SYNERGISTIC ACTIONS OF ALPELISIB AND MELATONIN IN BREAST CANCER CELL LINES WITH *PIK3CA* GENE MUTATION

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ABSTRACT

Aims: Breast cancer (BC) presents high mortality rate and about 30-40% have mutation in the *PIK3CA* gene. Alpelisib is a PI3K inhibitor that acts on p110α. The melatonin shown important anti-neoplastic effects and may increase the effectiveness of chemotherapy. This study evaluated the synergistic action of Alpelisib and Melatonin in BC lines carrying the H1047R mutation in *PIK3CA*, relative to the cellular dynamics and the *PI3K/AKT/mTOR pathway.*

Main methods: MDA-MB-468 (triple negative), MDA-MB-453 (triple negative, H1047R *PIK3CA*) and T-47D cells (luminal A, H1047R *PIK3CA*) were divided into four treatment groups: control; Melatonin (1 mM); Alpelisib (1 μM); and Alpelisib (1 μM) + Melatonin (1 mM). Cell viability and migration were investigated using the MTT assay and Transwell assay, respectively. Protein expression of PI3K, p-AKT, mTOR, HIF-1α, and caspase-3, was verified using immunocytochemistry.

Key findings: MTT assay revealed that MDA-MB-453 and T-47D showed reduction in cell viability in all groups, especially in the MDA-MB-453 treated with Melatonin + Alpelisib. MDA-MB-468 presents reduction in cell migration only with Melatonin, while in the lines with mutation, the treatment of Melatonin + Alpelisib caused inhibition of cell migration. PI3K, p-AKT, mTOR and HIF-1α were inhibited after treatment with Melatonin + Alpelisib in MDA-MB-453 and T-47D lines. The expression of caspase-3 increased in all groups in MDA-MB-453 and T-47D cells, being the increase more pronounced in the Melatonin + Alpelisib group.

Significance: These results indicate that the combined use of Melatonin and Alpelisib may be more effective in inhibiting BC in women carrying the *PIK3CA* gene mutation than either treatment alone.

Keywords: Breast Cancer, Alpelisib, Melatonin, *PIK3CA*, MDA-MB-453, T-47D.

1. Introduction

Breast cancer (BC) is the most common neoplasm in humans worldwide [1], [2]. Triple-negative breast cancer (TNBC) is highly heterogeneous, biologically more aggressive, and rapidly evolves with recurrence and involvement of lymph nodes and distant metastasis [3], [4]. Patients diagnosed with TNBC have a higher mortality rate within five years of diagnosis compared to other BC subtypes [5].

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian rapamycin target (mTOR) signaling pathway plays important role in unregulated growth, proliferation, cell migration, metabolism, angiogenesis and apoptosis. In BC, the PI3K/AKT/mTOR pathway can be deregulated by several mechanisms [6]. The oncogene phosphatidylinositol-4,5-bisphosphate 3-kinase alpha catalytic subunit (*PIK3CA*) encodes the p110α subunit of the PI3K protein [7].

Mutations in the *PIK3CA* gene are present in 25-46 % of breast malignancies, and are associated with chemoresistance, reserved prognosis and lower overall survival of patients [5]. Approximately 16 % of TNBC have mutations in *PIK3CA* [8], and the vast majority of *PIK3CA*-activated mutations occur in p110α, being more present in metastatic tumors [9], [10]. The mutation H1047R (3140A>G) in the gene *PIK3CA*, located in exon 20, chromosome 3 (3q26.32), presents a genetic alteration in 3140º nucleotide, i.e., meaning the exchange of histidine by arginine in the 1407th residue of the protein [11]. This mutation leads to increased function of the catalytic isoform p110α, manifested by an elevated lipid kinase activity, resulting in a hyperactivated PI3K pathway [7], [11]. Consequently, this induces an increase in phosphorylated AKT (p-AKT), which promotes

the downstream signaling activation leading to deregulated growth, proliferation and increased cell survival [7].

