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EFFECTIVENESS OF PALBOCICLIB IN REDUCING CELL MULTIPLICATION IN HUMAN AND MOUSE BREAST CANCER CELL LINES

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ABSTRACT

Palbociclib, a recently developed anti-cancer drug was evaluated to determine its effects on the cell proliferation patterns in vitro in two human breast cancer cell lines namely, MCF-7 (Estrogen Receptor positive) and MDA-MB-231 (Estrogen Receptor negative), and in one mouse breast cancer cell line, 4T1 (Estrogen Receptor positive). Approximately, 100,000 cells in two ml of culture medium in each well of 6-well plates were incubated with Palbociclib dissolved in dimethyl sulfoxide (DMSO) at 24-hour intervals for 24 to 96 hours at concentrations ranging from 62.5 nM to 1µM. The control received an appropriate amount of DMSO without Palbociclib. The numbers of viable and dead cells in each culture were recorded every 24 hours using the Trypan blue exclusion assay. The drug inhibited cell replication significantly (Student's t-test) in the majority of drug concentrations and durations in both human and mouse breast cancer cells. Increasing the concentrations of Palbociclib resulted in decreased rates of cell proliferation, which was found to be significant in most instances. In general, MCF-7 ER positive cells showed slightly more sensitivity, especially at lower concentrations of Palbociclib than MDA-MB-231 ER negative cells; however, these differences were found to be non-significant in most data points. The mouse cells generally exhibited less sensitivity to the drug. The percentage of viable cells treated with different concentrations of Palbociclib was comparable to those of control groups, suggesting the cytostatic mode of inhibitory action.

KEYWORDS: **Palbociclib, MCF-7 and MDA-MB-231 human breast cancer cells, 4T1 mouse breast cancer cells, cell multiplication inhibition, cytostatic, cytotoxic**

