Title:

Mechanistic investigation of bone marrow suppression associated with palbociclib and its differentiation from cytotoxic chemotherapies

Wenyue Hu1*, Tae Sung1, Bart A. Jessen1, Stephane Thibault1, Martin B. Finkelstein2, Nasir K. Khan3, and Aida I. Sacaan1

1. Drug Safety Research and Development, Pfizer Inc. San Diego, CA 92121

2. Drug Safety Research and Development, Pfizer Inc. Pearl River, NY 10965

3. Drug Safety Research and Development, Pfizer Inc. Groton, CT 06340

*Correspondence:

Wenyue Hu, Ph.D. DABT
Drug Safety Research and Development,
La Jolla Laboratories, Pfizer Inc.
10646 Science Center Drive
San Diego, CA 92121 USA
Telephone: 858-622-7530
Fax: 858-678-8290
Email: wenyue.hu@pfizer.com
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Statement of Translational Relevance

Palbociclib is a highly effective cyclin-dependent kinase 4/6 inhibitor approved for metastatic breast cancer. Though manageable, neutropenia is one of the most frequent adverse events associated with palbociclib in the clinic. The current investigative work sheds light on the mechanism of palbociclib-induced bone marrow suppression and differentiates the mechanism of bone marrow suppression from that induced by cytotoxic chemotherapeutic agents. These results potentially explain the rapid reversibility of palbociclib-induced neutropenia and its uncomplicated nature when compared to traditional cytotoxic agents. Moreover, these data support the current dosing regimen in the clinic, which provides time for bone marrow cells to resume proliferation during the one-week treatment-free period without impacting tumor efficacy. This investigative work provides information to the clinicians treating breast cancer patients in managing palbociclib-induced neutropenia and could have broader implications to future indications and combinations with palbociclib.
Abstract

Purpose: Palbociclib (PD-0332991) is the first selective cyclin-dependent kinase (CDK) 4/6 inhibitor approved for metastatic breast cancer. Hematological effects, especially neutropenia, are dose-limiting adverse events for palbociclib in humans.

Experimental Design: Reversible hematological effects and bone marrow hypocellularity have been identified in toxicology studies in rats and dogs following palbociclib treatment. To understand the mechanism by which the hematological toxicity occurs, and to further differentiate it from myelotoxicity caused by cytotoxic chemotherapeutic agents, an in vitro assay using human bone marrow mononuclear cells (hBMNCs) was utilized.

Results: This work demonstrated that palbociclib-induced bone marrow suppression occurred through cell cycle arrest, with no apoptosis at clinically relevant concentrations, was not lineage-specific, and was reversible upon palbociclib withdrawal. By contrast, treatment with chemotherapeutic agents (paclitaxel and doxorubicin) resulted in DNA damage and apoptotic cell death in hBMNCs. In the presence or absence of the anti-estrogen, palbociclib-treated hBMNCs did not become senescent and resumed proliferation following palbociclib withdrawal, consistent with pharmacologic quiescence. The breast cancer cells, MCF-7, conversely, became senescent following palbociclib or anti-estrogen treatment with additive effects in combination, and remained arrested in the presence of anti-estrogen.

Conclusions: Palbociclib causes reversible bone marrow suppression, clearly differentiating it from apoptotic cell death caused by cytotoxic chemotherapeutic agents. This study also distinguished the cell cycle arresting action of palbociclib on normal bone marrow cells from the...
senescent effects observed in breast cancer cells. These results shed light on the mechanism and support risk management of palbociclib-induced bone marrow toxicity in the clinic.

**Key words:**

Breast cancer, palbociclib, bone marrow suppression, CDK4/6 inhibitor, cytotoxic chemotherapeutic agents
INTRODUCTION

Cyclin-dependent kinases (CDK) are a family of serine/threonine protein kinases with more than 20 members that are characterized by the requirement of most CDKs for binding to cyclins or other protein partners to induce kinase activity. CDKs 1-4 and 6 regulate transition through specific checkpoints in the cell cycle, while CDKs 7-9 are involved in regulation of the transcriptional machinery. CDK5 is involved in neuron-specific functions while other family members have not been extensively studied (1, 2).

