UNIVERSITY OF NAPLES “FEDERICO II”

DOCTORATE

MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXIX CYCLE

TARGETING SUBTYPES OF BREAST CANCER CELLS WITH PALBOCICLIB, A CYCLIN-DEPENDENT KINASE INHIBITOR

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ABSTRACT

Background: Breast cancer is the leading cause of cancer death in women. Despite major advances in breast cancer research, the recurrence of cancer represents a serious obstacle to successful treatment; deregulation in cell cycle proteins have been implicated in different cancers including breast. A pharmacological approach to inhibit cyclin - dependent kinases 4 and 6 (CDK 4/6) using Palbociclib, a highly selective small molecule inhibitor, was used to evaluate the effects of this inhibitor on cell proliferation of 4 different breast cancer cells subtypes (MCF-7, MDA-MB-231,BT474Z, MCF-7/her2 transfected).

Methods: ERα and Cyclin D1 expression has been evaluated on 34 breast cancer specimens; 4 human immortalized breast cancer cell lines (BT474Z, MCF-7, MCF7/her2 transfected, MDA-MB-231) and one cell line derived from breast cancer tissue patient (K90) have been treated with increasing concentrations of Palbociclib (cyclin D1-CDK4/6 inhibitor) alone and in combination with AI Formestane; cell proliferation was evaluated by MTT assay and cell count. Cell cycle analysis and protein expression have been performed by Facs and WB.

Results: ERα-positive MCF-7 cells are more sensitive to Palbociclib compared to the other breast cancer cell subtypes; Palbociclib displays a cytostatic effect by inhibiting cell viability and proliferation; the inhibitory effect of Palbociclib is based on breast cancer cells subtypes through modulation of expression of G0/G1 regulators (cyclin D1, p27, Rb and phospo-Rb). The combination of Palbociclib with Formestane showed a greater inhibition of cell proliferation compared with Palbociclib alone.

Conclusion: This study suggests a potential role of CDK 4/6 inhibitor Palbociclib on different breast cancer subtypes and identifies Luminal A and Luminal B breast cancer as possible target of Palbociclib in clinical setting.

1.BACKGROUND

1.1Epidemiological data of Breast Cancer

Breast cancer is the second most common cancer in the world and, certainly, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 equivalent to 25% of all cancers. This neoplasia is the most common cancer in women both in more and less developed regions with slightly more cases in less developed (883.000 cases) than in more developed (794.000 cases) regions.
Incidence rates vary nearly four-fold across the world regions, with lower rates (27 per 100.000 in Middle Africa) compared to higher rates (92 per 100.000 in Northern America) depending on the specific geographical regions.

Breast cancer represents the fifth cause of death from cancer overall, it is the second cause of cancer death in more developed regions after lung cancer; however, it is the most frequent cause of cancer death in women in less developed regions. The range in mortality rates between world regions is less than that for incidence because of a better survival of breast cancer in developed regions, with rates ranging from 6 per 100.000 in Eastern Asia to 20 per 100.000 in Western Africa.

Figure 1 shows incidence and mortality of breast cancer according to the related countries [1].

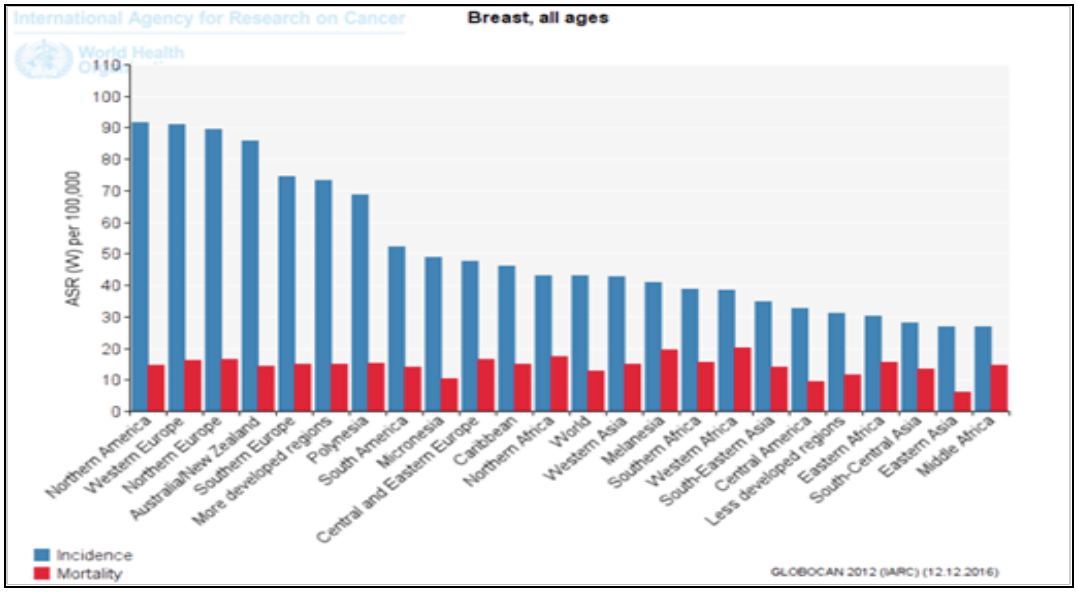

Figure 1.
Incidence and mortality of breast cancer in the world, the incidence is higher in more developed countries as Northern America and West Europe compared to less developed countries, mortality rate compared to incidence is higher in countries less developed such as West Africa and Melanesia [1].

1.2 Risk factors

The risk of developing breast cancer is due to genetic, endocrine, dietary, environmental factors, and to lifestyle habits. They can be divided into two groups: intrinsic (related to individual biological features) and extrinsic (closely related to lifestyle or medical intervention) factors. Sex, age, race, natural hormonal changes during life, and specific genetic alterations represent the most important intrinsic risk factors. Sex is crucial because the neoplasm is diagnosed predominantly in women and sporadically (less than 1 %) in men [2].

In addition, race also represents a very important intrinsic factor since differences in breast cancer incidence and aggressiveness have been reported. In fact, caucasian women are more susceptible to develop breast cancer compared to african-american and hispanic women, which, in turns, although having a lower incidence of breast cancer, are diagnosed at a younger age with an aggressive, often triple negative, breast cancer [3].

A direct correlation exists between the levels and duration of mammary gland exposure to estrogens and the onset of the disease. Estrogens modulate the various stages of female reproductive system development, the secondary sexual characteristics and stimulate the mammary gland during their entire fertile period. The risk of cancer is related to early menarche, age at first pregnancy, number of pregnancies to term, the length of breastfeeding and late menopause since in such physiological conditions changes in estrogen levels can cause a greater or lesser susceptibility to the development of breast cancer [4].

Although the majority of breast cancers are sporadic, 5% -7% are correlated to hereditary factors, a quarter of them determined by mutations of *BRCA-1 and BRCA-2 genes.* In women with mutations in the BRCA-1 gene the risk of developing breast cancer is equal to 65%; in women with mutations in the BRCA-2 gene it is of 40% [5] .

Other hereditary factors are:

- *Mutations in ATM (Ataxia Telangiectasia Mutated) or CHEK2 genes [6]*

- *Mutations in PALB2 gene [7]*

- Li-Fraumeni Syndrome (p53 mutation)

- Cowden syndrome (mutation of the PTEN gene)

- Ataxia-telangiectasia,Peutz-Jeghers syndrome

The most important extrinsic risk factors are related to lifestyle, such as dietary habits, and the use of oral contraception. Indeed, eating products that are rich in fat together with a low consumption of fruit and vegetables may be a factor promoting neoplastic trasformation in mammary gland [8]; on the contrary, a low fat diet contributes to decrease the possibility to develop cancer and reduces the risk of recurrence after the primary surgical

procedure [9]. Also, bad habits, such as excessive alcohol consumption, may increase the chance of developing breast cancer [10]. Finally, the use of oral contraceptives or hormone replacement therapy can alter the normal hormonal balance leading to breast tumor progression [11].

1.3 Breast cancer molecular subtypes

Breast cancer is a highly heterogeneous disease characterized by multiple tumoral subtypes with several biological features. The increased understanding of the process of tumorigenesis and the introduction of new technologies have allowed the identification of different subtypes of breast cancer by enabling more precise management of the neoplasm, but also increasing the level of complexity in the total understanding of the pathology. Biomarkers inclusive of estrogen receptor (ER)**,** progesterone receptor (PR),androgen receptor (AR) and human epidermal growth factor receptor 2 (HER2) are used for basic breast cancer subtyping.ER plays crucial role in breast carcinogenesis and its inhibition was the first step of endocrine therapy; ER-positive tumors are differentiated and less aggressive than ER-negative tumors.

ER-negative tumors do not respond to endocrine therapy whereas about 50% of ER-positive patients are responsive to inhibitor of estrogens [12] [13].

PR-positive tumors, in few cases (0.1% to 10%) are ER–negative [14] but most of PR-positive breast tumors (55% to 65%) are also positive for ER expression [15] [16].

HER-2 amplification or overexpression occurs in about 20% - 30 %of invasive ductal breast cancer; the definition of HER 2 state represents a routine investigation in clinical practice for the use of targeted agents[17] .

In addition, the definition of specific biomarkers of proliferation (Ki67,TOP2A) allowed a more accurate evaluation of the clinical implication.

Triple negative (ER-negative, PR–negative, HER2–negative) breast tumors represent the most aggressive and problematic tumors to cure, due to the absence of target receptors. Breast cancer patients with these characteristics are divided into basal and non-basal depending on the

presence of specific markers such as cytokeratins 5,14,17,18, EGFR, vimentin, P-Cadherin. Furthermore, the definition of EMT biomarkers (Zeb, Twist, Snail, Claudins) and biomarkers of stemness (CD44, CD24, EpCam, Aldh1, Muc1, Thy1) has increased the level of complexity in understanding breast cancer tumorigenesis [18].

In table 1 are summarized the main breast cancer subtypes.

SUBTYPE	ALIAS	BIOMARKER STATUS	GRADE	OUTCOME	ADDITIONAL FEATURES	PREVALENCE
Luminal	Luminal A	ER+;PR+;HER2; KI67-	1/2	Good	Luminal cytokeratin+ ;cell-cell adhesion gene high	23,7 %
	Luminal B	ER+;PR+;HER2; KI67-	2/3	Intermediate	Luminal cytokeratin+;cell-cell adision gene high	31-38%
HER 2 positive	Her2 overexpression	ER-;PR-;HER2+	2/3	Poor	Cell-cell adhesion high	11.2 %
Triple Negative	Basal	ER-;PR-;HER2-; basal marker +	3	Poor	Brca1-; CDK2Ahigh; RB low;cell-cell adhesion genes hight	12.3%
	Claudin-low	ER-;PR-;HER2-, EMT marker+; stem cell marker+; claudin –	3	Poor	Cell-cell adhesion genes low;claudins low	7-14%
	Metaplastic Breast Cancer	ER-;PR-;HER2-; EMT marker +; stem cell marker+	3	Poor	Cell-cell adhesion genes low;PIK3CA- ;AKT-;KRAS-	1 %
Molecular apocrine cancer (MAC)	Molecular apocrine cancer (MAC)	ER-; PR-; AR+	2/3	Poor	KI67+	13.2%

Table 1.

