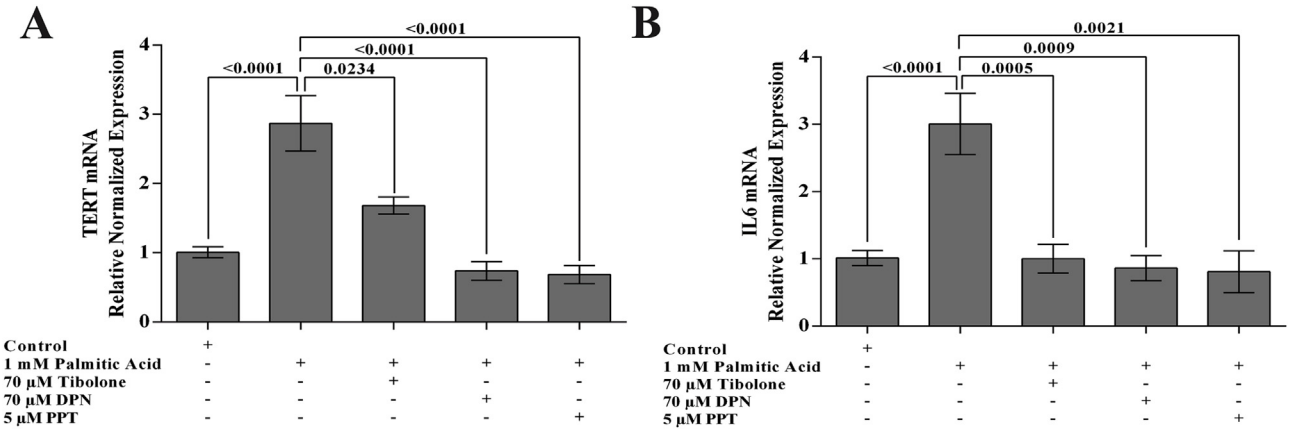


Fig. 6. Effects of DPN and PPT on TERT and IL6 gene expression. Total RNA was isolated and qPCR analysis was used to calculate the relative normalized expression for TERT and IL6 genes. GAPDH gene was used to normalize the data. **(A)** TERT expression was analyzed in cells pretreated with ERα agonist (PPT) at 5 µM (0.68 ± 0.13), ERβ agonist (DPN) at 70 µM (0.73 ± 0.13) and tibolone at 70 µM (1.6 ± 0.13) followed by 1 mM palmitic acid stimulation (2.9 ± 0.4). **(B)** Relative normalized expression for the IL6 gene in cells treated with ERα agonist (PPT) at 5 µM (0.80 ± 0.31), ERβ agonist (DPN) at 70 µM (0.86 ± 0.18) and tibolone at 70 µM (1 ± 0.21) followed by 1 mM palmitic acid stimulation (3 ± 0.45). Control sample consisted in 2.5% BSA and 2 mM carnitine. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis.

or 100 nM did not significantly affect the protective action of tibolone on cell survival against PA (Fig. 3B). When cells were pretreated with 10 µM PHTPP (an ERβ antagonist), the protective effect of tibolone on PA cells was abolished; however, significant differences were not observed between PHTPP + TIB + PA and TIB + PA groups, suggesting a partial interference by the blockade of ERβ (Fig. 3C). It is noteworthy that we did not observe any effect of DMSO (vehicle used for antagonists) at 0.15% or 0.01% in cells treated with PA and TIB + PA (Fig. 3D). In contrast to the PI uptake test, as for the mitochondrial membrane potential (Fig. 4A), we observed significant differences between TIB + PA and PHTPP + TIB + PA ($P = 0.0011$). The action of tibolone on mitochondrial membrane potential was reduced by ERβ