Given the pivotal role of CDKs in cellular proliferation, they have been among the first targeted therapy approaches pursued for the treatment of cancer (2, 3). One of the first CDK inhibitors, flavopiridol, a broad-spectrum ATP-competitive inhibitor of several CDKs, was described over 20 years ago. Flavopiridol has been tested in clinical trials for over a decade (3), and has demonstrated limited efficacy with a toxicity profile consisting of diarrhea, transient transaminitis, cytokine release syndrome, and tumor lysis syndrome (4). The nonclinical toxicity of another broad-spectrum CDK inhibitor, AG-012986, has been extensively profiled and demonstrated toxicities in multiple organs, including hematopoietic system (5), retina, peripheral nerves (6), gastro-intestinal tract and pancreas (7).

In contrast to the first generation broad-spectrum CDK inhibitors, palbociclib is an oral small molecule selective inhibitor of CDK 4 and 6 that specifically blocks the G1/S cell cycle transition and avoids other CDK targets that may induce apoptosis in quiescent cells (8). Palbociclib was recently approved by the US FDA in combination with letrozole for the
treatment of first line advanced breast cancer. The safety profile and oral dosing route enables a more convenient dosing regimen (three weeks on, one week off per cycle) than chemotherapeutic agents that are intravenously administered, that has facilitated the demonstration of efficacy in the treatment of breast cancer when given in combination with anti-estrogens (8, 9). In the PALOMA-1/TRIO-18 randomized phase II study, which evaluated the efficacy and safety of palbociclib in combination with letrozole in ER⁺/Her2⁻ breast cancer, hematological toxicities, especially neutropenia, were identified as dose-limiting toxicities and were the most frequently reported adverse events for palbociclib (10). Findings from this study suggest the neutropenia associated with palbociclib differs from that seen with cytotoxic chemotherapeutics in that it is transient, reversible, and does not commonly lead to febrile neutropenia (10). To aid in understanding the mechanism by which neutropenia occurs and why it differs from the myelotoxicity seen with cytotoxic chemotherapeutic agents, an in vitro bone marrow toxicity assay was utilized to evaluate the cellular mechanism and the reversibility of bone marrow suppression induced by palbociclib as a single agent or in combination with anti-estrogens (the currently approved indication). In addition, the differential effects of palbociclib and antiestrogens toward bone marrow cells and breast cancer cells, as well as their effects relative to cytotoxic chemotherapy agents in bone marrow cells, were investigated.
MATERIALS AND METHODS

Test Article

Palbociclib (PD-0332991) was manufactured by Pfizer Inc. Test articles, including paclitaxel, doxorubicin, and fulvestrant were purchased from Sigma (St. Louis, MO).

In vivo chronic toxicity and toxicokinetic study with palbociclib in dogs

To evaluate the toxicity of chronic administration of palbociclib in dogs, palbociclib was administered daily via oral gavage to 6 male and 6 female dogs per group at 0, 0.6 or 3.0 mg/kg/day for 10 cycles each consisting of three weeks of daily dosing and one week treatment free. Two of 6 dogs /group were assigned to a 12-week recovery period at the end of the 10-cycle dosing phase. Blood samples for hematology were collected in potassium EDTA via a jugular or cephalic vein twice prior to the dosing phase and at the end of each dosing and treatment-free period for cycle 1 (days 22 and 29), cycle 4 (days 106 and 113), cycle 7 (days 190 and 197), at the end of the dosing phase of cycle 10 (day 274), and week 7 and 12 (days 50 and 85) of the recovery period. Following overnight fasting, dogs were euthanized and necropsied at the end of the dosing phase and recovery period. Automated hematology measurements included red blood cell and platelet parameters and indices, and a differential white blood cell count. The bone marrow was collected with the sternum and proximal tibia, preserved in 10% neutral-buffered formalin, sectioned to slides, stained with hematoxylin and eosin (H&E) and examined by a board-certified veterinary pathologist.
In Vitro Cell Culture