Summary of molecular breast cancer subtypes with detailed expression patterns and clinical implications. [18].

1.4 Current breast cancer therapy

1.4.1 Endocrine Therapy

Endocrine therapy represents the first treatment option for estrogens receptor positive and HER2 negative breast cancer patients. Endocrine therapy, which applies to menopausal women, gives fewer side effects and better quality of life compared to chemotherapy.

In patients with breast cancer and premenopausal status the combination of LhRh analogue, which are able to block the production of ovarian estrogens by inhibiting hypothalamic pituitary, and tamoxifen represents the first line of treatment.

Tamoxifen, classified as a selective estrogens receptor modulator (SERM), contrasts proliferative stimulus and acts as an antagonist of ERα. Tamoxifen mainly inhibits the proliferation of ER-positive breast cancer cells by competing with estrogens binding to ERα [19]. In menopausal and post-menopausal women, aromatase inhibitors (AIs), which interrupt the production of estrogens in these women, are used as therapeutic agents for metastatic breast cancer. Moreover, these inhibitors block the enzyme aromatase, which turns the hormone androgen into estrogens in the body. The decrease of this synthesis determines a minor contribution of estrogens available to stimulate the growth of ER-positive breast cancer cells.

AIs are classified according to the date of discovery:

- AIs of first generation or inactivating steroidal enzyme are analogues of androstenedione that irreversibly bind to the same site of the aromatase molecule; Aminoglutemide was the first drug used in clinical practice.

- AIs of second generation (non-steroidal structure) reversibly bind to the heme group of the aromatase enzyme; formestane is the main drug in this group.

- AIs of third generation are anastrozole, letrozole, examestane; unlike others AIs, these compound are orally administered. Anastrozole and letrozole are non-steroidal inhibitors whereas

exemestane is a steroidal inactivator. These third-generation AIs have demonstrated greater efficacy than previous inhibitors, and fewer side effects.

Fulvestrant is a recent drug used for endocrine breast cancer treatment. It is an ER antagonist that disrupts ER dimerization and nuclear localization, by blocking ER-mediated transcriptional activity and accelerating receptor degradation. It has been proven to be effective on breast cancer progression disease after treatment with tamoxifen and aromatase inhibitors [20] [21].

1.4.2 Breast cancer therapy against HER2

Breast cancer therapy against HER2 are based on specific targeted agent:

-*Trastuzumab* is a human monoclonal antibody that binds tothe surface of HER2-positive cancer cells.Breast cancer *in vivo* models and clinical trials have demonstrated that trastuzumab has not only citostatic but also cytotoxic properties, due to the activation of immune system through the activation of natural killer cells, which leads to the lysis of cancer cells bound to trastuzumab [22].

-*Pertuzumab* is a humanized recombinant monoclonal antibody; it blocks the growth factor HER2 in combination with trastuzumab. This drug was developed to prevent the HER2 receptor binding to other HER receptors (EGFR / HER1, HER 3 and HER 4) [23].

-*Lapatinib* is a kinase inhibitor of the intracellular tyrosine kinase domains of both EGFR and HER2 receptors. In particular, lapatinib targets the intracellular tyrosine kinase domains of these two receptors and induces apoptosis and inhibition of tumor cell growth. Compared to Herceptin, which targets the HER2 protein on the outside of the cell membrane, lapatinib is a small molecule that enters the cell and blocks the transduction signaling, inhibiting cell growth and causing cell death [24].

1.4.3 Chemotherapy

Chemotherapy is administered in triple negative breast cancer patients; in ER-negative, PR-negative tumors overexpressing HER2, chemotherapy may be combined with anti-HER2 agents. Chemotherapy destroys breast cancer cells at the original tumor site and throughout the body. It represents a non-specific treatment that gives side effects. In fact, it can unintentionally strikes other types of rapidly dividing cells such as hair follicles, digestive tract, and bone marrow. Figure 2 shows a summary of therapeutic approach in breast cancer.

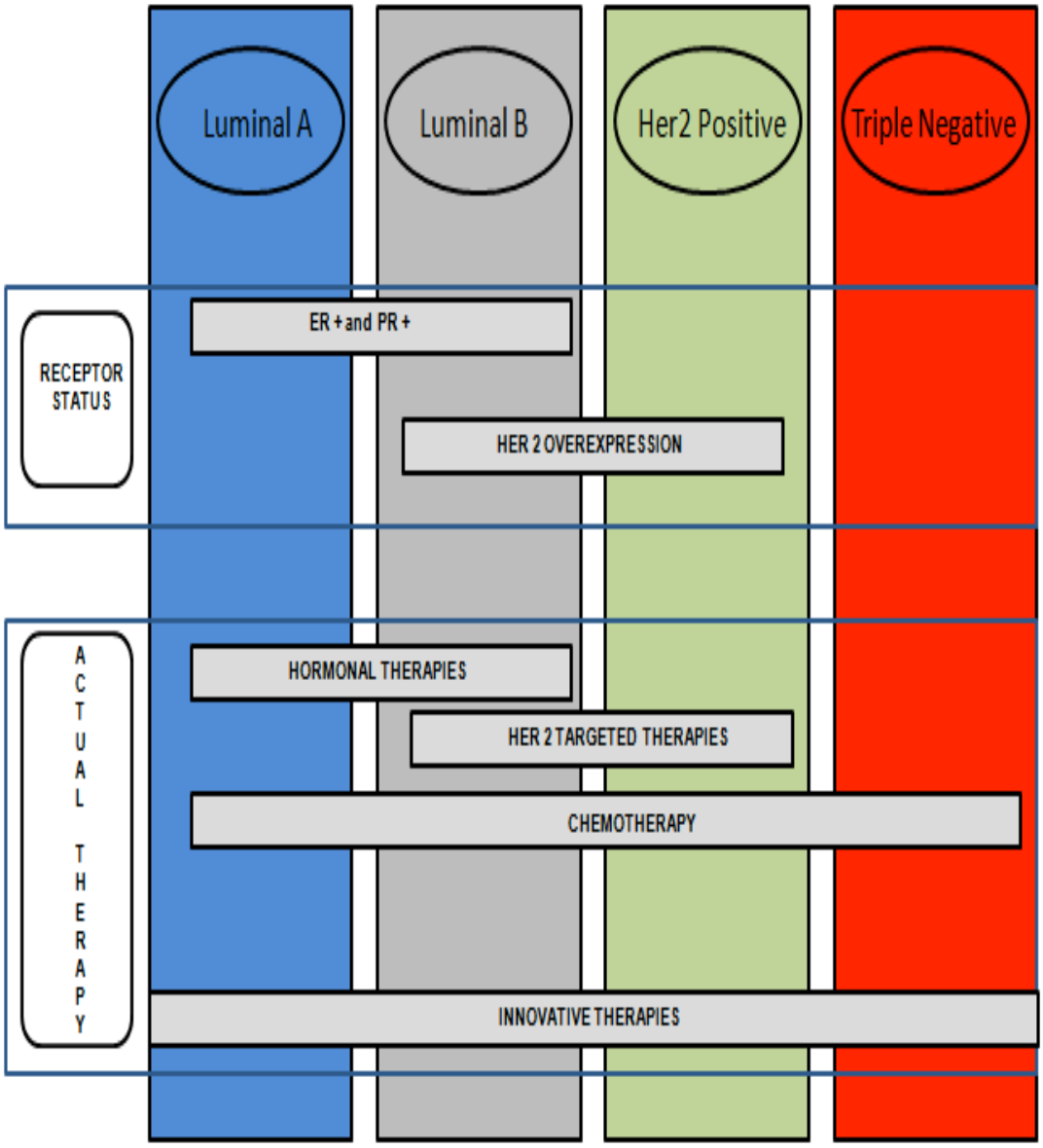

Figure 2.

Schematic representation of the therapeutic approach in the various subtypes of breast cancer.

1.5 Cell cycle control in breast cancer

Every cell that is part of a complex organism must decide his fate by proliferating or remaining in a state of quiescence; in an adult organism most of the cells are quiescent; on the contrary, specialized cells, such as hematopoietic system, and epithelium maintain active proliferation. Imbalance from a state of quiescence and active proliferation that occurs in cancer cells shows alteration in specific transduction pathway that leads to an unchecked proliferation.

Cell cycle is divided into four phases named G1, S, G2, M. In the S phase a cell generates a single copy of its genetic material; in M phase or mitosis it splits in two identical daughter cells.

G1 and G2 represent gap periods during which cells prepare themselves for the successful completion of S and M phases. In absence of a mitogenic signal cell can enter into a quiescence state (G0).

The central players of cell cycle are CDKs (cyclin dependent kinases) that form active heterodimeric complexes following the binding to cyclins.

There are four CDKs: CDK1 regulates the transition from G2 to M phase while CDK2, CDK4 and CDK 6 regulate the transition from G1 to S phase. CDKs are finely regulated by activators (cyclins) and inhibitors (CKI).

Different cyclins are required at different phases of the cell cycle. The three D type cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4 and to CDK6. Unlike the other cyclins, cyclin D is not expressed periodically, but is synthesized as long as growth factor stimulation persists. Cyclin E is another G1 phase cyclin; cyclinE – CDK2 complex regulates the progression from late G1 into S phase. In S phase, cyclin A binds to CDK2; instead in G2 and early M, cyclin A complexes with CDK1 promoting the entry into M phase. Mitosis is further regulated by cyclin B in complex with CDK1.

CKI are divided in two groups: the four member of the INK4 family (INK4A or p16, INK4B or p15, INK4C or p18 and INK4D or p19) that bind to the CDK4 and CDK6 kinases by hindering their association with D - type cyclins, and the three member of WAF/KIP1 family (WAF1 or p21, KIP1 or p27, and KIP2 or p57) that inhibit G0/G1 transition. An additional level of control is

conferred by specific kinases that regulate phosphorylation of specific threonine residues of activating (CAK) or inhibiting (WEE1, MYT1) CDK proteins.

Progression from G1 to S phase is controlled by the Retinoblastoma protein (pRb). Retinoblastoma prevents cell division by linking with E2F transcription factors and thereby inhibiting G1 to S transition. During G1 phase growth signals stimulate cyclin D to associate with CDK 4 or CDK 6. The complex cyclinD-CDK4/6 determines the Rb phosphorylation; this process stimulates the formation of other heterodimeric complex consisting of cyclinE-CDK2 by determining a state of complete Rb hyper-phosphorylation. Rb then releases E2F allowing cell cycle progression through the activation of S-phase factors.

In most breast cancer cases Rb does not shown genetic alteration; the minority of breast cancer (20%-30%) lost Rb expression; the incidence of Rb loss is dependent on the breast cancer subtype and is more common in triple negative breast cancer [25].

Also, Rb loss is associated to epithelial-mesenchymal transition and to metastasis formation [26].