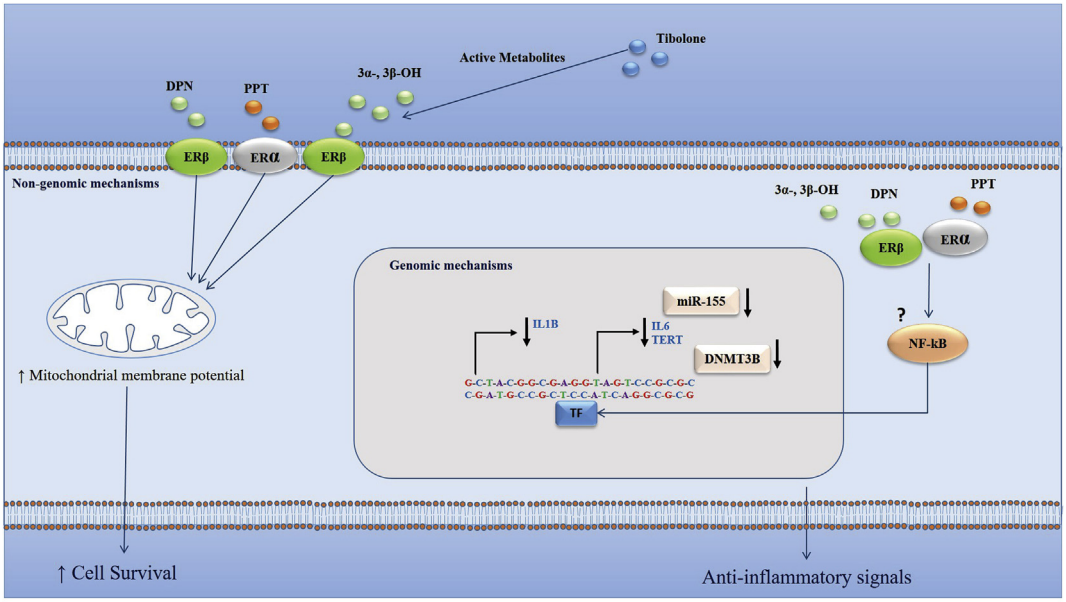


Fig. 7. Proposed model for tibolone and palmitic acid effects on our astrocytic cell model. Estrogen receptor agonists and tibolone reduced cell death by preserving mitochondrial membrane potential in PA cells. Our hypothesis is that these estrogenic compounds may attenuate gene expression (TERT, IL6, DNMT3B and MIR155-5p) by possibly reducing NF-KB activation upon PA.

antagonist (PHTPP) treatment (Fig. 4B).

3.3. Tibolone reduced inflammatory gene expression and affected epigenetic and telomere pathways, but did not ameliorate the negative effect of PA on astrocytic genes

ER activation results in transcriptional changes in cells through direct (dimerization and binding of ERs to target DNA) or indirect (interaction with other transcription factors activated by kinases) mechanisms (Arevalo et al., 2015). From our above results, suggesting that tibolone has estrogenic activity in our model, we hypothesized that tibolone could modulate the expression of genes that contain estrogen response elements (ERE) or that are regulated by indirect mechanisms through interaction of ERs with other transcription factors. Therefore, we performed an analysis of expression for 24 genes considering: 1) protein function; 2) previous evidence in astrocytes cells; and 3) presence of estrogen response elements (Table 1). The analysis was carried out to evaluate whether tibolone could prevent the effects of PA in our astrocyte cell model and to determine whether tibolone's protective actions involve epigenetic mechanisms.