Downstream of the PI3K/AKT/mTOR pathway are some proteins that influence breast cancer progression, such as the hypoxia inducible factor (HIF-1α). This protein plays a fundamental role in the adaptation of tumor cells to hypoxia, activating the transcription of target genes that regulate angiogenesis [12]. On the other hand, caspase-3 activity (CASP3) is a fundamental biomarker of apoptosis[13]. Pu et al. observed that high CASP3 expression is significantly associated with higher survival of BC patients [14].

PI3K protein is one of the main therapeutic targets for cancer treatment [15]. Alpelisib or BYL719 (marketed under the brand name Piqray®) is an oral PI3K α-specific inhibitor that selectively suppresses p110α; it has been approved for use by both the US Food and Drug Administration and Anvisa in 2019, based on the results of the phase III SOLAR-1 study (NCT027318) [16]. This drug acts by blocking the effects of PI3K enzymes thus reducing the growth and proliferation of tumor cells [17]. The drug is indicated for postmenopausal women and also for men with metastatic or advanced BC with mutation in *PIK3CA*, ER/PR+ and HER2- [18].

Melatonin (N-acetyl-5-methoxytryptamine) is best known as being synthesized and secreted by the pineal gland, mainly at night. The circadian production of melatonin is interrupted when exposure to light occurs during the dark period [19]. Melatonin has oncostatic, anti-metastatic and antiangiogenic effects as documented under many different experimental conditions [20], [21]. Many other cells also produce melatonin but not in a circadian nor do they release the synthesized product [22], [23].

Both Alpelisib and Melatonin alter the protein expression of the targets of the PI3K/AKT/mTOR pathway, in addition to the downstream proteins. The potentiation of

the oncostatic actions of Alpelisib would be desirable because, although partial responses and long-term stabilization have been observed in breast tumors with mutation in *PIK3CA*-treated with PI3K inhibitors, the majority of these cancers do not exhibit substantial regression [24]. Against this background, this study evaluated the synergistic action of Alpelisib and melatonin in BC lines with H1047R mutation in *PIK3CA*, with especial focus to cell viability and migration as well as expression of PI3K, p-AKT, mTOR, HIF-1α, and CASP3.

2. Materials and Methods

2.1. Treatments

Alpelisib pills (Piqray™ - Novartis, Basel, Switzerland) were kindly provided by Dr. Noam Falbel Pondé, Medical Director at IQVIA Biotech (Brazil), for the extraction of the active ingredient, in partnership with Prof. Dr. Luis Octávio Regasini of the Laboratory of Antibiotics and Chemotherapy (LAQ) of the Institute of Biosciences, Human and Exact Sciences of the São Paulo State University (IBILCE-UNESP). Pills containing 25mg of Alpelisib were minced and filtered with ethyl acetate. The extract was separated into organic and inorganic phases using water and ethyl acetate. Subsequently, sodium chloride and magnesium sulfate were added to absorb the water. A new filtration process was carried out and the solvent was then removed with the aid of a rotoevaporator. The resulting solution was cleaned in ultrasonic bath and finally dried at room temperature. Next, the pure solid of Alpelisib was diluted in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA) at a concentration of 1μM, as described by Baldassari et al. [25].

Melatonin was commercially obtained (Sigma-Aldrich, Missouri, USA) and diluted to 1mM in equal parts of absolute ethanol and 1X phosphate-saline buffer (PBS). The concentration of 1 mM of Melatonin was selected on the basis of results obtained from previous studies by our research group [20], [26].

2.2.Cell culture

MDA-MB-468 (HTB-132) cell line was purchased from the American Type Culture Collection (ATCC, Virginia, USA). The MDA-MB-453 cells were kindly provided by Prof. Dr. Sandra Martha Gomes Dias from the National Biosciences Laboratory (LNBio) of the National Center for Research in Energy and Materials (CNPEM). The T-47D cells were kindly provided by Prof. Dr. Gláucia Maria Machado-Santelli from the Laboratory of Cellular and Molecular Biology (LBCM) of the Institute of Biomedical Sciences (ICB) of the University of São Paulo (ICB-USP).

