Research Article

Verification of agomelatine in comparison with melatonin as a therapeutic agent to treat breast cancer

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Running title: Agomelatine and melatonin in breast cancer cells

Received: August 15, 2019; Accepted: December 21, 2020

ABSTRACT

The breast cancer (BC) has a high rate of morbidity and mortality; thus, the discovery of new therapeutic targets is of great interest for researchers. Previous studies have documented that melatonin, the main hormone synthesized by the pineal gland, plays important roles in the control of breast tumorigenesis. Similar to melatonin, agomelatine, a melatonin analogue can also perform its functions by binding to G protein coupled melatonin membrane receptors MT1 and MT2. In a series of studies carried out in two breast cancer cell lines (MCF-7 and MDA-MB-231 strains), the dose-responsive curves have been identified regarding cell viability, clonogenic survival, and cell migration. The results indicate that agomelatine has the potential to reduce the proliferative capacity in both cell lines, while melatonin significantly reduced the proliferative rate of triple-negative BC cells. Notably, agomelatine and melatonin showed the same inhibitory effect on BC cell migration. Collectively, agomelatine treatment caused a greater reduction in BC cell growth than that of melatonin which only suppressed the proliferative capacity of triple-negative BC. Also, melatonin and agomelatine have the same inhibitory response to migratory capacity of triple-negative BC cells. Based on the results from current study on BC cells, agomelatine could be considered as a promising adjuvant therapeutic agent compared to melatonin for BC treatment.

Key words: Melatonin, agomelatine, breast cancer cells, MT1 receptor, circadian rhythm.

1. INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed cancer in the female population, reported more than 1.5 million new cases every year worldwide (1). Over the past year, an estimated 626,679 breast cancer-caused deaths have been reported globally. (2). Given this

context, an important approach into BC-related studies is to identify the possible prognostic markers and the development of new therapeutic strategies that may inhibit tumor progression (3).

Currently, several studies have investigated the action of melatonin (N-acetyl-5-methoxytryptamine), the main hormone synthesized by the pineal gland, on the breast tumorigenesis (4). Melatonin plays an important role in the circadian cycle, acting as a major regulator of metabolic, physiological, and behavioural activities t, essential for the regulation of hypothalamic-pituitary-gonadal axis independent to the endocrine phenomena (5).

Studies have shown that melatonin has oncostatic and oncoprotective effects on breast cancer mainly through its ability to inhibit the levels and activity of the estrogen receptor alpha (ERα) via the MT1 receptor, while regulating the transactivation of estrogen-metabolizing enzymes and reducing the mitogenic response of cells in ER positive breast tumors (6,7). Subsequently, other studies using MCF-7 (ER-positive) cells corroborated the anti-estrogenic property of melatonin through its MT1 membrane receptor (8).

The action of melatonin through MT1 can trigger molecular signalings involved in the control of important events such as the BC cell proliferation, cell survival, metastasis, and drug resistance (7). Melatonin also possesses receptor independent effects on tumor cells (9); most of these effects were associated with chronodisruption, light-at-night, and melatonin suppression. Melatonin has been shown to effectively control breast cancer metastasis and wide spreading in both *in vitro* and *in vivo* conditions. These includes reduction in cell viability and invasion/migration processes in triple negative BC (10) and suppression of tumour growth and angiogenesis in these neoplasms (7-11).

Melatonin and agomelatine perform their functions by binding to MT1 and MT2, the G protein-coupled membrane receptors (10, 12). MT1 is expressed in human BC samples at different levels (7), and its higher expression levels were correlated with better prognosis when compared with negative MT1 breast tumors (13).

Deregulation of the circadian rhythms may imply in some pathologies such as psychotic disorders, post-infectious diseases, states of fatigue and chronic pain, as well as major and atypical depression, and seasonal affective disorder (11, 12). In this context, recent studies recommend the use of agomelatine, a melatonergic agonistic analogue, which has chronobiotic, anxiolytic, and antidepressant effects, and accelerates the resynchronization of essential biological circadian rhythms (14); these rhythms include body temperature and hormonal secretion, in addition to its effectiveness in disorders related to circadian cycle misalignment (15). Previous studies suggest that melatonin sensitizes tumor cells to cytotoxic effects of chemotherapeutic agents, thus presenting a synergistic effect (16-19). Similarly, agomelatine is currently used in a new therapeutic approach, which utilizes auxiliary attributes of seven non-tumor treatment drugs (called the ABC7 regimen) to disrupt the natural process of epithelial-mesenchymal transition (EMT) inherent in breast cancer (20). In addition, agomelatine has been used empirically to treat other cancers as an adjunctive therapy for glioblastoma multiforme (GBM), thus associating a new oncotherapeutic approach with antidepressants (21).