Human bone marrow mononuclear cells (hBMNCs: a heterogeneous population that includes hematopoietic lineage cells such as lymphocytes, monocytes, stem cells and progenitor cells) and CD34+ human bone marrow hematopoietic stem cells were primary cells purchased from Lonza (Walkersville, MD). The cells were cultured in the hematopoietic progenitor growth (HPGM) medium (Lonza) supplemented with 10% fetal bovine serum (FBS) and in the presence of the following cytokines (R&D systems, Minneapolis, MN): 25 ng/mL stem cell factor (SCF), 3 U/mL erythropoietin (EPO), 10 ng/mL granulocyte colony-stimulating factor (G-CSF), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 15 ng/mL thrombopoietin (TPO), 10 ng/mL interleukin 3 (IL3), 10 ng/mL interleukin 6 (IL6), and 25 ng/mL Flt3 ligand, in a 37°C 5% CO2 and 98% humidity incubator. Human peripheral blood mononuclear cells (hPBMCs) were primary cells purchased from Lonza, and cultured in the Roswell Park Memorial Institute media (RPMI).

MCF-7 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI media supplemented with 10% FBS, in a 37°C 5% CO2 and 98% humidity incubator.

In Vitro Testing of Lineage Specific Effects

The CD34+ hematopoietic stem cells were stimulated with the following cytokines for four days to induce lineage-specific differentiation: SCF, EPO, and IL-3 for erythroid lineage; SCF, G-CSF, GM-CSF, IL-3 and Flt3 ligand for myeloid lineage; SCF, TPO and IL-3 for megakaryocyte lineage. Cells were subjected to palbociclib treatment for 5 days and cell viability measurement was conducted.
In Vitro Cell Viability and Apoptosis Assay

The hPBMCs or hBMNCs cultured in RPMI or conditioned HPGM media were treated with test compounds at specified concentrations in a 3-fold serial dilution in triplicate. After 24 hours (hPBMCs) or five days (hBMNCs) of continuous exposure, cell viability was measured using the Cell Titer-Glo™ luminescent cell viability assay kit (Promega, Madison, WI). Apoptosis was assessed after 24 hours of compound treatment in hBMNCs using the Caspase-Glo® 3/7 activation assay (Promega) following the manufacturer’s recommended protocol. The bioluminescence was measured using a Safire2 microplate reader (TECAN, Switzerland). The dose response relationship was analyzed using Microsoft Excel.

In Vitro Cell Proliferation Assay

The effect on DNA synthesis was determined using the Click-iT® plus EdU flow cytometry assay kit (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. Briefly, cells were pulse treated with 10 μM 5-ethynyl-2’-deoxyuridine (EdU) substrate for 2 hours at 37°C prior to cell fixation and permeabilization. The reaction cocktail containing fluorescent dye picolyl azide was added to each sample, EdU incorporation was analyzed at 10,000 events per sample in triplicates using flow cytometry. The cell cycle analysis was conducted using propidium iodide (PI) DNA staining dye (BD Biosciences). Cells were fixed with 70% ethanol overnight at -20°C, followed by washing with PBS and ribonuclease digestion at 37 °C for 30 minutes. Cells were stained with 50 μg/mL PI and the percentage of cells in each cell cycle phase was quantitated using marker sets within the analysis system.
In Vitro Cellular Senescence Assay

Cellular senescence was measured using a quantitative flow cytometry assay (CBA-232) or a 96-well biochemical assay (CBA-231) (Cell Biolabs, San Diego, CA). In the quantitative assay, cells were pre-incubated with the senescence-associated β-galactosidase (SA-β-Gal) substrate for 4 hours at 37°C, followed by three washes with cold PBS. Cellular senescence was measured by flow cytometry at excitation of 485 nm and emission of 520 nm. In the 96-well senescence assay format, cells were washed with cold PBS and lysed with 1X cell lysis buffer, followed by centrifugation at 14,000 rpm for 10 minutes at 4°C. An aliquot of 50 µL of the cell lysate was combined with reaction buffer in a 96-well plate and incubated at 37°C for 2 hours. The reaction mixture was neutralized with stop solution and the fluorescence signal was detected in a TECAN multi-plate reader at excitation 360 nm and emission 465 nm. The data was expressed as the mean relative fluorescence reading normalized to total protein concentration, measured in triplicates.