For the in vitro study, MDA-MB-468 (triple-negative), MDA-MB-453 (*PIK3CA* H1047R mutation, triple-negative) and T-47D (*PIK3CA* H1047R mutation, luminal A) were cultivated in Dulbeco's Modified medium (DMEM) high glucose (Gibco™, Nova York, EUA), supplemented with 10 % Fetal Bovine Serum (FBS) (LGC Biotecnologia, São Paulo, Brazil) and 1 % Penicillin solution (10000 UI/mL)/Streptomycin (10 mg/mL) (LGC Biotechnology, São Paulo, Brazil). The cells in culture were kept in an incubator at 37 °C and 5 % CO_2 and on reaching 80 % confluence were divided into the four treatment groups: 1) control; (2) Melatonin (1 mM); (3) Alpelisib (1 µM); and (4) Alpelisib (1 µM) + Melatonin (1 mM).

2.3. Quantitative PCR (qPCR)

Initially, 5 x 10^5 cells were transferred into wells of culture plates with six wells in 2 mL of high glucose DMEM culture medium (Gibco™, New York, USA). The next day, the culture medium was removed and the cells were treated in duplicate for 24 h supplemented without or with Alpelisib or melatonin alone or in combination. After the treatment period, total RNA extraction was performed using the reagent TRIzol™ (Invitrogen, Massachusetts, USA) according to the manufacturer's instructions. The quantification of total extracted RNA, as well as its integrity and quality, were analyzed using the Nanodrop 2000™ (Thermo Scientific, Massachusetts, USA). The cDNA was synthesized by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Massachusetts, USA), following the manufacturer's instructions, in a total volume of 20 µL of reaction mixtures at a final concentration of 100 ng/µL.

Quantitative gene expression analysis was performed by StepOne Plus Real-Time PCR (Applied Biosystems™, Massachusetts, USA) and a negative control was included in each reaction. For this, TaqMan Gene Expression (Applied Biosystems™, Massachusetts, USA) assays were used: gene of interest *PIK3CA* (Hs00907957_m1); endogenous genes GAPDH – glyceraldehyde 3-phosphate dehydrogenase (Hs99999905_m1) and RPLP0 – ribosomal protein lateral stalk subunit P0, which were selected as reaction normalizers. The cycling conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 sec and 60 ºC for 60 sec. The value of the relative gene expression was determined by the quantification method relative to the mean of the normalizing genes (ΔΔCt). The samples were tested in triplicates and the experiments were followed by a negative control. The entire procedure was performed according to Moschetta et al. [27].

2.4. Cell Viability Assay (MTT)

To evaluate the viability of the cells after treatments, the colorimetric assay 3{4,5-dimetiliazol-2-il}-2,5difeniltetrazolium bromide (MTT) was performed according to Gelaleti et al. [28]. Initially, 5 x 10^4 cells/well of human BC lines were divided into 96-well culture plates containing 200 µL of high glucose DMEM culture medium (Gibco, New York, USA) supplemented and kept in an oven at 37 ºC overnight with 5 % CO_2. The treatments were performed in triplicate according to the groups for 24 h. At the end of the treatment period, the solutions used for cell treatments were discarded. Then, we added 100 µL of fresh culture medium and 10 µL of MTT solution (12 mM) to each sample, followed by incubation for 4 h in an oven at 37 °C and 5 % CO_2. Subsequently, for the solubilization of formazan crystals formed from the metabolization of MTT, 25 µL was removed from the supernatant and the cells were incubated with 50 µL of DMSO (Sigma-Aldrich™, Missouri, USA) for 10 min at 37 ºC and 5 % of CO_2. Finally, the absorbance reading was performed at 540 nm wavelength using the Multiskan™ FC Microplate Photometer (Thermo Scientific, Massachusetts, USA). Relative cell viability (%) was calculated based on the control cells (without treatment), attributed as 100 % cell viability.