We have analyzed genes belonging to astrocyte function (Fig. S3), steroid receptors (Fig. S4), inflammation and apoptosis (Fig. S5), telomere complex and DNA methylation (Fig. S6). Moreover, we analyzed the expression of four microRNAs regulating TERT and IL6 genes (Fig. S7). We found that tibolone attenuated the expression of IL6, ESR1, TERT, TERC, DNMT3B and MIR155-5p genes, which all were found increased by PA (Fig. 5, Table 2). However, tibolone was unable to counteract the negative effect of PA on two astrocyte genes (ALDH1L1 and CREB1). Indeed, tibolone reduced the expression of GLUL in comparison to controls. On the other hand, tibolone reduced the expression level of IL1β and TLR4 compared to control cells treated with either PA (Fig. 5 and Table 2) or tibolone alone (Fig. S8). Moreover, we performed a Pearson's correlation for those genes that were expressed differentially. This analysis showed a positive correlation between TERC and IL6 (r: 0.999, $P = 0.033$), ESR1 and IL6 (r: 0.998, $P = 0.037$) and TERT and TERC (r: 0.998, $P = 0.041$) (Table 3).

Since tibolone reduced the expression of some genes in PA cells, we performed a bioinformatics analysis to find possible transcription factors associated to gene regulation in our model. Genes were grouped into: (1) Those increased by palmitic acid and reduced by tibolone pretreatment (IL6, TERT, DNMT3B and ESR1), and (2) those down-regulated by tibolone pretreatment (APOE, GLUL, IL1B, TLR4 and DNMT1). EBF (Early B Cell Factor), NF-kB (Nuclear Factor Kappa B) and ZID (zinc finger with interaction domain) were the transcription factors with the strongest association identified for group 1 (Table 4). As for group 2, IRF (interferon-regulatory factor), PBX1 (PBX homeobox 1) and TITF1 (thyroid transcription factor 1) were the transcription factors with a significant association (Table 5).

3.4. Effects of DPN and PPT on TERT and IL6 gene expression

Genes can be differentially regulated by ERα and ERβ, where in some cases ERα can be a potent inductor of transcription, whereas ERβ might induce repressive effects on gene expression (M. M. Liu et al., 2002). In this regard, we assessed the effects of 70 μM DPN (ERβ agonist) and 5 μM PPT (ERα agonist) on expression of TERT and IL-6, as being the genes with the highest fold change expression observed in our study. 70 μM DPN and 5 μM PPT reduced TERT expression by 2.13 and 2.18-fold, respectively, in the PA-treated cells ($P < 0.0001$) (Fig. 6A). As for the IL-6 gene, PA upregulated its expression by 2.52-fold ($P < 0.0001$) in comparison to control, while DPN and PPT down-regulated IL-6 expression by 2.14-fold ($P = 0.0009$) and 2.2-fold ($P = 0.0021$) in PA cells, respectively (Fig. 6B). The data indicate that the actions of the ER agonists and tibolone are similar.

4. Discussion

In the present study, we have assessed which ER was involved in the protective effects of tibolone on astrocytic cells treated with PA. Then, we evaluated whether tibolone may modulate the expression of genes related to inflammation, astrocytic markers, apoptosis, steroid receptors, telomere complex and DNA methylation. Our findings indicate that although both agonists of either ERα or ERβ mimic the protective actions of tibolone against PA, only the ERβ antagonist was able to block the protective effect of tibolone, particularly, on the mitochondrial membrane potential analysis. It is noteworthy that tibolone reduces inflammatory gene expression and affects epigenetic and telomere pathways, but does not ameliorate the negative effects of PA on

astrocyte genes (Fig. 7).

High concentrations of PA induce several impairments in astrocytes; for example, PA reduces cell viability, increases inflammatory signals and affects glucose uptake and lactate release (Gupta et al., 2012; Patil et al., 2007). Interestingly, a previous study has demonstrated that astrocytes from different brain areas react differentially to PA stimulation (Oliveira et al., 2018). Moreover, PA upregulates BACE1 and amyloid beta (Aβ) protein production in neurons, with a direct involvement of astrocytes (Patil et al., 2007). Based on these previous findings, PA has been implicated in many pathological states such as neurodegenerative diseases (Hussain et al., 2013). Therapeutic strategies have been evaluated to reverse detrimental effects of PA in astrocyte cells, including the activation of estrogen receptors by estradiol (Frago et al., 2017; Morselli et al., 2014). Estradiol may reduce the expression of inflammatory markers and astrocyte activation via ERα (Morselli et al., 2014), but failed to prevent cellular apoptosis in hypothalamic astrocytes (Frago et al., 2017). In contrast, in the present study, we found that ERα agonist (PPT), ERβ agonist (DPN) and estradiol protected T98G cells from the deleterious effects induced by PA on cell viability, mitochondrial membrane potential and IL-6 cytokine expression (Fig. 7). Overall, these results may suggest that therapeutic strategies targeting estrogen receptors can be useful for neurological diseases.