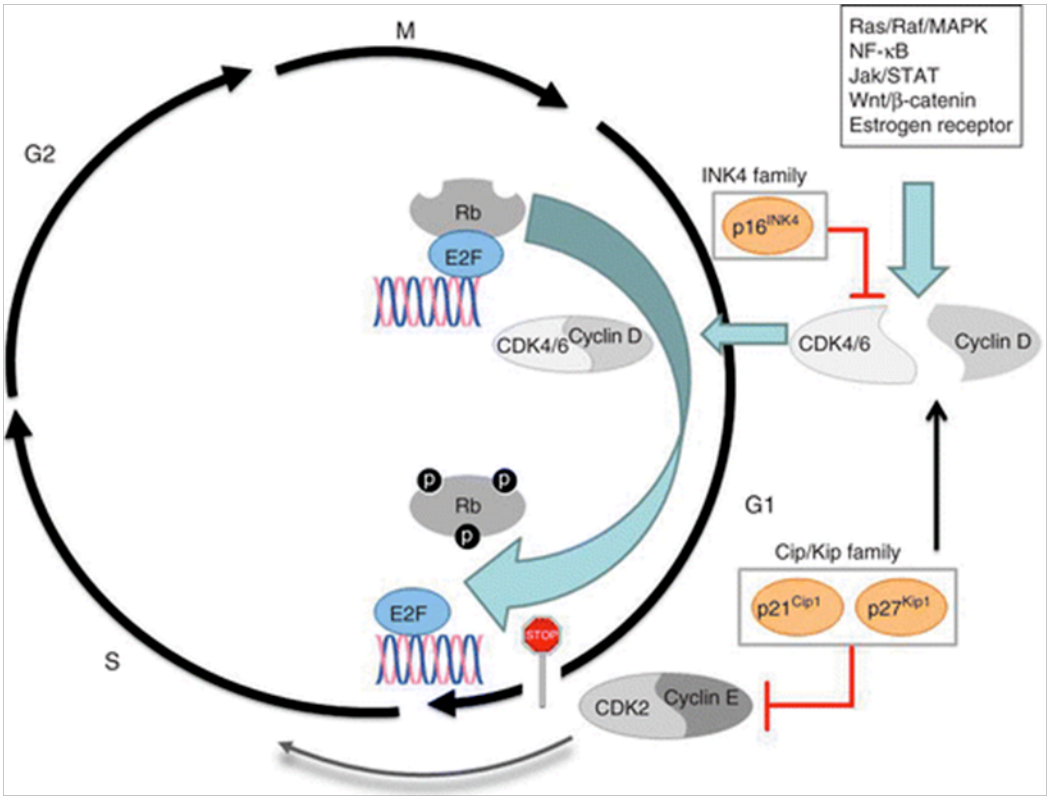

Figure 3.

Cyclin D-CDK4/6-Rb protein pathway regulate cell cycle progression. CDK: Cyclin dependent kinase; Cip/Kip: Kinase inhibitor protein family; E2F: Elongation factor 2; INK4: Inhibitors of CDK4; P: Phosphate; Rb: Retinoblastoma protein [27].

1.6 New therapeutic approach in breast cancer

About 75% of breast cancer is ER-positive. Estrogens can rise cell proliferation increasing the progression from G0 to G1 phase [28] [29].Currently, breast cancer therapy is based on the inhibition of estrogens synthesis (AIs), modulation of estrogen receptor (Tamoxifen) and its antagonists (Fulvestrant); this approach has resulted in a reduction of the proliferative stimulus at the level of breast tumor cells. Nevertheless, the development of resistance to endocrine therapy is a current real problem during endocrine therapy and novel studies on breast cancer drug resistance mechanisms are crucial for developing innovative therapeutic approaches. Cell cycle proteins are involved in the development of resistance to endocrine therapy; breast tumors ER-positive, Rb-negative mouse models are resistant to tamoxifene [30]. In addition, cyclin D1 overexpression has been implicated in tamoxifen resistance, since the formation of heterodimers cyclin D1-CDK4/6 induces the withdrawal of p21 and p27 with consequent activation of cyclin E-CDK2 complexes [31].

The combination of hormonal therapy with CDK4/6 inhibitors appears a promising strategy to overcome endocrine resistance [32].

1.6.1 Therapeutic strategies at the level of CDK

CDK are central players in the control of cell cycle progression.

The complexity of CDK regulation emphasizes possible opportunity to their therapeutic inhibition. The most direct and successful strategies involve inhibitors that can block CDK activity [33].

Other possible strategies include: 1. Prevention of cyclin–CDK interaction with peptide-mimetics, CKI degradation or CKI function restoration by gene therapy; 2. Prevention of CKI degradation: KIP1 could involve either blocking phosphorylation, which triggers its degradation, or its interaction with SKP2, member of the SCF complex; 3. Decreasing of the levels of cyclin D1 by diminishing specific transcription of the gene by antisense techniques; 4. Increasing of protein degradation by their specific proteasomes. 5. Downregulation of positive regulators, such as the CAK or the CDC25 dual specificity phosphatases and/or the activation of negative regulators, such as the dual specificity kinases WEE1 or MYT1 [34] [35].

1.6.2 Palbociclib

The use of cell cycle inhibitors has not been successful in clinical practice since the first-generation compounds, known as pan-CDK inhibitors, exhibited considerable toxicity. The discovery of new compounds with favorable pharmaceutical and physical properties increased the interest in these types of compounds; they are highly selective and this characteristic has allowed a re-evaluation of their possible use in clinical practice.

Palbociclib (also known as PD0332991) is one of the most promising CDK 4/6 inhibitor developed from a group of pyridopyrimidine; it is a non-competitive ATP inhibitor that inhibits CDK 4/6 at low nanomolar concentration in Rb proficient breast cancer cells (Figure 4) [36] .

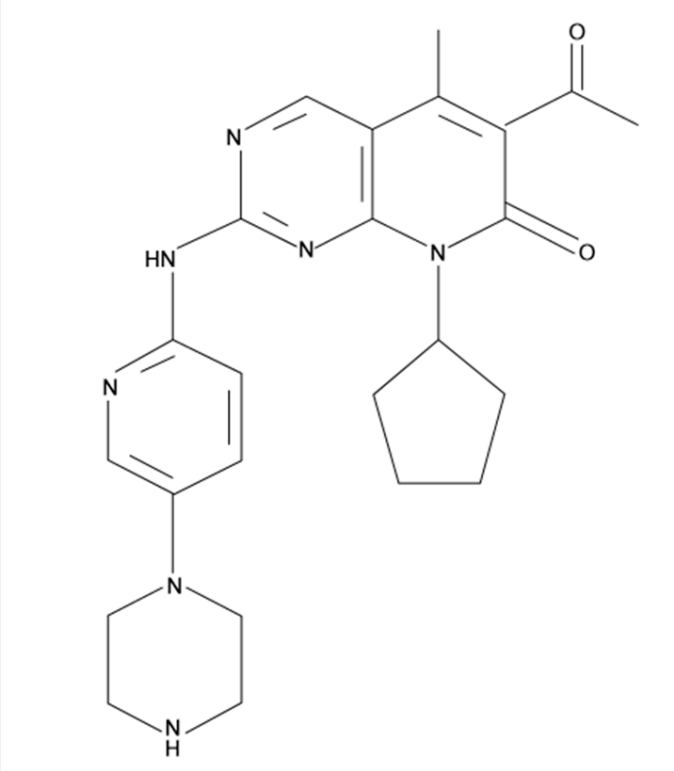

Figure 4.

Representation of the CDK 4/6 inhibitor Palbociclib. This compound consists of a group of piridopirimidine and is highly selective (order of nM) in the inhibition of cell proliferation [36].

Sensitivity to Palbociclib depends on molecular subtypes; Finn et al. have investigated the activity of Palbociclib in breast cancer cell lines representing several molecular subtypes and demonstrated that ER-positive breast cancer cell lines with luminal features are most sensitive to Palbociclib treatment compared to breast cancer cell lines with basal features, which are resistant [37].

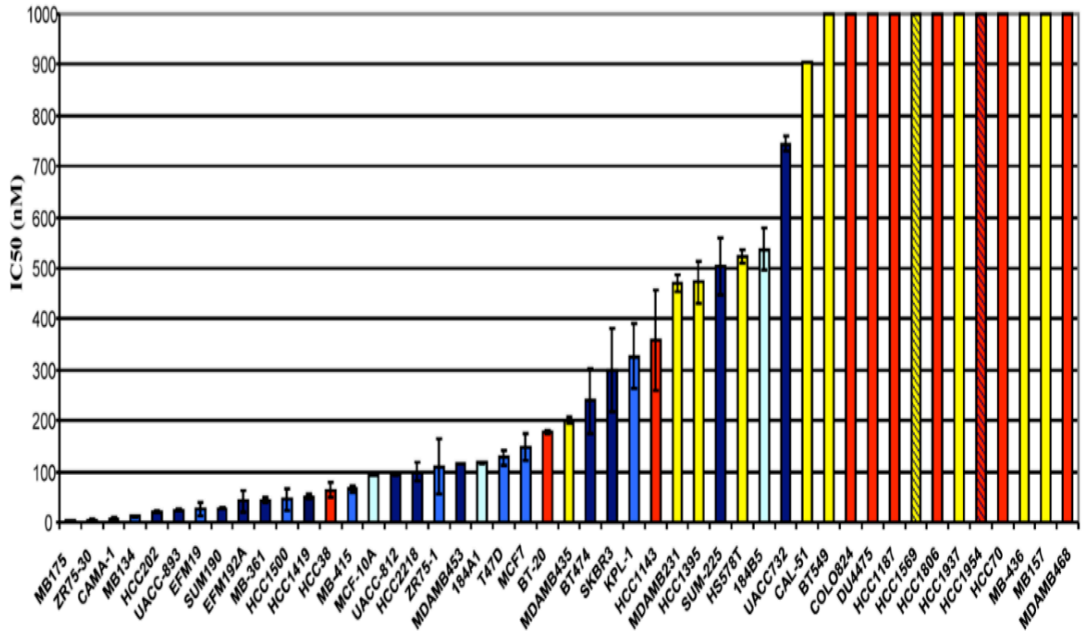

Figure 5.

Inhibitory concentration and breast cancer cell type. Bar graph representing different Cell lines are color coded by subtype: light blue, luminal; dark blue bars or stripes, HER2 amplified; yellow, nonluminal/undergone an epithelial-to-mesenchymal transition; red, nonluminal; turquoise, immortalized [37].

It has been demonstrated that Rb negative cells are resistant to Palbociclib [38]. The sensitivity to the drug is based on elevated level of Rb and cyclin D1 proteins and low levels of p16 protein [39]. Preliminary studies have evaluated the combined effect of Palbociclib with drugs currently used in breast cancer therapy. In ER-positive MCF-7 cells it shows a synergistic effect with tamoxifen and is able to re-sensitize the cells to tamoxifen. In HER2-positive breast cancer cell lines the synergistic effect of Palbociclib with Trastuzumab has been demonstrated [37].