The importance of the investigation of agomelatine in breast cancer pathogenesis is reinforced, and its potential may bring the dual benefits, i.e., control of tumor growth and improvement of life quality, since it alleviates the common depressive symptoms during breast cancer treatment (21-23). Therefore, the main purpose of this study was to investigate and compare the action of melatonin and agomelatine on cell proliferative capacity and its metastatic potential associated with their migratory capacity in two breast cancer cell lines (MCF-7 and MDA-MB-231 strains).

2. MATERIALS AND METHODS

2.1. Agomelatine extraction.

Agomelatine pills were used for the substance extraction, performed in partnership with the Laboratory of Antibiotics and Chemotherapeutics of the Institute of Biosciences, Humanities and Exact Sciences (UNESP). The 25 mg agomelatine pills obtained commercially were macerated with chloroform, and the resulting mixture was filtered and washed over additional chloroform. The extract was partitioned with water/chloroform. The resulting solution was dried at room temperature, and the solid was analysed by the Analytical Thin Layer Chromatography and Hydrogen Nuclear Magnetic Resonance (^{1}H NMR) to verify the extraction efficiency. Next, 150 mg of pure solid was obtained, and diluted in DMSO.

Melatonin was commercially obtained in powder (Sigma-Aldrich, St. Louis, MO, USA), and diluted in ethanol and PBS, so that the concentration did not exceed 0.5% in the culture medium.

2.2. Cell Culture.

The study was conducted using two BC cell lines: MDA-MB-231 (ATCC Cat # HTB-26MET, RRID: CVCL_VR67) and MCF-7 (ATCC Cat # HTB-22, RRID: CVCL_0031), supplemented with high glucose Dulbecco's Modified Eagle's Medium (DMEM) culture medium - DMEM - High Glucose (Cultilab) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), and 2% antimycotic and antibiotic solution (Sigma-Aldrich, St. Louis, MO, USA) and kept in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cell Viability Assay (MTT assay).

MTT assay was performed to determine cell viability of MCF-7 and MDA-MB-231 BC strains, and to identify proper dosage for melatonin and agomelatine to be used in the further experiments. The 4,5-dimethylthiazol-2-yl -2,5-diphenyltetrazolium bromide (MTT) salt was incubated with the cells and reduced to formazan crystals by cellular metabolic activity. The IC_{50} concentration was determined by dose-response curves.

2.3.1. Cell Plating.

Cells were distributed in 96-well plates at initial concentration of 1.0 x 10^4 cells per well for MCF-7, and 2.5 x 10^4 cells per well for MDA-MB-231. After 24 h incubation, the culture medium was discarded, and the cells were added to agomelatine dissolved in DMSO, while melatonin was dissolved in ethanol and PBS. They were diluted in the culture medium so that the concentration of diluents (DMSO and ethanol) did not exceed 0.5% in the culture medium during the treatment.

2.3.2. Treatment.

To determine the inhibitory concentration for 50% of a cell population (IC_{50}), the cell lines were treated with solutions ranging from 1,000 to 0.01 μmol L^{-1} for 48 h of incubation period. The culture media was then discarded and replaced with a MTT solution dissolved in DMEM (1 $mg.mL^{-1}$), followed by incubation for 40 minutes. The remaining medium was removed and replaced with DMSO, and the plates had their absorbance measured in a microplate reader at

562 nm (ThermoPlate® TP Reader). The percentage (%) of viable cells was calculated relative to the control group.

2.4 Clonogenic Survival Assay.

Cells were seeded in 6-well plates at a concentration of 500 cells per well in a total volume of 2 mL of medium and in culture for 10 days. They were then fixed with paraformaldehyde (4%) for 15 minutes and coloured with violet crystal for 15 minutes. Next, they were washed with milli-Q water and the colonies were quantified. Plate efficiency (PE) and survival factor (SF) were calculated according to Balça-Silva et al. (24) and Franken et al. (25).