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INTRODUCTION

Breast cancer is a significant cause of cancer death in females in the United States and the world. In 2016 it is predicted that there will be 246,660 new cases of breast cancer, and 40,450 deaths in the United States.[1] Traditional chemotherapies produce harmful and damaging side effects. One study found that in patients suffering from breast cancer or a malignant lymphoma, approximately 46% considered discontinuing their treatment by the sixth cycle. [2] It is imperative that effective treatments for breast cancer are developed, particularly ones that lessen the side effects usually associated with chemotherapy. Currently, cancers are generally treated with cytotoxic agents before or after surgery or radiation treatments. These chemotherapeutic agents not only kill cancer cells, but also attack other rapidly dividing cells like those in the bone marrow, lining in the mouth, intestines, and hair follicles, leading to serious side effects. A newer but less prevalent treatment option is to use a cytostatic agent that would halt tumor or cancer cell growth without markedly affecting other rapidly diving cells in the body. As a result, a tolerable treatment option will be available to both the patient and the physician.[3] Palbociclib, the current flagship drug for Pfizer, is currently in phase three clinical trials. The agent works by inhibiting CDK4 and CDK 6 kinases. By inhibiting these kinases, the cell division process cannot make the transition from the G1 to the S phase, effectively halting the cell division.[4,5] CDK 4 and CDK 6 bind with cyclin D to make the cyclin D-CDK4/6 complex. This complex functions in the cell division process by inhibiting the tumor suppressor gene Rb. Inhibition of Rb allows the expression of proteins necessary for the transition from G1 to S phase in the cell division cycle.[6] When Palbociclib blocks the action of CDK4 and CDK6, the cyclin D-CDK4/6 complex cannot be made and Rb remains active as a tumor suppressor halting unregulated cell division.[5] In a study by Cabrera et al., 7-month old mice with refractory dysplasia were given 150 mg/kg of Palbociclib by oral gavage. Palbociclib was able to successfully reverse the refractory dysplasia suggesting its success as a cytostatic treatment for halting abnormal cell divisions.[7] While the results suggest the potential of Palbociclib in inhibiting unbalanced cell divisions, the nature of abnormal cell proliferation in dysplasia differs from cancer. Baughn et al. showed Palbociclib's ability in inducing G1 arrest in multiple myeloma cells in vitro suggesting its effectiveness in halting uncontrolled cell proliferation.[5] Furthermore, at concentrations below 5μmol/L, Palbociclib acted as a CDK4/6 inhibitor but programmed cell death was not observed, signifying Palbociclib's potential as a cytostatic agent.[5] Finn et al. did not find evidence of apoptosis in a variety of human cancer cell lines including MCF-7 and MDA-MB-231 when exposed to Palbociclib for 5 days. Palbociclib has also been demonstrated to increase the effectiveness of cytotoxic drugs when used in combination with them. In another study, Finn et al. found that survival rates of women with ER+ and HER-2 negative breast cancer experienced longer survival times when Palbociclib was administered in combination with Letrozone than with either drug alone.[10] This suggests that Palbociclib can be used to boost the effects of cytotoxic drugs**.** In the present investigation, two human breast cancer cell lines (MCF7 and MDA-MB-231) and one mouse breast cancer cell line (4T1) were used. MCF-7 is a human breast cancer cell line, which is estrogen receptor positive (ER+), progesterone receptor positive (PR+) and does not over express HER-2 gene. The presence of the ER and PR allows MCF-7 to be a target for hormonal therapies.[11,12,13] MDA-MB-231 is characterized as a triple negative cell line because it lacks estrogen and progesterone receptors and does not over express HER-2.[14, 15, 16] The lack of these receptors limits the use of hormonal therapies. These triple negative cancers are also associated with poorer patient outcomes.[17] Palbociclib does not directly act through the ER or PR but rather as a kinase inhibitor. In combination with Paclitaxel, Palbociclib has shown potential in treating triple negative cell lines.[18] The 4T1 mouse breast cancer cells used in this study are estrogen receptor positive (ER+) cells. Xanthopoulos et al. and Goel & Majumdar reported that Tamoxifen, an estrogen antagonist, inhibited tumor growth and extended the life span of 4T1 ER+ inoculated tumor-bearing mice when compared to the control groups.[19,20] In our literature search, no published studies assessing the 4T1 mouse breast cancer cells' sensitivity to Palbociclib were found. 4T1 cancer cells are commonly used as a mouse model for aggressive stage IV breast cancer. The aim of our study to analyze the cell proliferation characteristics of two human and one mouse breast cancer cell types exposed to various concentrations of Palbociclib for different treatment durations in order to develop base line information for the potential use of this promising anticancer agent alone or in association with other chemotherapeutic drugs in treating breast and other cancer types in humans.

MATERIALS AND METHODS

Cells Lines and Materials

Human breast cancer cell lines (MCF-7 and MDA-MB-231) and mouse breast cancer cell line (4T1) cell lines were provided by Dr. Robert Kurt, Department of Biology, Lafayette College (Easton, PA, USA). MCF-7 is estrogen receptor positive (ER+), progesterone receptor positive (PR+) and does not over express HER-2. MDA-MB-231 is characterized as a triple negative cell line because it lacks estrogen and progesterone receptors and does not over express HER-2. 4T1 is an estrogen sensitive (ER+) mouse breast cancer cell line. The cells were cultured in 25 cm^2 tissue culture flasks (MedSupply Partners, Atlanta, GA) in 4 mL Dulbecco's Modified Eagle's (DME) (Life Technologies, Grand Island, NY) medium supplemented with 0.8% penicillin and streptomycin (Gibco, Grand Island, NY) and 10% Fetal Bovine Serum (DME-10). Cells were incubated in a 37°C humidified incubator with 7.5% CO_2 in air. Cells were lifted from the surface of 6-well plates using 0.25% buffered Trypsin (Life Technologies, Grand Island, NY). Trypan blue was used to assess viability (Gibco, Grand Island, NY). Palbociclib was purchased from Selleck Chemicals (Houston, TX. USA). The stock solution of Palbociclib was prepared by diluting 1mM Palbociclib in DME-10 for a final concentration of 10 μM and 1 μM. A control stock was prepared by diluting equivalent amounts of DMSO in DME-10. Both stocks (Palbociclib

and control) were stored at -20 C° (Selleck Chemicals, Houston, TX. USA).