In Vitro DNA Damage Assay

DNA damage response was determined by quantification of the γ-H2AX (pS139) phosphorylation using flow cytometry (BD Biosciences). Briefly, following treatment, cells were washed with cold PBS followed by fixation and permeabilization at 4 °C for 2 hours. Cells were incubated with Alexa Fluor 647-conjugated anti-H2AX antibody for 30min at 4 °C in the dark. The percent of cells stained positive for γ-H2AX (pS139) was determined from 10,000 events per sample, in triplicate.
In Vitro Assay to Assess Reversibility of Treatment Effects

To assess reversibility, hBMNCs were treated with test articles at specified concentrations as single agents for 5 days. At the end of treatment, cell viability was assessed in one plate using the Cell Titer Glo® kit (Promega), while cells in a second parallel plate were transferred to V-bottom plate and centrifuged into a pellet to allow for media removal. Fresh conditioned HPGM media was added to the wells, and the cells were re-suspended and cultured for an additional 4 days to assess reversibility of the treatment effect.

To assess reversibility following palbociclib and fulvestrant combinational treatment, hBMNCs or MCF-7 cells were treated with palbociclib at up to 1 µM with a 1 to 3-fold serial dilution, in the presence or absence of fulvestrant (300 nM in hBMNCs, or 6 nM in the MCF-7 cells) for 7 consecutive days. Cell viability was assessed in the first plate using the Cell Titer Glo® kit (Promega), while media was changed to fresh media (test article free) in the second plate or media containing fulvestrant in the third plate. Following an additional five days of culture, relative cell viability was measured via ATP content as an indication of cell regrowth from the second and third plate using the Cell Titer Glo® kit.

Data Analysis

Data values are expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s multiple comparisons test.
RESULTS

Selection of test article concentrations used in this work was based on plasma exposure for each agent at the corresponding therapeutic dose as reported in the literature, or internal data (palbociclib) (Supplementary Table S1). The concentrations used for each test agent in the in vitro assays and the exposure achieved for palbociclib in the in vivo dog study were within the therapeutic range calculated using clinical total AUC.

Hematological toxicity assessment in dogs following 9-month treatment with palbociclib

There were palbociclib-related decreases in all lineages (leukocytes, erythrocytes, thrombocytes), with the greatest effect observed in neutrophils. Absolute neutrophil counts (ANC) were decreased dose-dependently for samples following each cycle, down to 0.61x and 0.29x of pretest values for animals administered 0.6 and 3.0 mg/kg/day palbociclib, respectively (Figure 1). Samples from the end of the one-week treatment-free period during cycles 1, 4, and 7 (days 29, 113, and 197) of the dosing phase indicated the ANC had partially returned to pre-test levels. The ANC nadir appeared to plateau by the 4th and 7th cycle in animals administered 0.6 and 3.0 mg/kg/day palbociclib, respectively. ANC completely returned to pre-test levels by day 50 of the recovery period at both doses (Figure 1).

The bone marrow was examined microscopically from terminal tissue samples on day 274 (at the end of cycle 10) of the dosing phase and day 85 of the recovery phase. On day 274, slight to moderate decreased cellularity of the bone marrow was present in 3/8 animals administered 3.0
mg/kg/day palbociclib and involved all hematopoietic cell lineages. Compared to vehicle control, there was no morphologic evidence of increased hematopoietic cell death (Supplementary Figure S1). The bone marrow was normal for all dogs on recovery day 85.

**Evaluation of lineage-specific effects following treatment with palbociclib in CD34+ hematopoietic stem cells**

The CD34+ hematopoietic stem cells were stimulated with various cytokines to allow them to differentiate into erythroid, myeloid, or megakaryocyte-specific lineages, or a mixture of all lineages. The effect of palbociclib in each cell lineage was assessed and compared to all-lineage-differentiated bone marrow hematopoietic cells. Given the similar shapes of the concentration-toxicity curves and the overlapping data points, individual lineages were similarly sensitive to the anti-proliferative effects of palbociclib (Supplementary Figure S2). Therefore, all-lineage-differentiated hBMNCs were used in subsequent experiments.