Here, we found that ERβ is involved in the protective action of tibolone on T98G cells exposed to PA (Fig. 4B). Although our results show that activation of the ERα leads to a more efficacious response than the activation of the ERβ, these differences did not reach significance (Fig. 2C). These differences in response could be explained by alterations in the pharmacodynamics of each estrogenic compound, induced by changes in both receptor density and/or affinity (Salahudeen and Nishtala, 2017). In addition, in contrast to other studies using animal models (Crespo-Castrillo et al., 2018) or cells exposed to other stress conditions (Avila-Rodriguez et al., 2016), in the current study, higher concentrations of tibolone, estradiol and estrogen receptor agonists were used. This is due to the fact that physiological concentrations of these compounds did not have any effect on cell viability or mitochondrial membrane potential in our experiments (Gonzalez-Giraldo et al., 2017). Nevertheless, it is important to note that previous studies have also found that pharmacological concentrations (μM) of tibolone and estradiol have beneficial effects (Dodel et al., 1999; Maran et al., 2006). Indeed, effects on the regulation of gene expression by high concentrations of tibolone have been quite similar to those exerted by physiological concentrations of estradiol (Maran et al., 2006). Notably, the affinity of estrogenic compounds with estrogen receptors can be affected by sulfation mechanisms, which have been associated with the inactivation of estradiol (Falany and Falany, 1997) and tibolone (Falany et al., 2004). Therefore, high concentrations could be required to induce a response through ERs (Falany and Falany, 1997).

Here, although tibolone reduced the expression of inflammatory genes such as IL6, IL1β, TLR4 and MIR155-3p, in the presence of PA stimulation, it increased the expression of TNF gene. It should be noted that TNF has pleiotropic effects in the CNS, therefore its increased expression does not always involve negative effects (Probert, 2015). Inflammation is a process associated with several diseases, such as depression (Wohleb et al., 2016), Alzheimer and Parkinson's diseases, and stroke (Amor et al., 2014), whereby astrocytes play an important role on the induction of pro- and anti-inflammatory signals (Sofroniew, 2015). On the other hand, the Glutamate-ammonia ligase (GLUL) and APOE genes, that are involved in astrocyte functions, were affected by tibolone pretreatment. In here, differing from other studies demonstrating that ERs activation induced the expression of GLUL and APOE (Blutstein et al., 2006; Stone et al., 1997), we observed a significant reduction in the expression of these genes. These differences can be explained by a tibolone's inability to induce the transcriptional activity of ERs. Indeed, its effects can be explained by interaction with transcription factors that work as repressors or by the induction of kinase-activated transcription factors through activation of membrane receptors (ERα, ERβ and G protein-coupled ER (GPER) (Arevalo et al., 2015). Nevertheless, it should be noted that activation of ERα and ERβ induces a different effect on APOE expression (J. M. Wang, Irwin and Brinton, 2006).