Experimental Method

Experiments were set up in duplicate with 2 wells for each condition. Cells were dispensed in 6 well plates with each containing 2ml of DME-10 and 50,000 cells per ml. and incubated at 37°C in a humidified incubator with 7.5% CO_2 in air. Cells were treated with 1 µM, 500nM, 250 nM, 125 nM and 62.5 nM of Palbociclib or the control for duration of 96 hours and were studied at 24-hour time intervals. Samples were centrifuged at 73 x g for 10 minutes and counted using a hemocytometer. The viability was determined using the Trypan blue exclusion assay. Each condition was subjected to 4 trials and analyzed using a Student's t-test as well as an ANOVA with Bonferroni post-hoc tests.

Data Analysis

A Student's t-test was utilized to make pairwise comparisons between concentrations in each individual cell line and between cell lines. Additionally, a one-way ANOVA with Bonferroni post-hoc tests was also utilized. All statistics were calculated using SPSS version 22 and considered significant at p≤ .05). The charts were prepared by using Microsoft Excel 2011.

RESULTS

Palbociclib, compared to the untreated control groups, inhibited MCF-7 (ER+) breast cancer cell multiplication. When the Student's t-test was utilized, the Palbociclib treated MCF-7 breast cancer cells were statistically different from the untreated control groups at all concentrations after 24 hours (Table 1). At the 24 hours duration, statistical significance was observed only at two of the five possible concentrations (62.5 nM and 125 nM) (p≤.05). Similar inhibition of cell multiplication was also detected in the ANOVA and Bonferroni post-hoc tests (ANOVA and post-hoc test available upon request) where all concentrations of Palbociclib after the 24 hour treatment duration exhibited significantly reduced cell proliferation in MCF-7 compared to the control. Concentration dependence to Palbociclib was also observed; raising concentration levels of the agent led to lessened cell proliferation rates (Table 1). Similar to the MCF-7 cell line, Palbociclib significantly reduced cell proliferation in the MDA-MB-231 (ER-) human breast cancer cells (p≤.05). This was found to be significant at all concentrations after 24 hours and in four of the five concentrations at 24 hours (p≤.05) (no statistical significance at 250 nM) when compared to the control as shown in (Table 1). When utilizing the ANOVA with Bonferroni post-hoc tests no significance was observed in the 62.5 nM concentration until the 96-hour duration at which it was (p≤.05). In all other cases, proliferation was still significantly inhibited compared to the control at all durations above 24 hours. Like the MCF-7 ER+ cells, MDA-MB-231 ER- breast cancer cells also exhibited concentration dependency. The 4T1 mouse breast cancer cells (ER+), also had cell proliferation reduced in the presence of Palbociclib. In Student's t-test, the sensitivity of 4T1 cells to Palbociclib, at all concentrations and durations was statistically different from the control (p≤.05) (Table 1). However, when the data were analyzed using the ANOVA with Bonferroni post-hoc tests, inhibition in cell proliferation was observed only at the 72 hour duration in the 250 nM, 500 nM and 1 µM concentrations of Palbociclib (p≤.05). Like the other two human breast cancer cell lines, concentration dependency was also observed. Both human breast cancer cell lines (MCF-7 and MDA-MB-231) displayed time dependency in the level of cell multiplication in relation to the concentration of Palbociclib. As the concentration of Palbociclib increased, in general, there tended to be more differences between 24-hour time points in the number of cells present. This trend was not observed in the 4T1 mouse breast cancer cell line. Among the three breast cancer cell lines, when proliferation counts following Palbociclib exposure were examined as a percent of the control counts, MCF-7 cancer cells tended to multiplied the least compared to the control condition, followed by MDA-MB-231cells, while 4T1 cells exhibited the least reduction in cell multiplication (Fig 1). When percent of control counts were compared between cell lines via the Student's t-test, both human breast cancer cell lines had many pair-wise comparisons that were statistically different from the 4T1 mouse breast cancer cells (p≤.05) (Table 2). In all instances the 4T1 cells were less sensitive. When comparing human breast cancer cells to each other, there was only one instance at which MCF-7 cells were found to be significantly more sensitive than MDA-MB-231 cells (125 nM at 96 hours) (p≤.05) .The three breast cancer cell lines displayed a high level of cell viability when treated with Palbociclib. The viability percentages in treated cells were comparable to the untreated control groups (Fig 2). The figure displays the results of 500nM concentration at 72-hour treatment duration, a representative data point at which significant inhibition (p≤.05) of cell proliferation was observed in all the three cell lines. This high level of viability, points to the cytostatic nature of the agent leading to the halting of the cell proliferation, possibly stemming from the inhibition of CDK4 and CDK kinases.