2.5 Cell Migration Assay.

Cells were seeded in 24-well plate with culture medium without fetal bovine serum at a concentration of $1x10^5$ cells per well. Then, 300 µL of incomplete medium was added to the insert-added cell solution in triplicate for each experimental group. Subsequently, 500 µL of complete medium (10% fetal bovine serum) was added to the wells and the inserts were transferred to the wells. After 24 h, the migrated cells were quantified.

The inserts were washed with PBS and individually cleaned on the bottom with swab cottons. The culture medium was then discarded and 500 µL of paraformaldehyde (3.7%) was added to the empty wells and the inserts were transferred for cell fixation for 15 minutes. After fixation, paraformaldehyde was removed and 500 µL of the crystal violet dye was added, being the inserts transferred back to the wells for 15 minutes. Finally, the inserts were cleaned and photographed under inverted light microscope after drying. To obtain the average of each experimental group, the images were used to count the cells using the ImageJ software.

2.6 Statistical analysis.

The results were compared after determining normality using Student's t-test for two-variable comparison or analysis of variance (ANOVA) followed by Bonferroni's test for comparison of more than two variables. The statistical significance was set at P value < 0.05. All analyses were performed using GraphPad Prism 6 software.

3. RESULTS

3.1. Agomelatine and melatonin showed a dose dependent inhibitory effect on breast cancer cell viability.

The determination of IC_{50} was demonstrated based on dose-response curves for agomelatine in MCF-7 (Figure 1 A) and MDA-MB-231 (Figure 1 B) cell lines regarding the percentage of viable cells. The IC_{50} values were calculated and the average obtained in three independent samples was 0.300 $mmol.L^{-1}$ (Table 1). Of note, the IC_{50} concentration used for MCF-7 and MDA-MB-231 cells was pharmacologically equivalent after 48 h exposure to agomelatine

Based on previous studies from our laboratory (23), the concentration of 1 $mmol.L^{-1}$ melatonin was sufficient to reduce cell viability (< 50%) in aggressive BC cells. Thus, the concentration of 1 $mmol.L^{-1}$ melatonin was established to compare melatonin and agomelatine effects in the two BC cell lines.

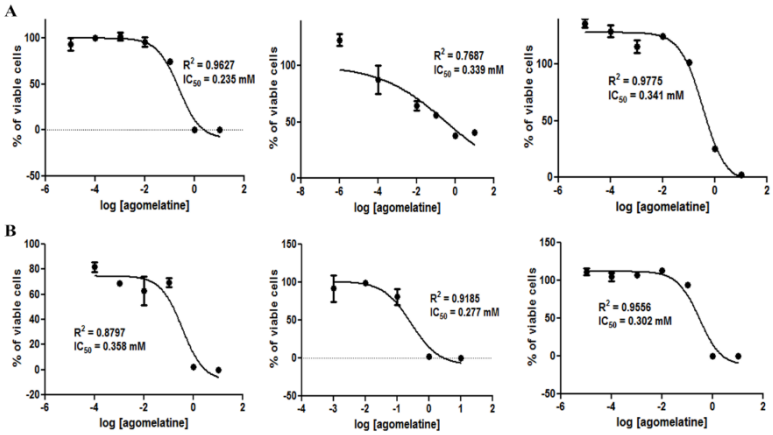

Fig. 1. Dose response curves to obtain IC_{50} concentration for agomelatine.

A) *Percentage of viable cells after agomelatine treatment (logarithmic scale) in MCF-7 cells. B) Percentage of viable cells after agomelatine treatment (logarithmic scale) in MDA-MB-231 cells. The final IC_{50} values were obtained as an average and the standard deviation of the three independent curves.*

Table 1. IC_{50} obtained for agomelatine in MCF-7 and MDA-MB-231 cell lines.

Substance	IC_{50} MCF-7 (mmol.L^{-1})	IC_{50} MDA-MB-231 (mmol.L^{-1})
Agomelatine	0.305 ± 0.05	0.312 ± 0.03

The inhibitory concentration of agomelatine obtained by IC_{50} after 48 h exposure.

3.2. Agomelatine promotes greater reduction in replicative BC cell growth than that of melatonin

After evaluating the clonogenic survival assay, treatment with agomelatine resulted in greater reduction in replicative cell growth than that of melatonin, which was evidenced in the two cell lines (Figure 2).