2.5. Cell migration assay

The cell migration assay was performed in the Boyden chamber, according to Galeti et al. [29]. The assay was developed in Costar™ 24-well plates (Corning, New York, USA) using Transwell™ inserts (Corning, New York, USA) with polycarbonate membranes of 8.0 µm and diameter of 6.5 mm. Initially, 1 x 10^6 cells of cell lines MDA-MB-468, MDA-MB-453 and T-47D were transferred to wells of culture plates (six wells

each). After 24 h, the cells were treated and at the end of the treatment period, the cells were trypsinized and counted in a Neubauer chamber to verify the number of viable cells. A 500 µL of culture medium supplemented with 10 % FBS (chemotoxic agent) was added to the bottom of the 24-well plate wells. Then, the inserts were positioned in each of these wells with the supplemented culture medium and 300 µL of culture medium without FBS was added inside the inserts containing 1 x 10^5 of the previously treated cells mentioned in the previous paragraph. After 24 h of incubation, the culture medium was removed and carefully absorbed with the aid of a cotton swab. The supplemented culture medium was also removed, and the cells contained in the inserts were fixed, transferred to other wells of the same plate containing 500 µL of paraformaldehyde at 4 %, for 15 min. Subsequently, the inserts were stained with violet crystal for 5 min to detect the migratory cells. The visualization of the migratory cells was performed in an optical microscope under a 10x objective. All experiments were developed in duplicate and the cell count was performed on Image J™ software.

2.6. Immunocytochemistry

After all treatments, the immunocytochemistry was performed to analyze the expression of PI3K (1:50; ab135958, Abcam), p-AKT (1:200; ab81283, Abcam), mTOR (1:200; ab84400, Abcam), HIF-1α (1:50; sc-59546, Santa Cruz), and CASP3 (1:50; ab4051, Abcam) using the REVEAL-Biotin-Free Polyvalent DAB kit (Bioscience, California, USA) according to Marques et al. [30]. First, 6 x 10^4 cells were transferred from BC cell lines to slide with coupled silicone, in 200 µL of high glucose DMEM culture medium (Gibco™, New York, USA). Then, the culture medium was removed and the treatments were applied as previously described for 24 h.

Briefly, the cells were washed with 1X PBS and fixed with paraformaldehyde 4 %. Then, the cells were permeabilized with 1X PBS/Triton X-100 at 0.5 % (Sigma-Aldrich, Missouri, USA) for 10 min. Peroxidase activity was blocked for 15 min and the Protein Block was applied and incubated for 10 min. The dilution of the specific primary antibodies was prepared according to Table 2 in 1X PBS solution and bovine serum albumin at 5 %. The specific primary antibodies were incubated at 4 °C overnight. The complement and the conjugate HRP were applied, followed by the DAB Chromogenic Substrate, which was diluted in DAB Diluent Buffer (1:50). Finally, the cells were stained with Harris hematoxylin. The assembly of the slides was performed in glycerol and sealed. All immunoreactions were accompanied by a negative control (no addition of the primary antibody). The slides were observed under the 40x objective microscope (Olympus BX53) and analyzed by optical densitometry. The analysis was developed in triplicate and the quantification of protein expression was performed using the Image J.

2.7. Statistical Analysis

The experiments were performed in duplicate or triplicate and the values were expressed as mean ± standard deviation. For the comparison of more than two parameters, Analysis of Variance (ANOVA) was used, followed by Tukey or Newman-Keuls test. $P<0.05$ was considered statistically significant and all analyses were performed using the GraphPad Prism 9 software.

3. Results

3.1. MDA-MB-453 and T-47D cells show high PIK3CA expression and PI3K protein levels

As expected, the BC lines, MDA-MB-453 and T-47D, carriers of the H1047R mutation, had higher *PIK3CA* gene expression and protein levels of PI3K (Figure 1A and B) compared to the MDA-MB-468 cell, which lacks this mutation ($p<0.05$).

3.2. Combined treatment of Alpelisib and Melatonin decreases viability in the MDA-MB-453 cell line

For the MDA-MB-468 cells, treatments with Melatonin and/or Alpelisib did not significantly reduced cell viability compared with control cells (Figure 2A). Conversely, for the triple-negative MDA-MB-453 cells, that carries the H1047R mutation, two of the three treatments (except for melatonin only) significantly reduced cell viability compared with control cells (Figure 2B). It is worth mentioning that the reduction of cell viability was more pronounced in the group treated with Alpelisib + Melatonin, reducing viability by about 35 % compared to the control. Regarding the luminal A cell line T-47D with the H1047R mutation, all treatments were able to significantly reduce cell viability compared to control cells (Figure 2C). However, there was no difference in cell viability between the group treated only with Alpelisib and that treated with Melatonin and Alpelisib,where these treatments reduced cell viability by approximately 58% and 59%, respectively.