Table 1
Cells per Well at 24-hour Time Points

MCF-7 Human Breast Cancer Cells						
Time (hours)	**Control**	**62.5 nM**	**125 nM**	**250 nM**	**500 nM**	**1 µM**
24	126.2±26.5	99.5±1.3*	103.5±3.9*	128.5±29.5+^	118.0±14.9+^	120.5±15.4+^
48	264.7±69.9	140.5±11.2*	122.5±1.5*+	118.0±17.0*+	113.5±15.7*+	108.0±8.5*+^
72	364.0±102.5	188.0±17.4*	165.5±12.2*+	162.0±26.0*	163.5±20.4*	122.3±8.7*+^=#
96	559.2±180.9	273.8±26.1*	217.3±24.8*+	166.5±49.6*+	135.5±18.1*+=	120.0±14.4*+^=
MDA-MB-231 Human Breast Cells						
Time (hours)	**Control**	**62.5 nM**	**125 nM**	**250 nM**	**500 nM**	**1 µM**
24	117.8±9.7	103±6.0*	104.5±5.0*	107±3.7	103.5±2.21*	102.5±4.4*
48	266.2±28.7	180.5±35.4*	157.5±32.6*	140.5±8.5*+	126.5±14.5*+^	121±25.7*+^
72	550.7±83.5	341±79.9*	281±51.6*+	220.5±33.4*+^	167.5±30.2*+^=	142.5±29.5*+^=
96	931.3±89.8	573±151.0*	443.5±135.1*+	290.8±71.2*+^	209.5±39.6*+^=	170±45.1*+^=#
4T1 Mouse Breast Cancer Cells						
Time (hours)	**Control**	**62.5 nM**	**125 nM**	**250 nM**	**500 nM**	**1 µM**
24	111.5±8.8	100±2.3*	99.5±2.2*	97±1*	96.5±2.6*	96.5±1.9*
48	313.5±122	246±68*	170.5±32.3*+	160.5±34.4*+	146±28.6*+	141.5±25.2*+^
72	601.5±103.9	470±83.8*	351±32.3*+	275±39.9*+^	261±12.2*+^	253±34.3*+^
96	791.8±171.8	638.5±108.1*	495±107.4*+	392±89.2*+^	379.5±88.8*+^	331.8±79.2*+^=#

Effects of Palbociclib on cell proliferation in MDA-MB-231 and MCF-7 human breast cancer cells and 4T1 mouse breast cancer cells treated with different concentrations of the agent for different durations. Analysis was completed with pairwise comparisons between concentrations with a students t-test. Cell counts are given in the thousands ± the standard error. Concentration values are the concentration of Palbociclib. Significance represents that p≤.05. A * indicates that a given concentration is statistically different from the control. A + indicates that the concentration is statistically different from the 62.5 nM concentration. A ^ signifies that the concentration is statistically different from the 125 nM concentration. A = signifies that the concentration is statistically different from the 250 nM concentration. A # signifies that the concentration is statistically different from the 500 nM concentration.