The combined effect of Palbociclib with chemotherapy is associated with the type of drug used and with the presence or absence of Rb. Indeed, when associated with carboplatin, Palbociclib has shown a myeloprotective activity in mice lacking Rb expression, by inducing hematopoietic progenitor cells in a quiescent state without altering the activity of the drug. In mice expressing Rb, the combination of the two agents compromised the drug efficacy compared with carboplatin alone [40].

1.6.3 Clinical development of Palbociclib

Palbociclib was evaluated in a open-label dose finding phase I study in patients with Rb-proficient solid tumor. Thirty-three patients were enrolled and dose limiting toxicities (DLT) were observe at 225 mg/day, 200 mg/day was selected as the maximum tolerated dose (MTD) [41].

PALOMA-1 study (NCT00721409) is a multicenter, randomized phase I/II study, where ER-positive, HER2-negative breast cancer postmenopausal patients have been treated with palbociclib (at a dose of 125 mg orally daily on the 3on/1off) combined with letrozole 2.5 mg daily continuously versus letrozole alone [42].

Eligible patients have received either regimen as first line treatment for metastatic disease. The analysis of 165 patients enrolled has been impressive, and PFS increased over three fold in patients treated in the combination arm (26.1 vs. 7.5 months). Combination therapy was very well tolerated and the most frequent side effects were uncomplicated neutropenia (61.4% in patients treated with both agents compared with 1.3% in patients treated with single-agent letrozole), leukopenia, anemia and fatigue. On the basis of these favorable results, the FDA designed palbociclib as a “breakthrough therapy” for metastatic breast cancer in 2015.

A confirmatory phase III PALOMA-2 trial (NCT01740427) patients with previously untreated, advanced ER-positive, HER2-negative breast cancer are randomized to the combination of palbociclib and letrozole vs. letrozole alone. The analysis in progress revealed that PFS increased in patients treated in the combination arm (24.8 vs. 14.5 months) [43].

The double-blind phase III PALOMA-3 trial (NCT0192135) evaluated the efficacy of palbociclib and fulvestrant in pre- and post-menopausal women with ER-positive, HER2-negative advanced breast cancer who had progressed on prior endocrine therapy. Patients were randomized 2:1 to palbociclib and fulvestrant or to placebo and fulvestrant. Pre and peri-menopausal women were also treated with the LhRh agonist, goserelin. At the first interim analysis, PFS was 9.2 months in the palbociclib and fulvestrant arm and 3.8 months in the placebo and fulvestrant arm.

On the basis of these results, palbociclib and fulvestrant represent a new treatment option for pre- and postmenopausal patients with ER-positive, HER2-negative advanced breast cancer who have progressed on prior endocrine therapy [44].

Currently, several trials evaluating efficacy of palbociclib and endocrine therapy are in progress in the adjuvant (NCT02040857) and neoadjuvant settings (NCT02400567, NCT01723774, NCT01709370) in patients with ER-positive, HER2-negative breast cancer.

2. AIM OF THE STUDY

The aim of the study was to evaluate the effect of the cell cycle inhibitor Palbociclib (PD 0332991) on the proliferation of different subtype of breast cancer cell. To this aim we chose four breast cancer cell lines (MCF-7, MDA-MB-231, BT474Z, and MCF7/her2 transfected, which are MCF7 cells stably expressing HER2) and one primary breast cancer culture (K90) obtained from residual breast carcinoma tumor tissue from patients undergoing surgery at the ”Azienda Ospedaliera Universitaria Federico II”. We identified breast cancer cells, representing different molecular breast cancer subtype, sensitive/resistant to drug treatment and evaluated whether the *in vitro* effect of Palbociclib was permanent or transient. Levels of cyclin D1, p27, and Rb phosphorylation (ser780) status have been investigated in Palbociclib treated compared with untreated breast cancer cells. Variations in cell cycle phases have been observed by Facs analysis, following treatment with the drug. Finally, it has been evaluated *in vitro* the combined effect of Palbociclib and Formestane on breast cancer cell proliferation.
In breast cancer, several specific molecular targeted-agents are used but the resistance to endocrine therapy still remains the main problem in the management of this disease.

3. MATERIALS AND METHODS

3.1 Breast cancer tissue collection

For this study 58 fresh tissue specimens were collected from breast cancer patients who underwent surgery at the "Federico II" University of Naples, General Surgery Unit, whose clinical and pathological features are summarized in the following table:

CLINICAL PARAMETERS	**NUMBER**
TOTAL NUMBER OF SAMPLES	58
AVERAGE AGE (AGES)	57.3
RANGE OF AGE(anni)	36-84
HISTOLOGY	
DUCTAL ORIGIN	48
LOBULAR ORIGIN	6
OTHER	4
GRADING	
I	1
II	19
III	38
NODAL STATE	
POSITIVE	26
NEGATIVE	32
TUMOR STAGE	
T1	28
T2	25
T3	3
T4	2

C-erbB2	
ABSENT	38
+	4
++	6
+++	10

Evaluation of protein expression (cyclinD1 and ERα) was carried out on 34 breast tissue specimens.

This study was authorized by "Federico II" University Institutional Review Board.

3.2 Cell lines

Breast cancer cell lines MCF-7, MDA-MB-231 and BT474Z were obtained from ATCC (American Type Culture Collection). MCF-7 / her2 transfected cells were provided by Dr. R. Schiff's group. MCF-7, MDA-MB-231 cells were maintained in standard medium consisting of DMEM (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma-Aldrich), 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), 15 mM HEPES (Sigma-Aldrich) and 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 ; BT474 cells were cultured in DMEM containing 10% FBS while MCF-7 / her2 transfected in DMEM containing 10% FBS, insulin (5ug/ml) and G418 (gentamicin).

Primary breast cancer cells (K90) were obtained directly from tumoral lesion [45] and maintained in standard medium consisting of DMEM/F12 1:1 (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma-Aldrich), 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), 15 mM Hepes (Sigma-Aldrich) and 5% foetal bovine serum (FBS) and at 37°C in a humidified atmosphere of 5% CO_2.

3.3 Drugs

Palbociclib (PD0332991) (Pfizer, NY, USA) was dissolved in DMSO and used at different concentrations ranging from 31.25 nM to 1 mM.

Formestane (Sigma-Aldrich) was dissolved in DMEM and used at different concentrations ranging from 25 nM to 600 nM.

3.4 MTT assay

Cell viability was determined using the colorimetric 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma Aldrich). Exponentially growing cells (1 x 10^4 cells/well) were seeded in 24-well plates and cultured at 37˚C, under 5% CO_2 for 24 h. Cells were treated with drugs for a maximum of six days.

MTT (0,72 mg/ml) was resuspended in DMEM and 500 µl of this resuspension was added to each well. After incubation at 37˚C for 4 h, 500 µl isopropyl alcohol-HCl was added in each well and were placed in agitation in the dark for 20 minutes. The OD value was measured at a wavelength of 570 nm and 690 nm. Triplicates for each point were counted in 3 different experiments.

3.5 Cell Count

Cells were seeded in triplicate at 5 x 10^3 to 15 x 10^3 cells per well in 24-well plates. The day after, Palbociclib was added at concentration of 31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 1 µM for 6 days. Breast cancer cells were counted after 1,3 and 6 days. After trypsinization, cells were collected in a solution of DMEM / trypan blue 1:1 and counted by haemocytometer chamber.

3.6 Cell cycle analysis

Breast cancer cells BT474, MCF-7, MCF7 /her2 trasfected and MDA-MB-231 were treated with Palbociclib at a concentration of 1 uM for 12 hours. Cells were trypsinized, collected in ice-coldPBS-10% FBS and centrifuged at 1500 rpm for 5 min; then, cells were washed twice with ice-cold PBS. For cell cycle analysis, cells were fixed in ice-cold ethanol (70%) and stained with 0.5 ml PI/RNase staining buffer (BD Pharmingen, USA) for 20

minutes at room temperature in the dark and analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

3.7 Western Blot

Western blotting was used to determine protein expression of estrogen receptor alpha and cyclin D1 on tissue specimens and to evaluate cyclin D1, p27, Rb and Rb phosphorylated in serine 780 in breast cancer cell lines analysed (BT474, MCF-7, MCF-7 /her2 transfected and MDA-MB-231).
Thirty-four breast cancer tissue specimens collected were processed as follows:

1. Mechanical shredding with a scalpel
2. Homogenization of the samples after the addition of lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP-40, 1mM EDTA) containing a cocktail of protease and phosphatase inhibitors [Complete-EDTA FREE (Roche)], 20 mM NaF, $Na_4O_7P_2$ 4 mM, β–glycerophosphate 5 mM and $Na_3VO_4$2 mM].
3. Incubation on ice for 30 minutes
4. Centrifugation at 4 ° C for 15 minutes at 13,200 rpm
5. Collection of the supernatant containing the protein fraction of the lysate

Breast cancer cells were seeded (5 x 10^^{5}) in petri dish 100 mm at 37˚C under 5% CO_2. After 24 hours, breast cancer cells were treated with Palbociclib (31,25 nM to 1 uM for 6 days). Subsequently, cells were trypsinized and washed in PBS; after centrifugation (1500 rpm for 5 minutes) cells were incubated in *Ripa lysis buffer* (Tris-HCl PH = 7.4 50 mM,Sodium Deoxycholate 0.25 %, 150 mMNaCl, 1 % NP - 40, 1 mM EDTA, NaF 1 mM, 1 mM Na_3VO_4, sodium pyrophosphate 100 mM, protease inhibitor) for 30 minutes on ice.
Finally, cells and tissues lysates were centrifuged at 12,000 rpm for 20 minat 4˚C. Protein concentrations were determined by Bradford assay. Fifty micrograms of protein from each total lysates were denatured in 4X sample buffer, loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide (SDS) electrophoresis gel. After electrophoresis, separated proteins were transferred onto PVDF membranes (Immobilion-P) followed by blocking with 5% non-fat milk in Tris-buffered saline [10 mMTris-HCl (pH

7.5), 100 mMNaCl, 0.1% Tween-20) for 1 h at room temperature. The membranes were then incubated with specific primary antibodies**:** Rb (Mouse Mab 4H1 # 9309 Cell signalling Technologies, USA) and p-Rb ser.780 (Rabbit Mab C84F6 # 3590 ,Cell signalling Technologies, USA), cyclin D1 (# sc-717 , Santa Cruz Biotechnology), ERα (# sc-8005, Santa Cruz Biotechnology, USA) and p27 (# sc-1641, Santa Cruz Biotechnology, USA) overnight at 4°C. After washing, the membranes were incubated with appropriate secondary HRP-conjugated antibodies and visualized by ECL. Each membrane was reprobed with anti-tubulin (Monoclonal Anti α Tubulin Clone DM 1 A # 9026 Sigma Aldrich, Milan) antibody to ensure equal protein loading.