3.3. Combination of Alpelisib and Melatonin decreases migration in the T-47D cell line

The cell migration for the MDA-MB-468 cell line showed a significant decrease of 55.04 % in the Melatonin-treated cells group (1 mM) compared to the control cells (Figure 3B). The same occurred with Melatonin treatment of the MDA-MB-453 and T-47D mutated cells showing a reduction of 54.3 % and 44.5 %, respectively (Figures 12C and 12D). Also, there was a significant reduction in the migration rates of mutation-bearing cells in all treated groups compared to control (Figures 12C and 12D). However, in the MDA-MB-453 cell line, no significant difference was observed between the groups Alpelisib and Alpelisib + Melatonin; these groups reduced cell migration by approximately 90 % compared to the untreated group (Figure 3C). For the T-47D cell line, there was no significant difference in the reduction of migratory cells between the groups treated with Melatonin and Alpelisib (Figure 3D). Notably, there was a marked reduction by about 72 % of migratory cells after treatment with Alpelisib and Melatonin compared to control.

3.4. PI3K protein expression is reduced in MDA-MB-453 cells after combination of Alpelisib and Melatonin

The PI3K expression in the MDA-MB-468 cells was significant decrease after treatment with Melatonin and Alpelisib + Melatonin compared to cells without treatment (Figure 4B). Treatment with Alpelisib resulted in significant rise of PI3K in the MDA-MB-468 cells compared to the control. In MDA-MB-453 cells, there was a decrease in PI3K protein expression in all treatment groups relative to the control group (Figure 4C), especially in cells treated with Alpelisib + Melatonin. Similarly, for the T-47D cell, the PI3K expression was significantly reduced in all treatment groups compared to the

untreated group (Figure 4D). However, no significant difference was observed by comparing the T-47D cell treated with Melatonin or Alpelisib only.

3.5. p-AKT protein expression decreases in T-47D cells after combining Alpelisib and Melatonin treatment

The p-AKT expression was significantly decreased in MDA-MB-468 and MDA-MB-453 cells after treatment with Melatonin or Alpelisib + Melatonin compared to untreated cells (Figures 5B and 5C), but this reduction was remarkably in the cells treated with Melatonin only. For the T-47D cells, there was a decrease in the p-AKT expression in all treatment groups compared to the control group (Figure 5D), especially in the cells treated with Alpelisib + Melatonin.

3.6. mTOR is reduced in MDA-MB-453 cells after combination of Alpelisib with Melatonin

In the MDA-MB-468 cells, mTOR expression was reduced by all treatments compared to the untreated group (Figure 6B), but this reduction was more pronounced in the Melatonin-treated group. Also in the MDA-MB-453 cells, we observed a significant decrease in mTOR protein expression in all treatment groups compared to the control cells, with an even greater reduction when they were incubated with Alpelisib + Melatonin (Figure 6C). In the T-47D cells, there was also a decrease in mTOR in all treatment groups compared to the controls (Figure 6D), being the reduction even more evident after treatment with Melatonin or Alpelisib + Melatonin.

3.7. HIF-1α expression decreases in MDA-MB-453 and T-47D cells after Alpelisib and Melatonin treatment

MDA-MB-468 cells showed a reduction in HIF-1α expression after treatment with Melatonin compared to the untreated group (Figure 7B). Conversely, in the MDA-MB-453 and T-47D cells that carry the H1047R mutation, there was a drop in HIF-1α expression in all treatment groups, with a greater reduction when treated with Alpelisib + Melatonin (Figures 7C and 7D).