Cell Proliferation Rates of Cells Treated with Palbociclib as a Percent of Control Cell Proliferation

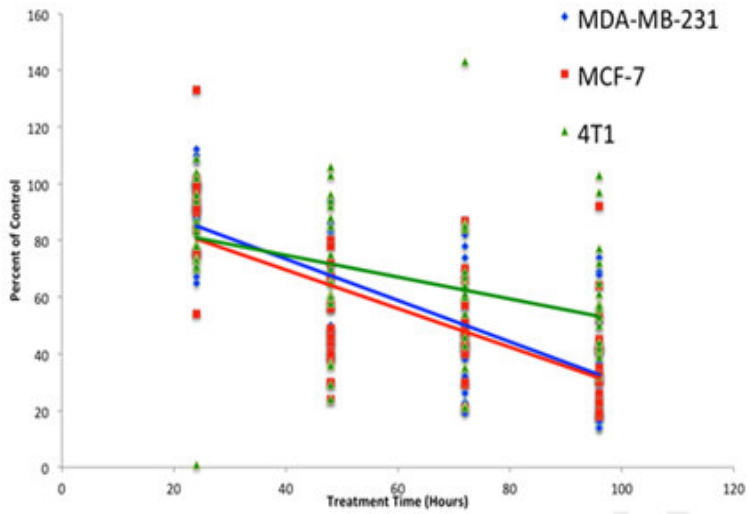

Figure 1

Percent Control cell proliferation rates in three breast cancer cell lines. Cell counts are represented as scatter plot points. Points were calculated as the percentage of the control cell counts. The number of cells in a well in a given duration/condition was divided by the number in the control condition. Trend lines are added to represent the percentage of the control over time. The MCF-7 human breast cancer cells experienced the greatest inhibition followed by MDA-MB-231 and the mouse 4T1 breast cancer cells. While the difference is small, the data trends in the direction of slightly increased sensitivity of MCF-7 compared to MDA-MB-231 human breast cancer cells to Palbociclib.

Table 2

MCF-7 Human Breast					
Time (hours)	**62.5 nM**	**125 nM**	**250 nM**	**500 nM**	**1 µM**
24	85.5±10.63	95.5±7.72	97.5±24.08	85.5±12.82	87.25±13.50*
48	63.25±6.69*	55.5±8.11	40.25±4.09*	46.5±9.84	44.25±8.23
72	63.5±9.95*	50.5±5.74	44±5.05	44.5±2.53	35±11.75
96	62.75±10.73*	37±3.81#^*	31.75±5.69*	26.75±3.84*	24±3.49*
MDA-MB-231 Human Breast Cells					
Time (hours)	**62.5 nM**	**125 nM**	**250 nM**	**500 nM**	**1 µM**
24	96±4.14	97.75±5.39	89.25±8.24	86.75±7.00	86±7.29^
48	75.5±8.77	66.5±7.44	50±3.89^	61±10.32	49±6.04
72	71.75±8.53^	60±8.60	36±5.02^	34.75±7.12^	30.25±5.29^
96	63.5±7.00^	48.7±5.00#^	28.25±5.12^	25.5±5.61^	21.25±4.50^
4T1 Mouse Breast Cancer C					
Time (hours)	**62.5 nM**	**125 nM**	**250 nM**	**500 nM**	**1 µM**
24	91±5.05	91±7.29	88.5±6.51	88.5±8.54	63.25±21.37^*
48	88.75±10.34*	68.5±12.24	63.25±11.48^*	62±14.54	58.25±12.04
72	87±2.08^*	63±10.36	50.25±10.63^	49±9.46^	46.25±9.38^
96	84.5±9.60^*	62.75±3.64^*	49.5±3.28^*	44.5±3.01^*	42±4.42^*