**Effects of palbociclib, paclitaxel and doxorubicin on hBMNCs and MCF-7 cells**

Treatment of hBMNCs with palbociclib caused concentration-dependent inhibition of DNA synthesis measured by EdU incorporation (Figure 2A). A 25-80% reduction in EdU incorporation was observed with palbociclib at 0.1 to 1 µM. Upon further cell-cycle analysis using propidium iodide DNA content staining, treatment of hBMNCs with palbociclib caused a concentration-dependent increase in G1 phase, and decreases in the S and G2/M phases, consistent with G1 cell cycle arrest (Supplementary Table S2). Palbociclib treatment at
concentrations up to 1 µM caused no induction of apoptosis, DNA-damage response, or cellular senescence, as measured by caspase 3/7 activity, γ-H2AX phosphorylation, or SA-β-galactosidase activity, respectively (Figure 2B, 2C, and 2D). While the cytotoxic chemotherapeutic agents, paclitaxel and doxorubicin, also caused concentration-dependent inhibition of cell proliferation (Figure 2A), they additionally caused significant concentration-dependent increases in caspase 3/7 activities (up to 2.5-, and 3.5-fold over that in the vehicle control, respectively), at concentrations comparable to clinical exposure (Figure 2B). Both agents also caused concentration-dependent induction of DNA-damage (Figure 2C), but minimal to no effect on cellular senescence as measured by SA-β-galactosidase activity (Figure 2D).

As a comparison to normal hBMNCs, the mechanism of palbociclib and cytotoxic chemotherapies in the breast cancer cell line (MCF-7) was evaluated using the same assay endpoints. Palbociclib and cytotoxic chemotherapy treatments in MCF-7 cells induced concentration-dependent inhibition of cell proliferation (Figure 3A) and minimal induction (paclitaxel only) of apoptosis as measured by caspase 3/7 activation (Figure 3B). However, in contrast to hBMNCs, palbociclib and cytotoxic chemotherapeutic treatments resulted in significant concentration-dependent induction of cellular senescence measured by SA-β-galactosidase activity (Figure 3C).

**Reversibility of bone marrow suppression following treatment with palbociclib or cytotoxic chemotherapeutic agents**
Reversibility of test article-induced toxicity in hBMNCs was assessed by removing the media containing test article and replacing it with fresh media for an additional 4 days. Cell growth post-media change was calculated by subtracting cell viability values at the end of the 5 day treatment period from that at the end of the 4 day recovery phase (Figure 4). The hBMNCs treated with up to 1 µM of palbociclib showed similar levels of cell growth post media change as the vehicle control, indicative of full reversibility of cell cycle arrest. By contrast, hBMNCs treated with paclitaxel and doxorubicin showed minimal recovery over the same period for the test concentrations indicated.

**Combination treatment of palbociclib and fulvestrant: reversibility of effects in hBMNCs or MCF-7 cells**

Palbociclib in combination with anti-estrogens is approved (in combination with letrozole) or being tested in phase 3 clinical trials (in combination with fulvestrant) for the treatment of breast cancer. Therefore, we investigated the effect of combining palbociclib and fulvestrant on hBMNCs, contrasted to MCF-7 breast cancer cells, and assessed reversibility following treatment withdrawal of palbociclib or both agents.

Combining palbociclib (up to 1µM) and fulvestrant (300 nM) did not alter palbociclib induced cell arrest of hBMNCs. Furthermore, following a 5-day treatment-free recovery period, hBMNCs regained their proliferation ability to a comparable level as the vehicle control whether fulvestrant (300 nM) was present or absent in the culture media during the recovery phase (Figure 5A). By contrast, inhibition of tumor cell growth was additive in MCF-7 cells following treatment with palbociclib and fulvestrant. When MCF-7 cells were treated with the combination of palbociclib and fulvestrant (6 nM), cells remained arrested with partial recovery
observed following the 5-day treatment-free recovery period (both palbociclib and fulvestrant were removed), while minimal to no recovery was observed when fulvestrant (6 nM) remained present during the recovery phase (Figure 5B).