3.8 RT-PCR analysis

Total RNA was isolated from sample and control cells using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Purity of RNA was checked by measuring the absorbance ratio at 260/280 nm in a Beckman Coulter spectrophotometer (Beckman Coulter, Fullertone, CA, USA) with appropriate purity values between 1.8 and 2.0. RNA was stored at -80°C. The integrity of RNA was assessed on standard 1% agarose/formaldehyde gel. The reverse transcription of 1.5 μg of total RNA was performed with SuperScript III reverse transcriptase Kit (Invitrogen, USA) according to the manufacturer's instructions. Multiplex PCR was performed in 50 μL reactions using the PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, USA) and gene specific sets of primers, including those for the internal standard β-actin. Agarose gel electrophoresis and staining with 0.3 mg/ml of ethidium bromide (Sigma, St. Louis, MO) were carried out to assess templates products. Primer sequences and PCR reactions steps are the following:

Primers **ER α**:

- Forward: 5'-CCCTACTGCATCAGATCC-3'
- Reverse: 5'-CCTGGCGTCGATTATCTG-3'

→ Product of Aplification: ERα (441 bp)

Conditions:

95°C x 5'
95°C x 1'
56°C x 1'
72°C x 1'
} 35 cycles
72°C x 10'

Primers **PR**:

- Forward: 5'-CGCGCTCTACCCTGCACTC-3'
- Reverse: 5'-TGAATCCGGCCTCAGGTAGTT-3'

→ Product of amplification: PgR (121 bp)

Conditions:

95°C x 5'
95°C x 30"
65°C x 30"
72°C x 30"
} 35 cycles
72°C x 10'

Primers HER-2:

- Forward: 5'-TGCGGCTCGTACACAGGGACTT-3'
- Reverse: 5'-TGCGGGAGAATTCAGACACCAACT-3'

→ Product of amplification : Her-2 (420 bp)

Conditions:

95°C x 5'
95°C x 1'
65°C x 1'
72°C x 1'
} 30 cycles
72°C x 10'

Primers β-Actin:

- Forward: 5'-TGACGTGGACATCCGCAAAG-3'
- Reverse:5'-CTGGAAGGTGGACAGCGAGG-3'

→ Product of amplification : β-Actin (205 bp)

3.9 Statistical analysis

Data are expressed as the mean derived from triplicate replications in each experiment. Significance was accepted at the $p < 0.005$.

4.0 RESULTS

4.1 Levels of cyclin D1 correlated with level of expression of estrogen receptor alpha in breast tumor tissues

Cyclin D1 is essential for the regulation of breast cell division. Estrogen binding of ERα drives cyclin D1 transcription, activation of CDK4/6, phosphorylation of Rb and cell cycling [46]. However, cyclin D1 can activate ERα, transcription and cell division independently of estrogen and CDK. This independent activity is not inhibited by anti-estrogens strategies and is associated with endocrine resistance [47].

Given the relationship between the estrogen receptor, which acts as activator of proliferation, and cyclin D1 that promotes cell cycle progression, estrogen receptor and cyclin D1 protein expression have been evaluated in tumor tissues specimens derived from breast cancer patients undergoing surgery at the Surgery Unit of “Azienda Universitaria di Napoli Federico II”.

Thirty-four fresh-frozen tissue samples were analysed by Western blot to evaluate estrogen receptor alpha and cyclin D1 expression (figure 6). MCF-7 was used as breast cancer control cells and α-tubulin was used as loading control.

Brest tumor tissue specimens 4, 6, 7, 8, 15, 16, 17, 18, 22, 23, 24, 25, 27, 28, 33, 34 resulted ER - positive; on the contrary breast tissue specimens 1, 2, 3, 5, 9, 10, 11,12, 13, 14, 19, 20, 21, 26, 29, 30, 31, 32 resulted ER-negative. The evaluation of cyclin D1 expression revealed that breast tumor tissue specimens 1, 2, 4, 5, 6, 7, 8, 9, 11, 15, 16, 17, 18, 20, 22, 23, 24, 25, 27, 28, 33, 34 are Cyclin D1-positive; conversely breast tumor tissue specimens 3, 10, 12, 13, 14, 19, 21, 26, 29, 30, 31, 32 are Cyclin D1-negative. Densitometric analysis revealed that ER–positive tumor tissue specimens analysed express discriminable levels of cyclin D1; on the contrary ER-negative breast tumor tissue specimens do not express cyclin D1, except for the sample 20. Analysis performed revealed a correlation between ER-α and Cyclin D1 expression. This result indicates that activation of the estrogen receptor is closely associated with cyclin D1 expression which represents its main downstream effector.

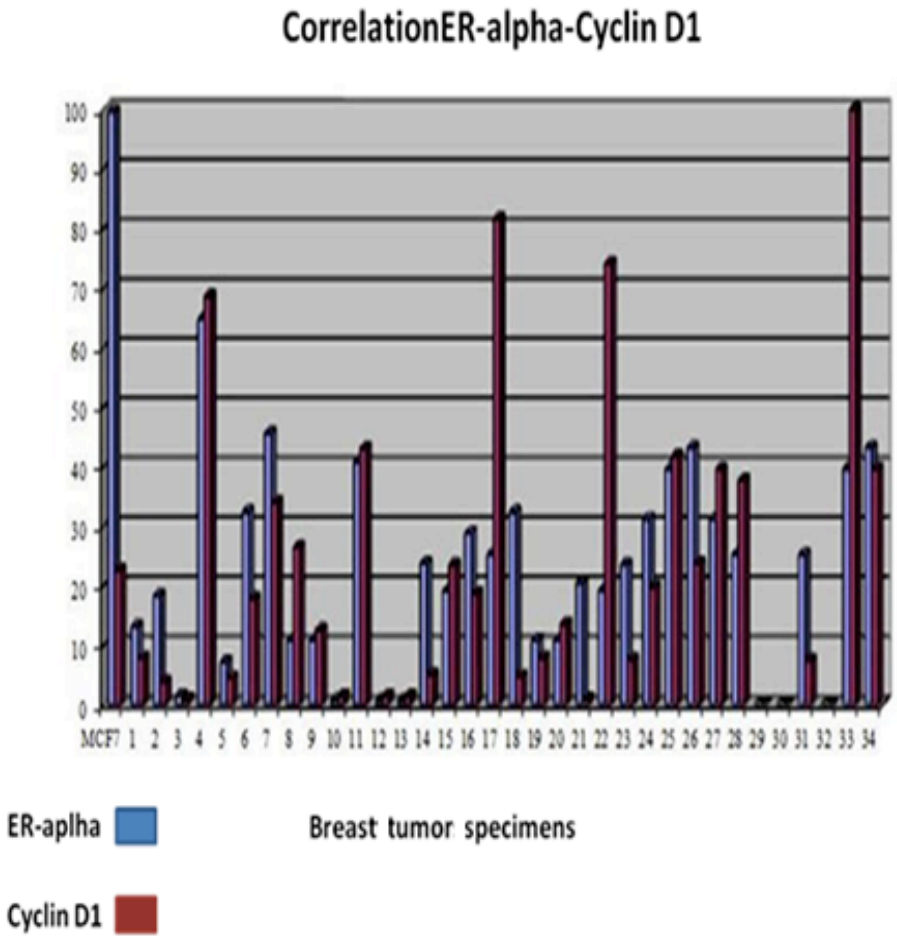

***Figure 6**. Quantitative analysis of estrogen receptor alpha and cyclin D1 protein expression on thirty four breast tumor tissue specimens were evaluated. Protein expression was normalized on α-tubulin expression; samples analyzed showed concomitant expression of estrogen receptor α and cyclin D1.*

4.2 Palbociclib inhibits cell proliferation of human breast cancer cell lines

To evaluate the role of Palbociclib on cell proliferation, the effects of the drug have been tested on a panel of 4 human breast cancer cells, consisting of MCF-7, MDA-MB-231, BT474, MCF7/her 2 transfected.

The selected breast cancer cells display characteristics of molecular subtypes of breast cancer; MCF-7 is a breast cancer cell line derived from invasive breast ductal carcinoma, from pleural effusion. They are ER-positive PR-positive and HER2-negative.

BT474 cell line was isolated from invasive ductal breast carcinoma; It is ER-positive, PR-positive and HER 2-positive.

MCF7/her2 transfected was provided by Dr. R. Schiff's group (Department of Medicine, Baylor College of Medicine Houston, Texas).These breast cancer cells are MCF-7 stable transfected with a vector that allows HER2 expression, and maintained in selective medium with geneticin.

MDA-MB-231 are breast cancer cells isolated from pleural effusion having ER-negative, PR-negative,HER2-negative and representing a triple negative in vitro model.

To confirm the receptor expression features of breast cancer cell lines, gene expression of different receptors used in the breast cancer classification such as ERα, HER 2 and PR, have been evaluated by RT-PCR (Figure 7). MCF-7, BT474, MCF7/her2 transfected resulted ERα and PR positive; on the contrary MDA-MB-231 resulted PR and ERα negative. The evaluation of HER 2 confirmed MCF-7 and MDA-MB-231 as HER2-negative; as expected BT474 and MCF7/her2 transfected were HER2-positive.