Data percentage of the control cell counts were utilized to determine the comparative sensitivity of the three cell lines. The number of cells in a well in a given duration/concentration was divided by the number in the control condition. Analysis was completed with pairwise comparisons between concentrations with a students t-test. Concentration values are the concentration of Palbociclib. Significance represents that p≤.05. A * indicates that the MCF-7 cancer cell value and 4T1 cancer cell value are statistically different from one another. A ^ indicates that the MDA-MB-231 cancer cell value and 4T1 cancer cell value are statistically different from one another. A # indicates that the MDA-MB-231 cancer cell value and MCF-7 cancer cell value are statistically different from one another. Both human cancer cell lines were statistically different from 4T1 cells in many instances. The two human breast cancer cell lines were only statistically different at 125 nM at 96 hours.

Viability of Cells Treated with Palbociclib

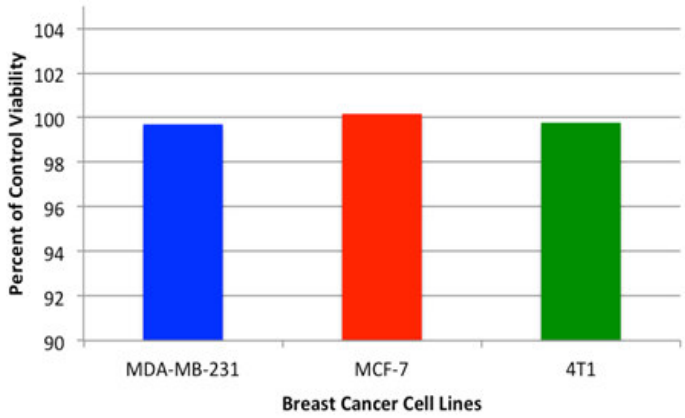

Figure 2

Comparative cell viability (not proliferation) of the three treated cell lines versus untreated controls at 500 nM and 72 hours treatment duration. Viability was determined using a trypan blue exclusion assay and calculated by dividing the percent of viable cells in the experimental condition by the percent of viable cells in the control groups. This concentration as well as treatment time best represents the general inhibitory effects of Palbociclib on cell proliferation in the three breast cancer cell lines. The cell viability in all breast cancer cell lines was comparable to the controls, suggesting that Palbociclib's inhibitory effect on cell proliferation is possibly mediated via the cytostatic pathway.

DISCUSSIONS

Palbociclib is an anticancer drug, that functions by inhibiting the activity of the CDK 4/6 kinases, which suppresses the cell cycle [4]. The hallmark feature of Palbociclib is its cytostatic effects on cancer cells and lessened cytotoxicity [5]. Through inhibition of the CDK4/6 kinases, the chemical agent is able to arrest cell proliferation at the G1 to S phase transition [6]. This study shows that Palbociclib at various concentration levels (62.5 nM to 1000 nM) inhibited cell proliferation at different rates in the three breast cancer cell lines, namely the human MCF-7 (ER+) and MDA-MB-231 (ER-) breast cancer cells and the 4T1 mouse mammary cancer cells in vitro. In general, the chemical extended the lag phase of cell division, and the extension of the lag phase depended on the concentration of the chemical used. All concentrations and durations of Palbociclib treatment inhibited cell proliferation at different rates (after 24 hours) in the three breast cancer cell types when compared to the controls (Table 1). The three cell lines exhibited some levels of concentration dependency and showed a higher level of inhibition in