3.8. CASP3 expression is increased in MDA-MB-453 and T-47D cells after combination of Alpelisib and Melatonin

The MDA-MB-468 cells showed a significant decrease in CASP3 after treatment with Alpelisib or Alpelisib + Melatonin compared to the untreated cells (Figure 8B). In the cells harboring the H1047R mutation, i.e., MDA-MB-453 and T-47D, a significant increase in CASP3 expression was observed in all treatment groups, mainly after exposure to Alpelisib + Melatonin (Figures 8C and 8D).

4. Discussion

The main purpose of this study was to determine if there is a synergistic action of Alpelisib and Melatonin in BC cell lines carrying the gene mutation H1047R in *PIK3CA*. We used MDA-MB-453 and T-47D cells since they have high *PIK3CA* gene expression.

Alpelisib is mainly indicated for women with advanced BC, ER/PR+ and HER2; this profile corresponds to the phenotype of the T-47D cell. However, interesting results

were also observed in the triple-negative MDA-MB-453, especially when Alpelisib was associated with Melatonin. This fact corroborates the initial hypothesis that Melatonin may potentiate the action of this drug in women with TNBC who do not yet have a fully established treatment protocol.

The mechanism of Alpelisib's action in breast neoplastic cells is already well established and documented. However, there are several mechanisms by which Melatonin exerts its anti-proliferative roles [26], antiangiogenic [31], anti-metastatic [20] and pro-apoptotic [32] actions and some hypotheses have already been raised. Thus, it has been observed that Melatonin binds and activates two distinct types of membrane-bound G-protein-coupled receptors, MT1 and MT2, as well as the orphan nuclear receptors RZR/RORα, members of the superfamily of steroid/thyroid hormone receptors [33]. The MT1 and MT2 are expressed in human cells and neoplastic cells, while BC cells express the MT1 receptor [26].

The Melatonin receptors are common for ER+ BC cells. These receptors have low expression in breast cells that have a triple negative receptor phenotype [34]. However, by activating MT1, Melatonin suppresses cancer development through a broad spectrum of mechanisms with and without ER involvement [35]. Thus, Oprea-Ilies et al. [36] demonstrated that even in triple negative receptor breast tumors, MT1 positivity was associated with a smaller tumor stage and size at the time of diagnosis.

Melatonin exerts antitumor properties by a set of rather complex mechanisms which do not involve the receptor pathway [37]. Via non-receptor-mediated processes, melatonin modulates redox homeostasis, especially at the level of the mitochondria, to inhibit cancer cell proliferation [38]. While melatonin is a potent antioxidant in normal cells, in pathological cells it can also function as a pro-oxidant agent [39].

In this study, both Melatonin and Alpelisib reduced cell viability and migration in the mutation-bearing cells, MDA-MB-453 and T-47D. However, when Alpelisib was used in conjunction with Melatonin, the decrease in cell viability was more pronounced in the MDA-MB-453 cell; conversely, the reduction in cell migration was more pronounced in the T-47D cell when compared to the exclusive use of Alpelisib.

The efficacy of Melatonin in suppressing ER+ breast cancer is especially obvious where it has a marked inhibitory effect on the proliferative response of cells [26]. This corroborates with the results obtained in the analysis of cell viability of the T-47D cells, which included both ER+ and PR+ cells; here, we observed a reduction of approximately 41 % of the viability of cells treated with Melatonin only.

There was, however, no difference in the viability of T-47D cells when compared to the Alpelisib-treated cells or those incubated with Alpelisib + Melatonin; this was not unexpected since Alpelisib is an effective treatment for this cancer type. Thus, melatonin presumably had no synergistic effect because Alpelisib has already reached the full therapeutic potential in T-47D cells.

Wang et al. [32] analyzed cell proliferation in the triple-negative cell line MDA-MB-361 and the results showed that treatment with Melatonin at the pharmacological concentration (0.6-1 mM) for 72 h inhibited cell proliferation in a dose-dependent manner. Moreover, the treatment of this same cell line with 10 μM of PI3K inhibitor-IV reduced cell proliferation and this inhibition exhibited a non-statistically significant increase when used in combination with melatonin [32].