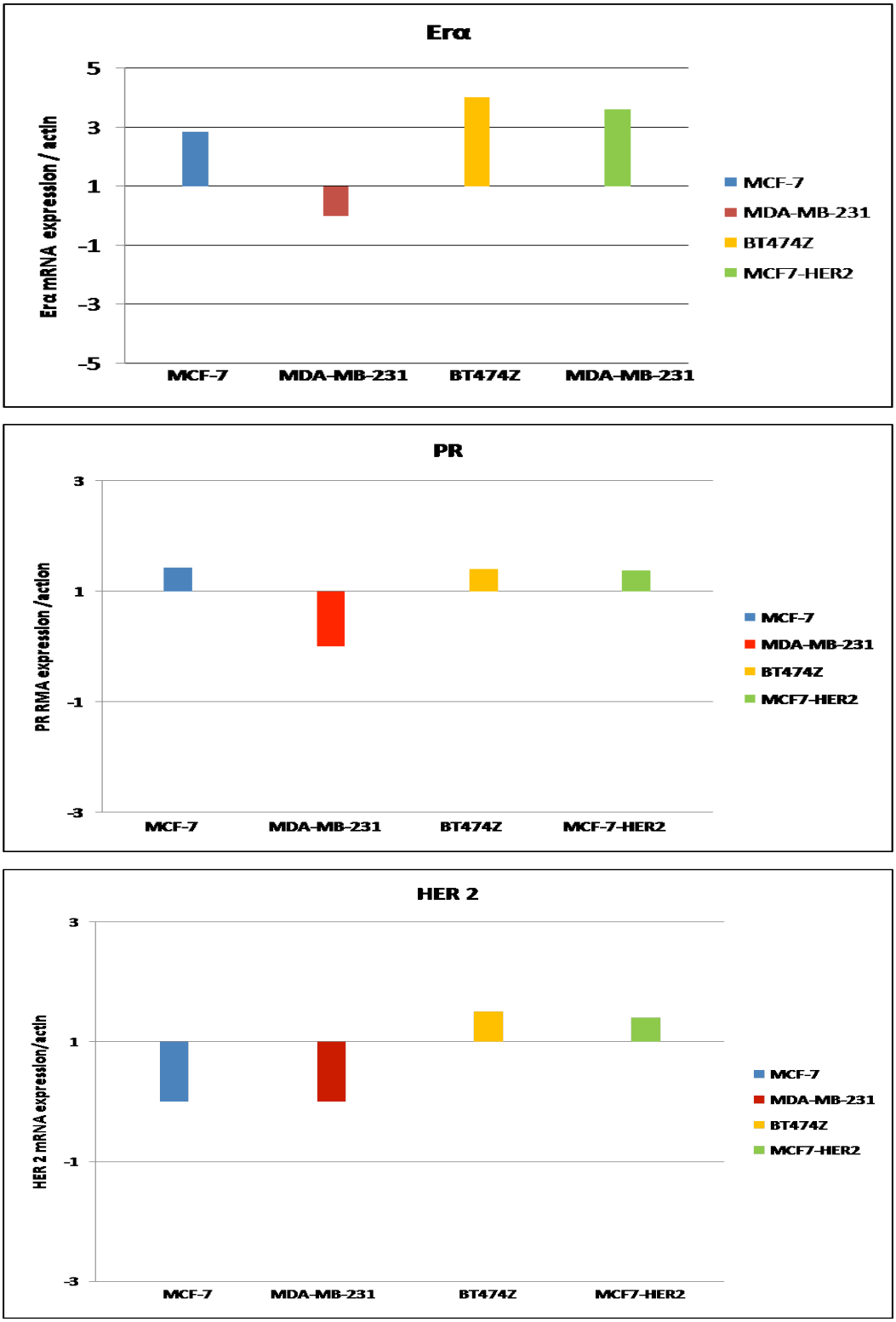

Figure 7. *mRNA expression (ERα, PR, HER 2) of 4 breast cancer cell subtypes selected.*

To evaluate the effects of Palbociclib on cell proliferation, breast cancer cells were seeded for 24 hours in multiwells of 24 in triplicate. Once attached, cells were treated i*n vitro* with scalar concentrations of drug (1 μM to 31,25 nM). MTT assay and cell count were performed at 1 day, 3 days and 6 days of treatment.

MTT assay revealed that Palbociclib displayed anti-proliferative activity after 3 days in four different breast cancer cell lines at the different tested concentrations.

Palbociclib does not alter MCF-7 cell proliferation after 24 hours; a dose dependent effect was observed after 3 days of treatment with a range of inhibition from 1% at 31,25 nM to 56% of inhibition at 1 μM compared to not treated control cells. Dose dependent effect persists after 6 days of treatment with a maximum of 80% inhibition of cell proliferation at 1 μM, compared to untreated cells.

MDA-MB-231 cell proliferation is not altered when these cells are treated with palbociclib for 24 hours. The effect on proliferation becomes substantial after 3 days of treatment with a reduction of cell proliferation by 25 % at 250 nM of drug concentration, and an inhibitory effect on cell growth up to 57 % at 1 uM. Interestingly, after 6 days of treatment, palbociclib displays the strongest inhibitory effect on cell growth (63 %), at the maximum dose.

In BT474 Palbocilib determines a reduction of cell proliferation (52 % of cell growth inhibition at 1 μM dose) after 3 days of treatment compared to untreated control cells. After 6 days of treatment a 66 % of inhibition was observed.

Palbociclib determines a reduction of cell proliferation on MCF-7/ her2 transfected with a cell growth inhibition of 60% after 6 days of treatment at the concentration of 1 μM .

Cell count was performed after 1, 3 and 6 days after CDK4/6 inhibitor treatment. After 1 days of treatment at 1 μM of Palbociclib, MCF-7 show a cell growth inhibition of 30%; 68% of growth inhibition was observed after 3 days while 92% after 6 days of treatment. MDA-MB-231 resulted more resistant to Palbociclib compared to other breast cancer cells analyzed with 27% of cell growth inhibition after 1 day, 43% after 3 days and 63% after 6

days of treatment. BT474 showed a slight inhibition of cell growth (3%) after 1 day; 52% of inhibition was observed after 3 days of treatment and 81% was observed after 6 days of treatment. MCF-7/ her2 transfected showed 16% of cell growth inhibition after 1 days of treatment, 67% of inhibition after 3 days and 84% after 6 days of treatment with Palbociclib at 1µM (Figure 8).

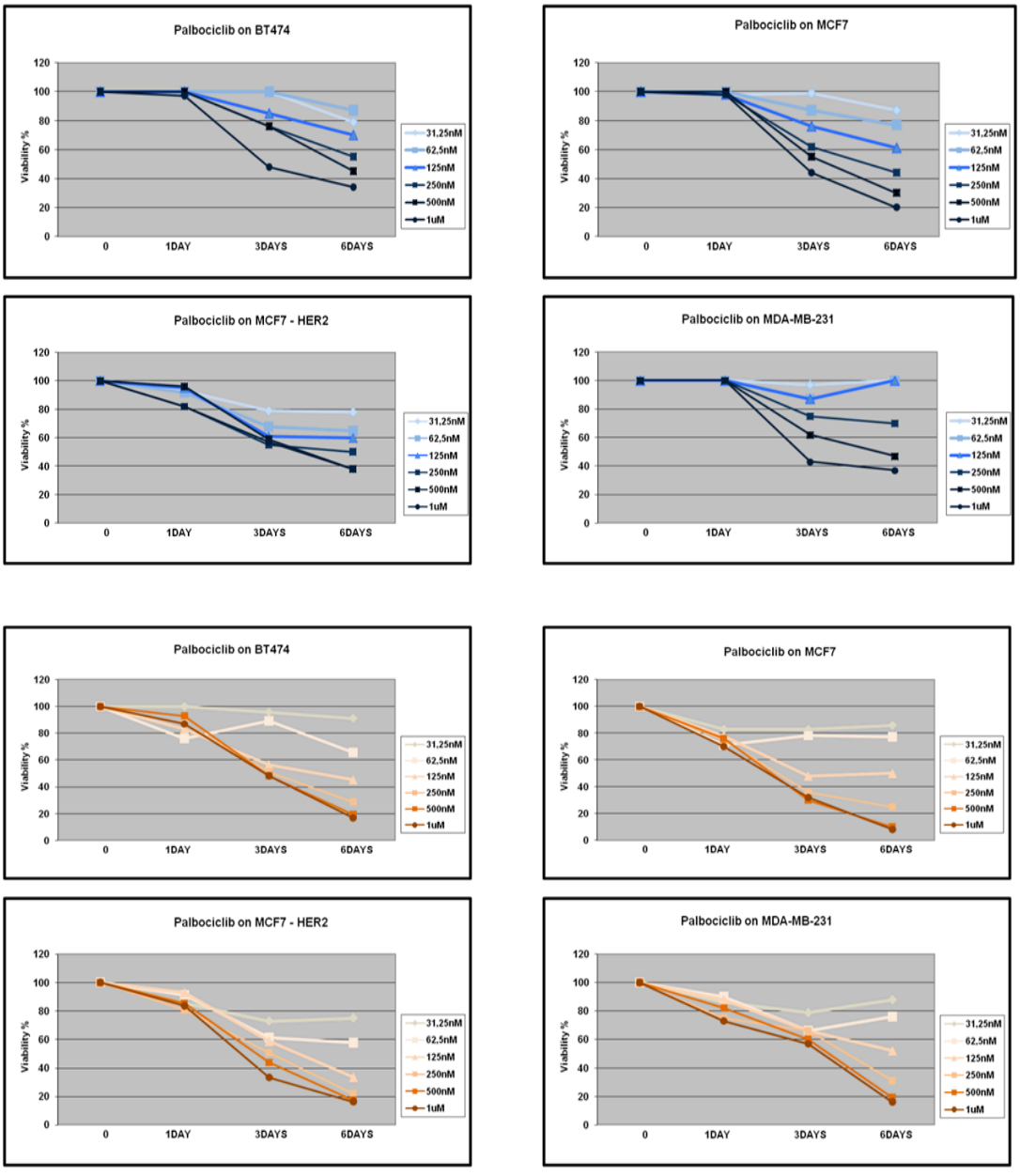

Figure 8. *Study of cell proliferation In breast cancer cells (BT474; MCF-7; MCF-7 / her2 trasfected; MDA-MB-231) following treatment with Palbociclib ; MTT assay (blu) and cell count (orange) were performed at different concentration (31.25 nM - 62.5 nM - 125 nM - 250 nM - 500 nM - 1 uM) of the CDK4/6 inhibitor for 1, 3 and 6 days. MCF-7 resulted more sensitive to treatment compared to other breast cancer cells subtypes.*

Based on MTT data it was possible to calculate the IC 50 (Table 3).
MCF-7 cell line (Luminal A) resulted more sensitive to the treatment with an IC 50 equal to 201.5 nM. On the contrary, MDA-MB-231 cell line (Triple negative) was the most resistant to treatment with an IC 50 of 608 nM; MCF7/her2 transfected (Luminal B) show a IC 50 of 262 nM while BT474 (Luminal B) show a IC50 of 384 nM.

BREAST CANCER CELL LINES	IC 50 VALUE	CLASSIFICATION
MCF-7	201 nM	LUMINAL A
MCF-7-her2/trasfected	262 nM	LUMINAL B
BT474Z	384 nM	LUMINAL B
MDA-MB-231	609 nM	TRIPLE NEGATIVE

***Table 3**. Inhibitory concentration and cell type. Breast cancer cell lines MCF-7 cells (Luminal A) are sensitive to treatment with the CDK4/6 inhibitor Palbociclib; MDA-MB-231 cells (Triple negative) are more resistant compared to other breast cancer cell lines analysed. The expression of HER 2 receptor in MCF-7 / her2 transfected determines a greater resistance to in vitro treatment than MCF-7 (262 nM vs 201 nM); BT474 cells (Luminal B) have a IC 50 value of 384 nM.*

4.3 Palbociclib inhibits cell proliferation with a cytostatic effect

In order to assess whether Palbociclib led to a cytostatic or cytotoxic effect on different breast cancer subtypes, the resumption of cells proliferation after *in vitro* treatment with Palbociclib was evaluated.

Residual breast cancer cells (20% of MCF-7, 37 % of MDA-MB-231, 34 % of BT474 and 24% of MCF-7/ her2 trasfected), after 6 days of treatment with Palbociclib at concentration of 1 μM, were detached and seeded again.

Cell proliferation was evaluate by cell count after 1,3 and 6 days from seeding; breast cancer cells not previously treated were used as controls. All breast cancer cells analyzed, resumed to proliferate in a manner comparable with control cells, never treated with Palbociclib. The same sensitivity to the drug was observed when treating re-proliferating breast cancer cells again with Palbociclib at 31,25 nM, 62,5 nM, 125 nM, 250 nM,500 nM and 1μM for 6 days; Figure 9 shows the cytostatic effect of Palbociclib.