cell multiplication with increasing concentrations of Palbociclib and treatment durations. To add to the body of knowledge on these cell lines, information was gathered examining how growth evolved over the 96 hour period. Generally, these two human breast cancer cell lines displayed a comparatively lesser time dependency as the concentration of the chemical increased. This is possibly due to the delayed entry of cells into the exponential phase of the growth resulted from higher concentrations of the drug, leading to noticeable changes in cell proliferation over time. Elevated concentrations of the agent possibly contributed to the longer lag phase and a delayed entry of cells into the exponential phase of cell growth. When percent of control counts was compared, the MCF-7 and MDA-MB-231 breast cancer cells, in many instances, showed statistically greater inhibition of proliferation than 4T1 cancer cells according to the Student's t-test. Between the two human breast cancer cell lines, MCF-7 cells exhibited, to some extent, more sensitivity to Palbociclib, although the differences were not significant at most points. In ANOVA with Bonferroni's post-hoc tests, Palbociclib at its lowest concentration (62.5 nM) retarded cell proliferation significantly in MCF-7 ER+ cells at all durations after 24 hours, while MDA-MB-231 ER- cancer cells did not exhibit inhibition until much later at 96 hours. This might suggest a greater sensitivity of MCF-7 ER+ cells to Palbociclib at lower concentrations compared to MDA-MB-231 ER- breast cancer cells. Studies by Finn et al. observed a greater sensitivity of ER+MCF-7 human breast cancer cells compared to ER- MDA-MB-231 cells.[8] A link between hormonal dependence especially the estrogen signaling and the expression of cyclin D1 was reported.[21] The increased sensitivity of ER+ MCF-7 is possibly due to this link. Past studies have observed a synergism between hormone receptor targeting drugs and CDK 4/6 inhibitors such as Palbociclib.[10] Although Palbociclib exerted differential inhibitory effects on cell proliferation as reported in previous studies, our study detected only a minor sensitivity difference between the two ER+ and ER- human breast cancer cell lines. Similar reductions in sensitivity difference was also observed between certain ER+ and ER- human breast cancer cell lines by Finn et al.[8] While Finn et al. noted ER+ cells to have much greater sensitivity to Palbociclib, the ER- MDA-MB-231 cells still exhibited cell growth inhibition in the presence of the agent.[8] Although hormone receptors may have a link to Cyclin D, this link may not be integral for cell proliferation in all cancer cells. Even though MDA-MB-231 cells lack the estrogen receptor, a CDK 4/6 inhibitor might still suppress the cell proliferation. In previous studies the ER+ 4T1 mouse breast cancer cells responded positively to estrogen antagonists such as Tamoxifen and other SERMs (selective estrogen receptor modulator).[19, 20] To our knowledge, no studies involving the effects of Palbociclib on 4T1 cell proliferation were reported. The present study revealed sensitivity of ER+ 4T1 cells to Palbociclib at similar but somewhat lessened rates than the human breast cancer cell lines. This suggests the potential of 4T1 breast cancer cells for use as a mouse model for human breast cancer research. Viability was high for all three treated cell lines. When the cell viability in the experimental groups was compared to the controls only a negligible difference was detected in all treatments and durations. This implies that Palbociclib exerts its inhibitory effects via a cytostatic pathway. This finding is in conformity with the report published by Baughn et al., who found Palbociclib to be relatively non-toxic in myeloma cells.[5] Studies by Finn et al. detected no evidence of apoptosis in Palbociclib treated breast cancer cells.[8] However, further studies should be initiated involving different apoptotic assays to detect if Palbociclib induces cell death via programmed cell death pathways. [22] Our investigation revealed the effectiveness of Palbociclib in reducing cell multiplication of both human and mouse breast cancer cells. The high level of viability observed in the treated breast cancer cells demonstrates the possible cytostatic nature of Palbociclib, implying the reduction of damaging cytotoxic side effects of the agent. The non-toxic nature of Palbociclib on human and mouse breast cancer cell lines suggests its use as an effective anti-cancer drug in treating human breast cancer with possibly less harmful side effects.

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CONCLUSIONS

The three breast cancer cell lines, MDA-MB-231, MCF-7, and 4T1, all exhibited sensitivity towards Palbociclib *in vitro.* In general, human cancer cell lines compared to mouse 4T1 cells were observed to be more affected by the drug with only a slight difference in sensitivity between the two human cell lines. Additionally, no cell lines displayed a decrease in viability in the presence of Palbociclib suggesting its cytostatic as opposed to cytotoxic nature. Research could be furthered by examining the exact mechanism of action in these cell lines to explain differences in sensitivity.

CONFLICT OF INTEREST

Conflict of interest declared none.

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