On the contrary, our results showed that Melatonin potentiated the effect of Alpelisib in the triple-negative MDA-MB-453 cell, reducing cell viability by about 35 %. Thus, the actions of melatonin used in conjunction with the specific inhibitor of PI3K-α were synergistic, since MDA-MB-453 cells have the *PIK3CA* mutation and Alpelisib is

a target of PI3K [11]. To date, no study evaluated the combined action of Alpelisib and Melatonin on the cell viability of BC cells.

Alpelisib reduced the migratory capacity of cells with the H1047R mutation. However, this reduction was even more pronounced in T-47D, when these cells received the treatment combination with Alpelisib + Melatonin. Thus, we once again observed the synergistic effect of Melatonin on the PI3K α-specific inhibitor in cell lines carrying the *PIK3CA* mutation.

Since the triple-negative MDA-MB-468 cells do not have the mutation in *PIK3CA*, our results showed that Alpelisib did not alter cell viability and migration. However, Melatonin reduced the migration of MDA-MB-468 cells by about 55 %, as noted by Jardin et al. [40]. These authors showed that Melatonin significantly reduces the migratory capacity of MDA-MB-468 cells. They also mentioned that Melatonin induces similar effects in MDA-MB-231 cells, suggesting that these actions may be specific in TNBC cells [40]. Similarly, herein we observed that after 24 h of treatment with Melatonin, there was a significant attenuation in the invasion and migration of triple-negative MDA-MB-231 cells [20].

Inhibition of PI3K represents a potentially promising strategy for the treatment of BC [24]. Chen et al. [41] showed that Melatonin suppresses the PI3K/AKT/mTOR signaling pathway in a time-dependent manner, inhibiting phosphorylation of PI3K, AKT and mTOR. Frigieri et al. [42] suggest that Melatonin activates their receptors, reducing the expression of *PI3K/AKT* genes; this reduction would presumably be a major contributor to the beneficial effect of Melatonin in reducing carcinogenesis.

The present findings showed that Melatonin decreases the PI3K in all cell lines. This change was more pronounced in the MDA-MB-453 cells, when Melatonin was associated with Alpelisib. These data are similar to those of Vora et al. [24] in which

PI3K inhibitors were not as effective as each agent by itself. Thus, we suggest the synergistic effect of Melatonin and Alpelisib on the PI3K inhibition in the TNBC cell line carrying the H1047R mutation.

Herein, we report that melatonin inhibits the protein expression of phosphorylated AKT, both in the non-mutated cell line (MDA-MB-468) and in the mutated cell lines (MDA-MB-453 and T-47D). These results support previous reports showing that AKT expression is modulated by Melatonin [42].

In breast adenocarcinoma, Proietti et al. [43] observed that treatment with Melatonin significantly reduced phosphorylated AKT, but not an increased expression of PTEN, which may be a precursor event, before the negative regulation of phosphorylated AKT. Yuan et al. [44] showed that treatment with Alpelisib significantly reduced phosphorylated AKT levels in T-47D cells, as observed in our study, phosphorylated AKT was dramatically reduced in T-47D cells when they are treated with Melatonin + Alpelisib. Again, these results indicate that Melatonin in association with Alpelisib have synergistic actions.

The activation of mTOR plays an important role in protein synthesis and protein activation for cell cycle control. However, it also participates in the synthesis of tumor growth factors, contributing to the pathogenesis of cancer [42]. According to Jung et al. [45], Melatonin (12 mM) negatively regulated the phosphorylation of mTOR in the TNBC MDA-MB-231 cell line, which does not carry the H1047R mutation. Likewise, the present study revealed that treatment with Melatonin in TNBC cells (MDA-MB-468), which also lacks the H1047R mutation, reduced the expression of mTOR.