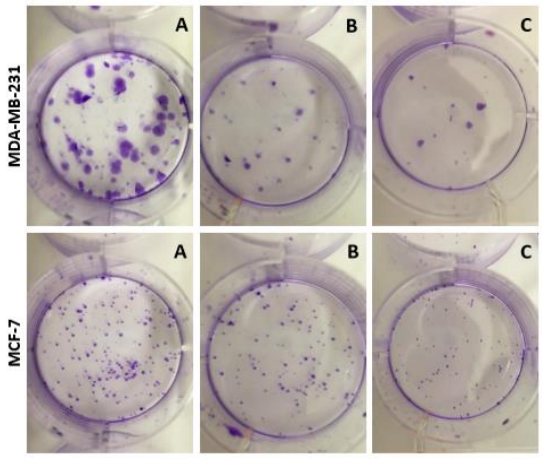

Fig. 2. Melatonin and agomelatine significantly affect MDA-MB-231 and MCF-7 cell growth.

The images show a reduction in the proliferative potential of both tumor cell lines after treatment with 1mmol L^{-1} melatonin and 0.300 mmol L^{-1} agomelatine; (A: control; B: melatonin; C: agomelatine). The growth reduction is even accentuated after agomelatine exposure.

After counting the colonies, the plating efficiency (PE) and the survival factor (SF) were calculated. In fact, agomelatine showed lower values of PE and SF in MCF-7 and MDA-MB-231 cell lines than melatonin (Table 2). The colonies formation assay revealed that both melatonin and agomelatine significantly reduced the number of MDA-MB-231 cells. Conversely, the number of MCF-7 cells was only significantly reduced after agomelatine exposure (Figure 3).

Table 2. PE and SF in the two cell lines after treatment with agomelatine (0.300 mmol L^{-1}**) and melatonin (**1mmol L^{-1}**).**

Variable	Group	MCF-7	MDA-MB-231
-	Control	33%	14%
Plating efficiency	Agomelatine	27%	3.6%
	Melatonin	34%	7.6%
Surviving factor	Agomelatine	81.8%	25.7%
	Melatonin	103%	54.2%

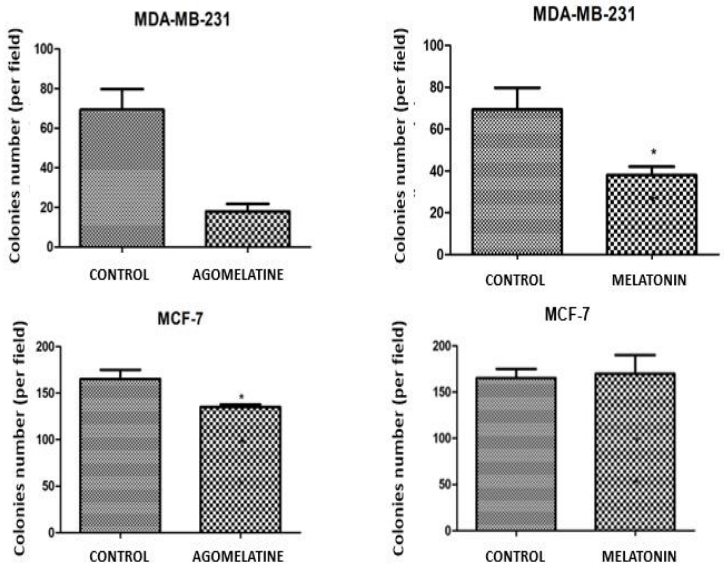

Fig. 3. Number of colonies in the two BC cell lines after treatments.

*Agomelatine treatment (0.300 mmol L^{-1}) showed a statistically significant reduction in colony proliferation in both BC strains, especially in MDA-MB-231 cells. Melatonin treatment (1mmol L^{-1}) was effective to significantly reduce the number MDA-MB-231 cell lines. * P <0.05 vs control group. The results were presented as Mean ± SEM. Data were performed in three biological and technical replicates.*

3.3. Melatonin and agomelatine similarly inhibited the migratory capacity of triple-negative BC cell.

After 24 h of exposure period, a significant reduction in MDA-MB-231 triple-negative cell migration was observed following both melatonin and agomelatine treatment, as shown in Figure 4. Notably, agomelatine and melatonin showed a similar potential to inhibit the migratory capacity of aggressive triple negative BC cells.