Telomeres are repetitive sequences in the end of chromosomes and their main function is to preserve chromosome stability (Stewart et al., 2012). Telomere maintenance is performed by telomerase and other proteins conforming a shelterin complex, such as TERF1, TERF2 and TIN2 (Martinez and Blasco, 2011). Telomerase is encoded by TERT and TERC genes (Rebhan et al., 1997). As for TERT, several non-canonical functions have been identified, including reduction of oxidative damage and apoptosis in neurons (Gonzalez-Giraldo et al., 2016) and the regulation of gene expression (Martinez and Blasco, 2011), such as IL6 and TNF (Ghosh et al., 2012). In the current study, it was observed an increase of TERT and TERC expression induced by palmitic acid, which was attenuated by tibolone pretreatment (Fig. S5). The promoter region of TERT has binding sites for ERs, a mechanism by which ERα has been associated to increased expression of TERT (Cha et al., 2008). Against our initial hypothesis, we observed that stimulation of ERs with DPN, PPT and tibolone reduced TERT expression in cells treated with PA. This fact suggests that ER activation in our model might lead to induction of other signaling mechanisms in T98G cells, which do not involve a genomic action of ERs (Arevalo et al., 2015). It has been demonstrated that TERT is induced by inflammatory stimuli via activation of NF-kB (Gizard et al., 2011); therefore, NF-kB inactivation could lead to reduction of TERT expression (Zuo et al., 2011). Nonetheless, as this is the first study reporting a regulation of TERT by a fatty acid, further studies are necessary to address the role of telomerase under PA stimulation.

We found that PA induced the overexpression of DNMT3B gene, which is responsible for the *de novo* methylation, and its expression was attenuated by treating cells with tibolone. Furthermore, we observed that tibolone reduced the expression of DNMT1 in comparison to PA only. DNA methylation is an important mechanism for the regulation of astrocytic functions and it has been associated with molecular mechanism in diseases. Increased DNA methylation of some genes is induced by stress and is reduced during astrocyte differentiation (Neal and Richardson, 2018). Changes in methylation status for genes related with glutamate ionotropic kainate receptors, actin-binding related proteins, adhesion molecules at junctions and signal transduction have been observed in astrocytic cells from patients with depression and suicide behavior (Nagy et al., 2015). Indeed, analysis of DNA methylation for pro-inflammatory cytokines has been performed in AD patients, whereby methylation status was inversely correlated to gene expression for IL6 and IL1β (Nicolia et al., 2017). In addition, it has been shown that PA induces changes on gene expression through DNA-related methylation mechanisms (Su et al., 2015), suggesting that DNA methylation may be involved in the detrimental effects of PA.

Bioinformatics analysis for genes encoding proteins (ESR1, IL6, TERT, DNMT3B) showed that NF-kB is one of the transcription factors that might be regulating their expression, especially for IL6, TERT and DNMT3B genes. Previous studies have demonstrated that PA induces NF-kB activation, thus increasing cytokine expression (Z. Wang, Liu, Wang, Liu, Zhao, et al., 2012). For instance, NF-kB can be activated through several cellular receptors such as toll like receptors (TLRs) and CD36 (Rocha et al., 2016), which are involved in PA effects on astrocytes as well (Gupta et al., 2012). Conversely, NF-kB activation is attenuated by tibolone and estradiol under stress conditions (Hidalgo-Lanussa et al., 2018). NF-kB plays an important role on several mechanisms in the brain, such as inflammation, neuroprotection, myelination and synaptic function (Kaltschmidt and Kaltschmidt, 2009). This transcription factor has been postulated as an important therapeutic target, since it has been involved in several neurological diseases (Camandola and Mattson, 2007; Koo et al., 2010).

In summary, our results suggest that i) protection against PA in

T98G cells by tibolone is in part mediated through the activation of ERβ and ii) tibolone has anti-inflammatory effects and can modulate pathways associated to DNA methylation and telomeric complex. However, future studies are necessary to elucidate the role of epigenetic mechanisms and telomere-associated proteins on tibolone actions. Altogether, it is hypothesized that, in T98G cells, estrogen receptor agonists and tibolone reduced cell death by preserving mitochondrial membrane potential in PA-treated cells. Moreover, these estrogenic compounds might reverse the augmented expression of TERT, IL6, DNMT3B and MIR155-5p upon PA by a possible inhibitory action on NF-kB signaling (Fig. 7).

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.02.017.

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