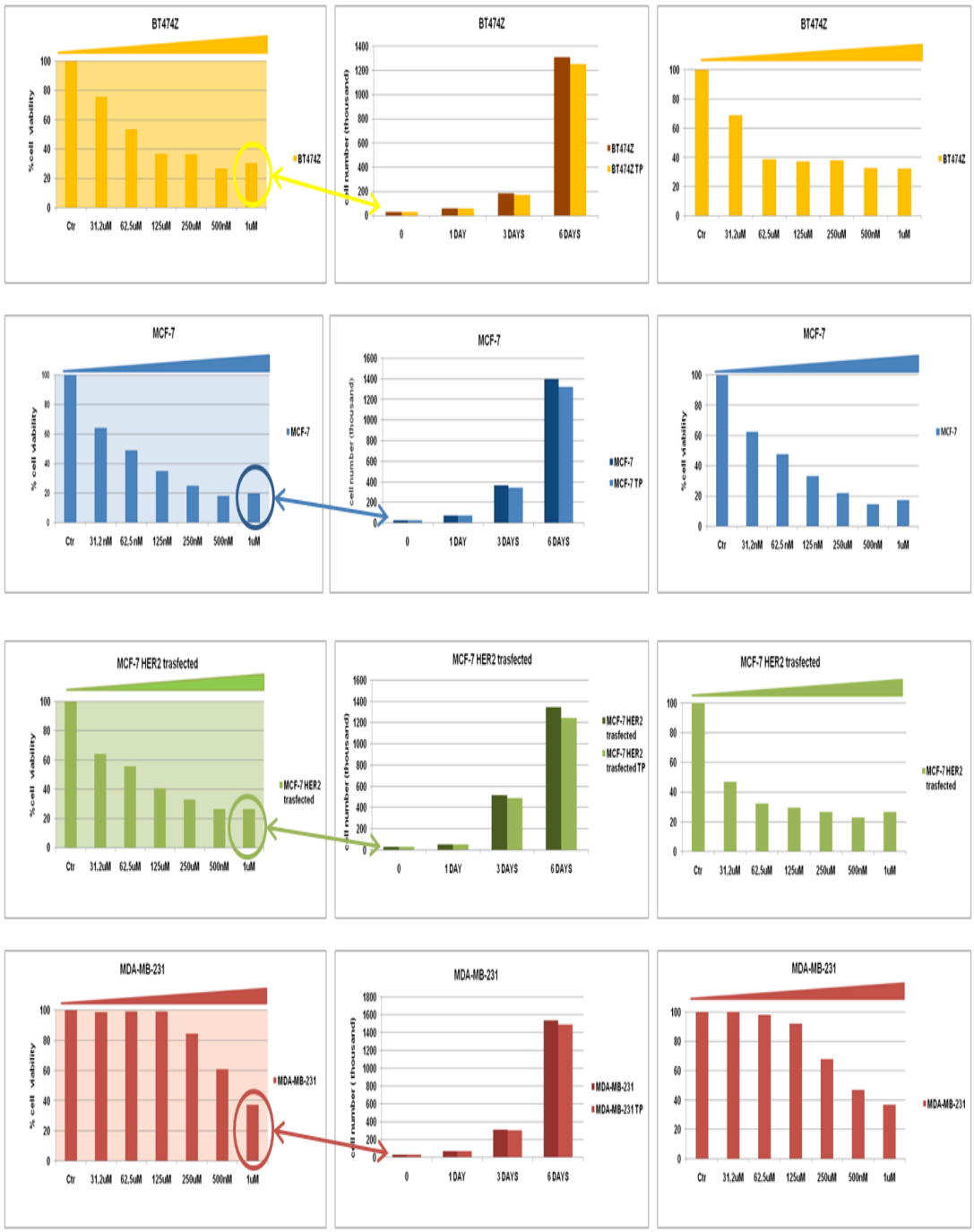

Figure 9. *Palbociclb determines a cytostatic effect in breast cancer cell line analyzed. After 6 days of treatment with the inhibitor at 1 uM cells were tripsinized and seeded again (arrow in the figure). Cell proliferation resumed and was comparable with control cells not previously treated with the inhibitor. On the right Palbociclib inhibits cell proliferation of treating breast cancer cells again. BT474Z are orange, MCF-7 are blue, MCF-7/her2 trasfected in green and MDA-MB-231 are in red .*

4.4 Effect of Palbociclib in cell cycle protein modulation (cyclin D1, p27, Rb, pRb) depends of breast cancer cells subtypes

To evaluate the effect of Palbociclib on cell cycle, I compared breast cancer cell lines untreated and breast cancer cell lines treated with Palbociclib at 1 μM for 12 hours performing flow cytometry using propidium iodide.

In all breast cancer cells analyzed G0/G1 phase increased following treatment with the drug; In BT474Z control cells, percentage of G0/G1 phase was 59,42%; in BT474Z treated for 12 hours with Palbociclib the percentage of cells in G0/G1 increased to 71,2%. The percentage difference between treated and untreated cells was 11,78%. In MCF-7, G0/G1 phase was 77,65% in control cells; following treatment for 12 hours with Palbociclib (μM) G0/G1 percentage was 97,97%. In addition, MCF7/her2 control cells showed 33,97% in G0/G1 phase; the increase of percentage of G0/G1 phase in MCF7/her2 treated cells was 7,08% compare to control cells not treated. In triple negative MDA-MB-231 G0/G1 percentage in treated cells was 82,94 % compared to cells not treated with the drug (62,07%).In this case, percentage of difference between treated and untreated cells was 20.87%. Furthermore, a decrease of S phase was observed following treatment with Palbociclib at 1 μM in BT474 (8,47% vs 17,9%), MCF-7 (1.74% vs 15,81%) and MDA-MB-231 (4,09% vs 13,21%). In MCF-7/her2 transfected percentage of S phase in control cells untreated was 15,53% while in treated cells with the inhibitor (1μM) was 11,93%.

We did not observe obvious differences of percentage in G2/M phase in BT474Z (11,46% vs 12,45%) and MCF-7/her2 transfected (17,59% vs 16,54%) treated and not treated cells with Palbociclib (1μM) for 12 hours ; on the contrary MCF-7 (0,25% vs 6,25%) and MDA-MB231 (10,45% vs 23,04%) showed a decrease of percentage in G2/M phase in treated cells compared to untreated breast cancer control cells.

These results demonstrate that Palbociclib determines a cell cycle arrest in G0/G1 phase in all the four breast cancer cells analyzed (BT474Z, MCF-7, MCF7/her2 transfected and MDA-MB-231) (Figure 10).

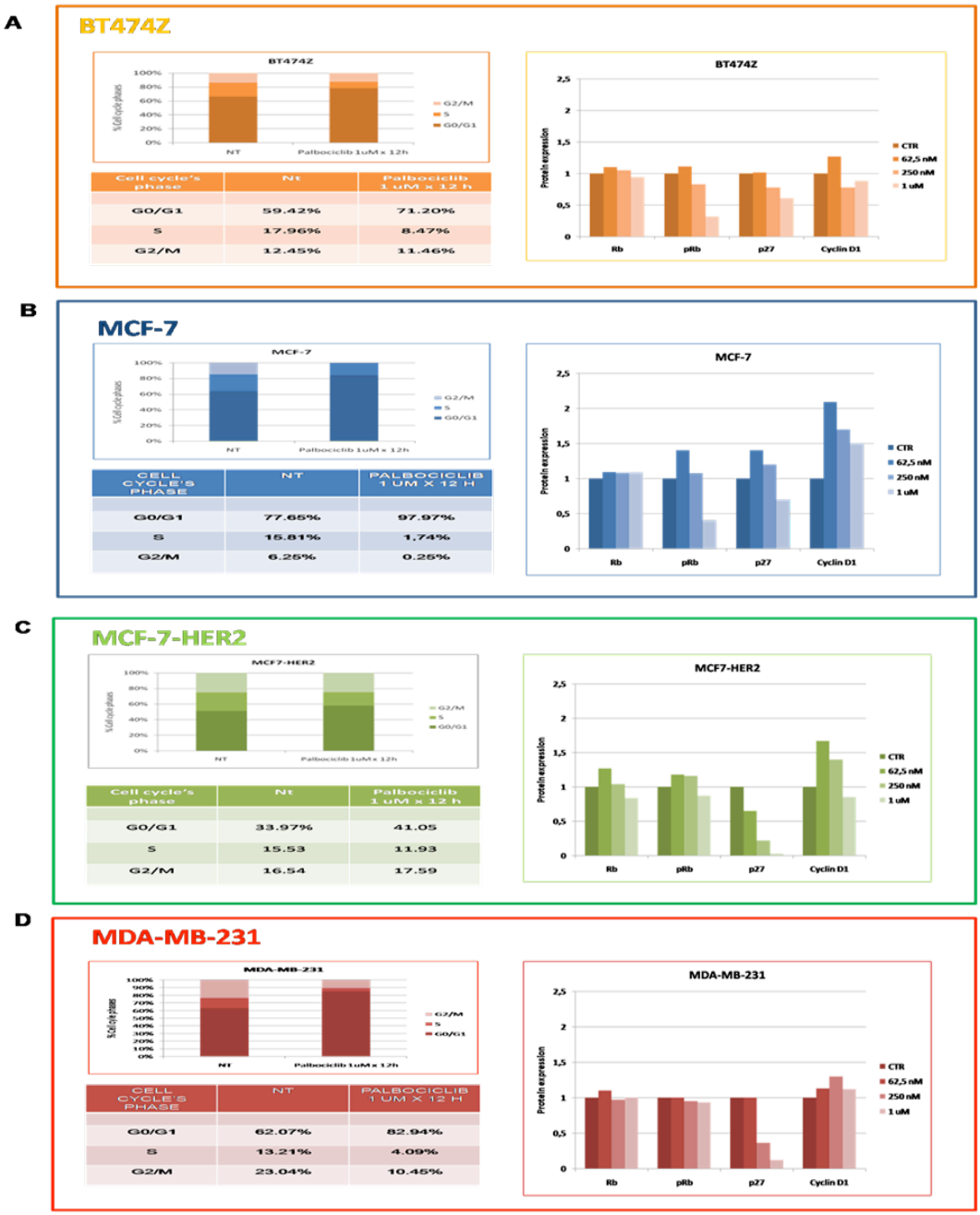

Figure 10. *Cell cycle analysis (left panels) and levels of expression of cyclin D1, p27, Rb and pRb in breast cancer cells (right panels). G0/G1 arrest was observed in breast cancer cells BT474Z (A), MCF-7 (B) MCF-7-HER2 (C) and MDA-MB-231 (D) treated compared to control cells .*

Rb is a tumor suppressor that plays a critical role in overcoming the restriction point from G0 to G1 phase. During the G1 phase of the cell cycle, the cyclin D1- CDK4/6 complex stimulates the phosphorylation of tumor suppressor Rb promoting cell cycle progression. The effect at different concentration (62,5 nM, 250 nM, 1 uM) of Palbociclib after 6 days of treatment on total Rb and pRb (phosphorylation of serine780), Cyclin D1 and p27 expression was determined in breast cancer cell lines BT474Z, MCF-7, MCF-7/her2 transfected and MDA-MB-231 breast cancer cell lines (Figure 10, right). No modulation in the expression of total Rb was found in the 4 breast cancer cells treated with the inhibitor of the complex CDK 4/6-cyclin D1; conversely, in the phosphorylation of Rb at the level of serine 780 differences were evident in BT474 and MCF-7 treated with Palbociclib at concentration of 1 uM for 6 days compared to their untreated controls.