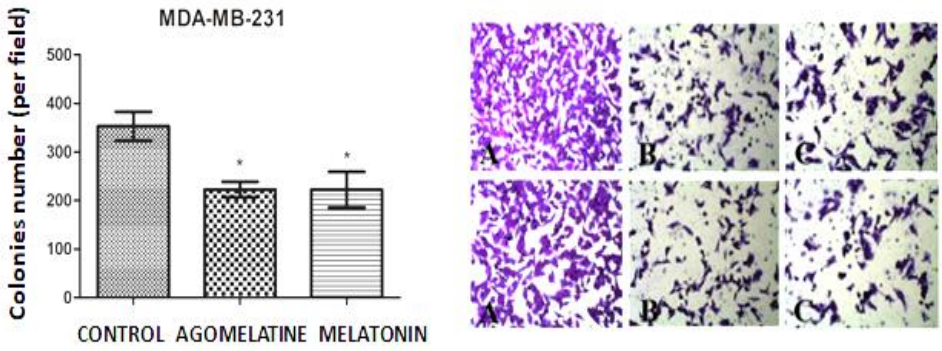

Fig. 4. Migratory capacity of MDA-MB-231 cells after agomelatine and melatonin treatments.

*The inserts were photographed using the inverted light microscope. Group A: control; Group B: treated with agomelatine at 0.300 mmol L-1; Group C: treated with melatonin at 1 mmol L-1. The similarity of the reduction in cell migration after treatment with 1 mmol L-1 melatonin and 0.300 mmol L-1 agomelatine was observed with a significance of * p = 0.0041. One-way ANOVA complemented with Bonferroni's test. Results were presented as Mean ± SEM. Data were performed in three biological and technical replicates.*

4. DISCUSSION

The oncostatic and antiproliferative actions of melatonin have been well documented (4, 6). By activating MT1 and MT2 receptors, melatonin influences microtubule formation and cytoskeleton organization in ER positive tumor cells, inducing cellular stress that culminates in reduced cell adhesion - one of the factors responsible for cell invasion and migration - characteristics of tumor cells (7). In addition, melatonin acts in an antiestrogenic manner through MT1, via the enzymes responsible for estrogen synthesis, while preventing the binding of the estradiol-RE complex to the estrogen receptor binding site (ERE) in DNA (7).

ER positive cells have characterized as the high MT1-expressing cell line, and the action of melatonin on this cell line is mainly mediated by its membrane receptors. In ER negative cells, which show reduced expression of membrane MT1 receptors, melatonin may act by crossing the cell membrane due to its lipophilic aspect (27), thereby interacting with intracellular molecules.

Jablonska *et al.* (28) reported that MT1 receptor expression is higher in ER positive and HER2 positive tumors, and their results showed that the lowest level of MT1 expression was observed in MDA-MB-231 strain. Girgert *et al*. (29) obtained similar results in a study with different strains, thus revealing that there was higher MT1 expression in MCF-7 than those in other strains with lower ER expression.

In previous studies, Treeck *et al*. (30) observed that MT1 expression is significantly lower in ER-positive MCF-7 and in OVCAR-3 than that in MDA-MB-231 cells. This divergence indicates that the effect of estradiol is clearly dependent on MT1, albeit the mechanisms are not yet fully elucidated (29). Being a more specific ligand to the MT1 receptor than melatonin, agomelatine may be another viable option as a new therapeutic agent for the breast cancer. The results obtained in the clonogenic survival assay allow us to conclude that agomelatine is more potent than melatonin in inhibiting the viability of MCF-7 cells, while both melatonin and agomelatine similarly inhibited the migratory capacity of MDA-MB-231 strain. To obtain a better comparison of the action between these two molecules, we have also investigated the effects of agomelatine and melatonin on proliferative capacity in the *in vitro* condition, in addition to metastatic potential associated with the migratory capacity of mammary tumor cells.

The results indicate that agomelatine has the potential to reduce the proliferative capacity in both mammary tumor cell lines, and the treatment with the agent revealed a greater reduction in replicative cell growth, with a statistically significant reduction in both cells. Melatonin also, showed its efficacy in reducing the colony proliferation of MDA-MB-231 cells.