**Effects on cellular senescence following treatment with palbociclib, fulvestrant, or both agents together, in hBMNCs or MCF-7 cells**

To further investigate the mechanism for the difference observed in the reversibility between hBMNCs and MCF-7, cellular senescence was evaluated in both cell types following treatment with palbociclib, fulvestrant or a combination of both agents. No significant increase in SA-β-galactosidase activity was observed following treatment of hBMNCs with palbociclib (100 - 300 nM) or fulvestrant (30 nM) alone, or in combination (Figure 6). By contrast, treatment of MCF-7 cells with palbociclib (100 - 300 nM) or fulvestrant (6 nM) caused significant increases in SA-β-galactosidase activity over vehicle control, with additive effects observed when combining the two agents (Figure 6).
DISCUSSION

Mechanism of bone marrow toxicity

Hematological toxicity is a common side effect of cytotoxic chemotherapeutic agents and is often dose-limiting, resulting in dose reduction, dose delay or discontinuation of treatment. It can be a result of direct damage or consumption of peripheral blood cells or decreased production of blood cells in the bone marrow, commonly referred to as bone marrow suppression or myelotoxicity (11). In vitro treatment of hPBMC with palbociclib showed no direct cytotoxicity up to 10 µM, supporting that the mechanism is through bone marrow suppression (Supplementary Figure S3).

The mechanism of bone marrow suppression resulting from chemotherapy treatment can be multi-faceted, including direct cytotoxicity to bone marrow cells, inhibition of bone marrow precursor or progenitor cell proliferation, or interference with hematopoietic growth factor and receptor signaling subsequently affecting the downstream differentiation processes (12). When tested in differentially stimulated hematopoietic stem cells, palbociclib demonstrated similar effects on erythroid, myeloid and megakaryocyte-specific lineages. This result suggests that the higher frequency of decreases in neutrophils seen in the clinic, as compared to other blood cell types, is not due to preferential inhibition of myeloid cell production in the bone marrow. Rather, the relatively low production rate, long maturation time, and short lifespan of neutrophils compared to the other cell types may explain the higher frequency of neutropenia observed following palbociclib treatment in the clinic (13).
Dose-dependent decrease in ANC was observed nonclinically with palbociclib following a three week on/one week off regimen, which can progress to neutropenia at supra-pharmacologic exposures. However at pharmacologic exposures, bone marrow can resume production sufficient to sustain adequate ANCs. In a chronic (9 months) dog study with palbociclib at 0.6 mg/kg/day and 3 mg/kg/day, dose-dependent decreases in ANC were observed at the end of each dosing period, which returned partially to pretreatment levels by the end of the one week treatment-free period for each cycle. The ANC nadir never fell below 1500 neutrophils/µL for any dose group, and did not decrease overtime, suggesting a plateauing of the nadir. Palbociclib treatment at 0.6 mg/kg/day or 3 mg/kg/day achieved unbound AUC$_{24}$ of 112 ng·h/mL or 954 ng·h/mL, approximately 0.4 or 3-fold clinical exposure at 125 mg dose QD, respectively. The preclinical findings in dogs were consistent with clinical observations, as demonstrated using a pharmacokinetic-pharmacodynamic (PK-PD) model, where ANC nadir was reached approximately 21 days after palbociclib treatment initiation in which the neutropenia rapidly reversed and was non-cumulative (14).

In addition to characterizing the bone marrow toxicity in vivo, the mechanism of bone marrow suppression associated with palbociclib treatment was investigated using an in vitro model consisting of hBMNCs with assay endpoints including cell viability, cell cycle progression, apoptosis, DNA damage, and cellular senescence. Palbociclib caused cell cycle arrest at G1/S phase, consistent with the intended pharmacology of CDK4/6 inhibition (9). In hBMNCs, palbociclib treatment did not induce apoptosis, DNA damage, or cellular senescence. By contrast, cytotoxic chemotherapeutic agents, including paclitaxel and doxorubicin, caused bone
marrow toxicity primarily through DNA damage and apoptotic pathways. Since hematopoietic stem cells are usually resistant to chemotherapy treatment, bone marrow suppression should be reversible upon treatment cessation. The timing and degree of reversibility is closely related to the mechanism of toxicity: temporary cell cycle arrest can be lifted rapidly, whereas permanent DNA damage or cell death of bone marrow precursor or progenitor cells may require a longer time interval to allow the bone marrow to resume adequate production. This is due to the reliance on replenishment of bone marrow from stem cells following cytotoxic chemotherapy treatment rather than the reversal of quiescence in partially differentiated cells following cell cycle arrest.