A decrease of Rb phosphorylation was observed with exposure to Palbociclib at 1 uM in BT474 and MCF-7; no change in MDA-MB-231 and in MCF-7/her2 transfected was observed in the Rb phosphorylation status.

Cyclin D1 protein expression is modulated differently depending on the breast cancer cells subtype; in MCF-7 and in MCF-7/ her2 transfected cyclin D1 expression increased in treated cells compared to untreated cells. In BT74Z cells (Luminal B) Cyclin D1 expression decreased in treated cells (250 nM and 1 μM) compared to untreated control cells. MDA-MB-231 (Triple negative) showed a specific expression of cyclin D1 characterized by the formation of a doublet.

In MDA-MB-231 and MCF-7/her2 transfected treated with Palbociclib, p27 protein expression was reduced compared to untreated control cells; on the contrary no significant changes were observed in treated and untreated breast cancer cells MCF-7 and BT474Z.

These results showed that in breast cancer cell lines more sensitive to the drug such as MCF-7 (Luminal A) and BT474Z (Luminal B) expression of endogen inhibitor p27 persists in treated cells; on the contrary, in cells resistant to Palbociclib p27 expression was reduced in treated compared to untreated cells.

4.5 The inhibition of cell proliferation increases if Palbociclib is associated with Formestane

In breast cancer, the inhibition of estrogen synthesis is an effective treatment for breast patients in menopause; however, patients may develop drug resistance.

The effect of combination of AI Formestane and the CDK4/6 inhibitor was estimated on *in vitro* cell proliferation.

MCF-7, BT474, MCF-7 / her2 transfected, are ER-positive but Aromatase-negative; MDA-MB-231 is ER-negative and expresses low level of aromatase, enzyme capable of converting androgens to estrogens.

In order to find the right candidate for the the Palbociclib-Formestane combination study, it was evaluated the estrogen receptor alpha and aromatase (CYP19A1) expression of 11 breast primary breast cancer cells obtained directly from fresh tumor tissue specimens [45].

K 90 primary breast cells were selected as ideal candidate for the analysis since they resulted to be positive for the expression of estrogen receptor and aromatase; MCF-7 was used as negative control (Figure 11A).

K 90 and MCF-7 were seeded (15 x 10^3 in triplicate in 24 multiwells) and, after 24 hours, the cells were treated with increasing concentrations (25 nM, 50 nM, 100 nM, 150 nM, 300 nM, 600 nM) of Formestane (AI) alone, Palbociclib alone (1 uM) and with a combination of Formestane (at increasing concentrations) and Palbociclib (1 μM) for 6 days.

MTT assay was performed to measure cell viability.

In MCF-7, Formestane alone is not able to inhibit cell proliferation whereas Palbociclib alone, as previously shown, greatly inhibits MCF-7 cell proliferation (80% inhibition). Moreover, combined effect of Palbociclib and Formestane does not show a greater effect than Palbociclib alone.

Interestingly, in K 90, Formestane alone displays a dose dependent effect with 61% of inhibition of cell proliferation at 600 nM compared to not treated control cells; palbociclib alone reduces the cell proliferation of 41% compared to untreated control cells.

The combined effect of the two compounds greatly inhibits (81,7%) breast cancer cell proliferation (Figure 11B).

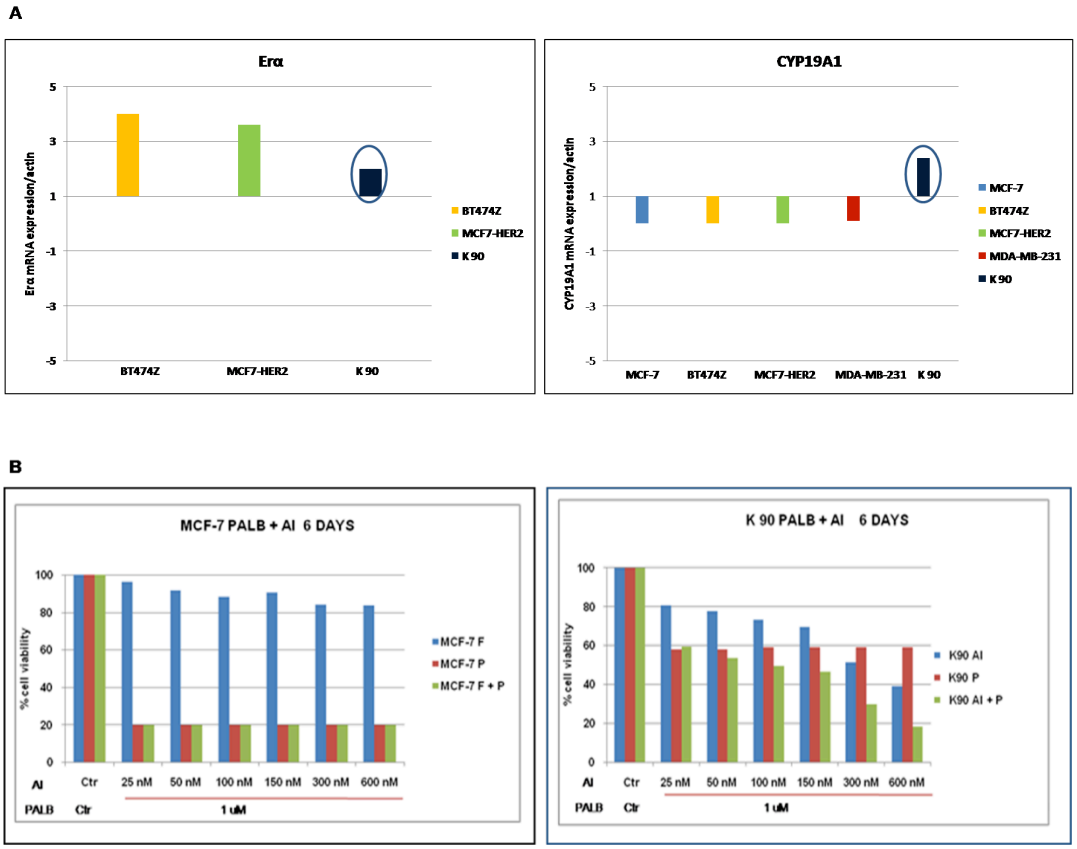

Figure 11. *In (A) selection of primary breast cancer cell K 90; MCF-7, MDA-MB-231, BT474 and MCF-7/ her2 trasfected don't express aromatase. K 90 expresses estrogen receptor and aromatase (circle blu in figure); IN (B) on the left Formestane has not effect alone in MCF-7 (blu bars) and the effect on cell proliferation is due to Palbociclib ; on the right K 90 were treated with different concentration of Formestane alone (25 nM to 600 nM), Palbociclib alone (1 uM of concentration) and Formestane at different concentration plus Palbociclib at 1 uM. The combined effect determines a greater inhibition of cell proliferation compared single drug.*

On the basis of these results a dual targeted strategy, that determines the arrest of the proliferative stimulus induced by the inhibition of estrogen synthesis and the inhibition of the cell-cycle progression in G0/G1 phase could give important indications for use in clinical practice.

5. Discussion

In this project, I describe Palbociclib, PD 0332991, as a potent and highly selective inhibitor of CDK 4 and CDK 6 and show that the suppression of the cell cycle regulators in human breast cancer cells results in significant antiproliferative activity. PD 0332991 was tested on 3 breast cancer cells subtypes (MCF-7, BT474Z, MDA-MB-231) representing 3 main subtypes of breast cancer (Luminal A, Luminal B and triple negative) and on MCF-7 her 2/transfected to evaluate a possible role of HER 2 in response to Palbociclib.

In the first part of the study I observed a concomitant expression of level of cyclin D1 and ERα in breast cancer tissue specimens; the correlation of this expression prompt me to evaluate the *in vitro* effect of Palbociclib (cyclin D1 – CDK 4 inhibitor) in ER-positive and ER-negative breast cancer cells lines to understand the effect on cell proliferation. Palbociclib induced an inhibition of cell proliferation on the 4 breast cancer cell lines analysed; MCF-7 cells were the most sensitive to treatment with IC 50 of 201 nM.

The effect exerted by the drug is cytostatic; indeed, the different subtypes of breast cancer cells treated for 6 days with the inhibitor grew if they were cultured again. In addition, cell cycle regulatory proteins are modulated by the drug depending by cell subtype and the inhibition of cell proliferation can be increased if the drug is associated with AI.

As observed in the present study and by Finn and colleagues [37] the drug displays a better inhibitory effect on luminal breast cancer cells growth; on the contrary basal type breast cancer cells are more resistant to the drug. These data are supported by the inhibition of the Rb phosphorylation observed in MCF-7 and BT474 but not in MDA-MB-231. Further studies are needed to understand the lack of inhibition of Rb phosphorylation in certain cell types of breast cancer; a possible mutation in the binding site of CDK4/6 or overexpression of complex CDK1/2-cyclin E, inactivating checkpoint at the level of the G1 phase may be a possible hypotheses. Also, amplification or overexpression of cyclin D1 is more frequently associated to Luminal A

subtype and the effect of estrogens is tightly connected to cyclin D1 expression [48] . The *in vitro* effect of the Palbociclib on cancer growth is cytostatic. However some studies reveal a possible cytotoxic effect. Uras and colleagues [49] have demonstrated a cytotoxic effect of the drug on leukemia cells; indeed CDK4/6 kinase inhibition induced cell cycle arrest and apoptosis in FLT3-ITD leukemic cells; Hu W and colleagues [50] have shown no change in apoptosis in Human bone marrow mononuclear cells following treatment with Palbociclib. These works suggest that the drug activity can depend on the cellular type.

In breast cancer, Palbociclib has showed synergistic effects with drugs such as tamoxifen and trastuzumab and a more complex effect with DNA-damage agents [37]; in the present study Palbociclib along with AI is able to greatly inhibit cell proliferation of primary K 90 breast cancer. Further studies are needed to evaluate the effects of Palbociclib in combination with other drugs used in breast cancer and to evaluate its possible clinical use in the different stages of this disease.

6. Conclusions

The purpose of this study was to identify subtypes of breast cancer more sensitive to the treatment with palbociclib. The results clearly show an inhibitory effect on the proliferation of Luminal A (ER- positive) and Luminal B (ER-positive, HER2- positive) breast cancer cells.

However, *in vitro* and *in vivo* combination studies with specific targeted drugs (Trastuzumab, AIs , Fulvestrant) are needed to obtain information to help to guide patient selection in the clinic.

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