We observed a decrease in mTOR protein expression in all treatment groups in cell lines carrying the mutation in *PIK3CA* (MDA-MB-453 and T-47D). The MDA-MB-453 cells had a more pronounced reduction when treated with Melatonin + Alpelisib, thus

confirming the synergistic effect in the negative regulation of mTOR. This is the first study that evaluated the joint action of Alpelisib and Melatonin on mTOR expression. Wang et al. [32] reported that Melatonin can inhibit the mTOR signaling via its MT1 receptor. According to Jung et al. [45] Melatonin negatively regulates the phosphorylation of mTOR in the MDA-MB-231 cells, which lacks the H1047R mutation. Similarly, the present study revealed that melatonin reduced mTOR expression in MDA-MB-468 cells, which are also devoid of the H1047R mutation. Relative to Alpelisib, Yuan et al. [44] observed that this drug is able to inhibit proteins downstream of mTOR, such as p-S6K and p-S6, in TNBC cells (MDA-MB-231 and Hs578T). However, the inhibition was more pronounced in ER+, such as T-47D cells. According to some authors [43], [44], [46], [47], Alpelisib exerts an important effect on proteins of complex 1 of mTOR, and the sensitivity of tumor cells to Alpelisib depends on this effect. Thus, Vora et al. [24] suggest that the sensitivity of T-47D and MDA-MB-453 cells to Alpelisib may be dependent on its ability to suppress mTOR signaling.

HIF is critical in modulating tumor angiogenesis [48]. The anticarcinogenic properties of Melatonin are mediated by tumor metabolism suppression and signaling pathways such as PI3K/AKT and HIF-1α [49]. Melatonin has been shown to reduce HIF-1α expression in several types of cancer, including BC [26].

The results of the present study revealed that Melatonin reduces the protein expression of HIF-1α in MDA-MB-468 cells, as also observed by Marques et al. [30] This group documented that Melatonin (1 mM) positively regulated Mir-152-3p mediated reduction in HIF-1α protein levels in MDA-MB-468 cells [30]. This reinforces that Melatonin has a prominent role in controlling the growth of the MDA-MB-468 cells. Since the combined treatment reduced the expression of HIF-1α in cell lines MDA-MB-

453 and T-47D, we presumed that melatonin is capable of potentiating the actions of the PI3K inhibitor, Alpelisib, in cells with H1047R mutation.

The pro-apoptotic CASP3 plays an important role in cellular apoptosis and therefore has become a target for cancer treatments [50]. This study showed a higher expression of CASP3 in cells with H1047R mutation, MDA-MB-453 and T-47D when treated with Melatonin. Likewise, Wang et al. [32] reported that Melatonin stimulates caspase3/9 activities and cleavage, thus inhibiting cell proliferation and inducing apoptosis in MDA-BC cells. Alpelisib also acts on CASP3 expression. Maeda et al. [11] using the Caspase-Glo 3/7 assay, observed that Alpelisib inhibited cell proliferation by suppressing AKT phosphorylation and inducing apoptosis in canine hemangiosarcoma cell lines with *PIK3CA* mutation. In this study, Alpelisib + Melatonin showed a synergistic effect on the expression of CASP3, which promotes apoptosis of cells with *PIK3CA* mutation.

Conclusion

In general, we observed the potentiation of the effect of Alpelisib by Melatonin, particularly in the TNBC carrying the H1047R mutation in *PIK3CA* (MDA-MB-453); in this case, the combined therapy exaggerated the reductions in cell viability and migration over that caused by either treatment alone. This combination of drugs reduced the protein expression of PI3K, p-AKT, mTOR, HIF-1α while increasing the expression of CASP3. These results may encourage clinical studies with BC carrying the H1047R mutation where the combination of melatonin and Alpelisib could be useful In addition to having synergistic actions with Alpelisib, melatonin commonly reduces the collateral toxicity of chemotherapies and improves patient wellbeing.

Ethical approval and consent to participate

No applicable

Consent for publication

Yes

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Credit authorship contribution statement

BLVG, JC and DAPCZ conceived the study, designed the experiments, and drafted the manuscript. BLVG carried out the experiments. MGMP helped in migration assay. LGAC, NFP, RJR and JC assisted in the analyses of data and with the production of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no competing interests.

Data availability

Data will be made available on request

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Abbreviations

BC, breast cancer; TNBC, triple negative breast cancer; *PIK3CA*, Oncogene Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; PI3K, Phosphoinositide 3-kinase; AKT, Protein kinase B; mTOR, Mammalian target of rapamycin; HIF-1α, Hypoxia-Inducible Factor 1 alpha; CASP3, caspase-3; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide)

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