Our results showed some difference from Mao *et al*. (31), who demonstrated that melatonin had little influence in the proliferation rate of MDA-MB-231 cells, even though the expression of MT1-associated G proteins was elevated. According to the authors, these divergences suggest that the same set of G proteins could regulate cell proliferation differently in these strains due to changes in ERK1 and ERK2 signaling pathways.

Based on the results from previous studies, the better performance of agomelatine may be associated with its higher affinity for melatonergic receptors and a longer half-life compared to melatonin (9). S26131, a dimer formed by the binding of two agomelatine molecules, shows a 200 times higher affinity for MT1 than other ligands, including melatonin, as well as greater MT2 receptor binding capacity (32).

The *in vitro* and *in vivo* studies using various antidepressants (including agomelatine) were conducted by Bielecka-Wajdman *et al*. (21). They suggest a high influence on immune activities and intracellular signaling pathways which may not be neutral in relation to the cancer progress and therapy. It has also been shown that the same antidepressants can promote or inhibit tumor growth while modulating the harmful effects of anticancer drugs. In this context, agomelatine was also effective in reducing cell migration.

We further observed a significant reduction in MDA-MB-231 cell migration in both molecule-treated groups. Our results show that agomelatine presents the similar capacity as melatonin to inhibit BC cell migration, thus corroborating the possible similarity in their therapeutic action. The cell migration assay was also performed with the MCF-7 cell line. Although the protocol was performed and repeated, no results could be obtained for this cell line; these failures are believed to be due to the fact that this is a luminal histological subtype A - positive estrogen receptor (ER), and/or positive progesterone receptor (PR), and negative epidermal growth factor 2 receptor (HER2 -). This subtype has a low proliferation rate and presents good prognosis, which may have influenced its action in this trial (26).

Several advantages of agomelatine have been described and these include: 1) Agomelatine is approved by Health Canada and by European Medicines Agency (EMA) and commercialized as an antidepressant. As such, it is a standardized product sold without medical prescriptions, exempt from stringent approved drug standards; 2) agomelatine has a considerably more restricted affinity for MT1 and MT2 receptors than its natural ligand; 3) agomelatine has a much longer half-life in the organism than that of melatonin; 4) absorption is more uniform and reliable than melatonin (33, 34).

Our experimental data confirmed that agomelatine has antiproliferative and antimetastatic effects, which are also observed in melatonin-treated cells, revealing a synergistic potential among them in breast cancer therapy. The mechanisms involved in the relationship between these agents and their active receptors, however, need further studies for better elucidation. Recent studies have been successful in discovering and testing the MT2 receptor-specific ligands; however, there are still few suitable ligand options for MT2 and none has been tested *in vivo* (9).

In summary, this study provides important information on the action of agomelatine and melatonin in breast cancer cells, confirming the anti-proliferative and pro-apoptotic action of these agents. Melatonin and agomelatine similarly inhibit the migratory capacity in triple-negative breast cancer cells, being both helpful against aggressive breast cancer. Additional studies involving other tumor subtypes are needed to elucidate their effects considering specific receptor expression, activity, and distinct circadian gene machinery. These approaches may stimulate preclinical studies enabling its use as therapeutic agents.

ACKNOWLEDGEMENTS

We thank to FAMERP for the infrastructure and professional support, and to São Paulo State Research Support Foundation (FAPESP - process number 2017 / 18832-3) for the financial support. Also, we would like to give a special thanks to Prof Dr Luis Octavio Regasini, from Laboratory of Antibiotics and Chemotherapeutics (LAQ), Institute of Biosciences Humanities and Exact Sciences (Ibilce), for the partnership during agomelatine extraction procedure.

AUTHORSHIP

AOCG was responsible for the experimental design, performed the experiments, analyzed obtained data and the article writing. JGO and MGMP contributed to the experiments and data analysis. MBS contributed with agomelatine extraction procedure. LGC and JC contributed to the data analysis and the article writing. DAPCZ was responsible for experimental design, data analysis and the article writing.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Please cite this paper as:
Galeti, A., de Oliveira, J., Pinheiro, M., dos Santos, M., Colombo, J., Chuffa, L. and Zuccari, D. 2021. Verification of agomelatine in comparison with melatonin as a therapeutic agent in breast cancer. Melatonin Research. 4, 1 (Jan. 2021), 141-151. DOI:https://doi.org/https://doi.org/10.32794/mr11250087.