Using an in vitro system, we demonstrated that hBMNCs treated with up to 300 nM of palbociclib resumed cell proliferation to the same degree as vehicle control treated cells upon treatment withdrawal, consistent with the reversal of G1/S arrest. By contrast, cells treated with the cytotoxic chemotherapeutic agents at clinically relevant concentrations demonstrated minimal levels of reversibility upon treatment cessation under similar assay conditions. The in vitro results are consistent with the in vivo findings in dogs treated with palbociclib, as well as clinical observations: patients treated with palbociclib in the phase II clinical trial demonstrated quick recovery from neutropenia during the one week treatment free period (14) whereas the neutrophil levels in patients treated with cytotoxic chemotherapeutic agents usually take at least three to four weeks to return to pretreatment levels (10).

**Differences between normal bone marrow and tumor cells**

Palbociclib in combination with anti-estrogen agents is currently being used for treating advanced breast cancer. We therefore characterized the effects of palbociclib alone or in
combination with fulvestrant in the breast carcinoma cell line, MCF-7, and contrasted the results to those observed in normal bone marrow cells. In tumor cells, palbociclib or chemotherapeutic agents caused inhibition of cell proliferation and induced cellular senescence without apoptosis. This is in direct contrast to normal human bone marrow cells, where chemotherapeutic agents, but not palbociclib, induced significant apoptosis. The lack of effect on apoptosis in tumor cells is explained by the observation that MCF-7 cells have lost caspase-3 activity owing to a 47-base pair deletion of the CASP-3 gene (15).

Palbociclib induced cellular senescence in MCF-7 cells at clinically relevant concentrations. When combined with fulvestrant, the magnitude of senescence induction was greater than that achieved by either palbociclib or fulvestrant alone. This demonstrates a significant contrast to normal bone marrow cells, where neither palbociclib nor fulvestrant, as single agents or in combination, induced significant senescence. The difference in senescence induction between normal bone marrow and MCF-7 cells can be explained by the differential regulation of senescence pathway in those cell types. Normal human cells undergo a finite number of divisions before entering a state of irreversible growth arrest termed “replicative senescence”, which is triggered by erosion and dysfunction of telomeres (16, 17). Whereas most human somatic cell types have little to no detectable levels of telomerase activity, while hematopoietic stem and progenitor cells express low to moderate levels of telomerase which help them ameliorate replicative senescence (18). Therefore, palbociclib treatment in bone marrow hematopoietic cells induces temporary cell cycle arrest, but not permanent cellular senescence. By contrast, many tumor cells bypass replicative senescence through different mechanisms. The tumor suppressor gene p53 and the cyclin-dependent kinase (CDK) inhibitors p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} have been reported to play pivotal roles in orchestrating replicative and stress-induced senescence (19, 20).
By inhibiting cyclin-dependent kinases, p16 activates the G1-S checkpoint, and this response is often considered to be critical for establishing a senescence-like growth arrest. Several reports demonstrated that the majority (~85%) of human cancer cell lines, including MCF-7, do not express p16 due to deletion, mutation, or silencing of the INK4A locus (21). This may explain the resistance of the MCF-7 cell line toward the normal senescence process. However, treatment of MCF-7 cells with palbociclib caused inhibition of CDK4/6 activity and the downstream inhibition of the phosphorylated form of the retinoblastoma protein (pRb) as well as repression of FoxM1 (another substrate of CDK4/6 implicated in the regulation of senescence) (22), eventually leading to senescence. It was previously reported that palbociclib caused strong reduction in FOXM1 protein and triggered senescence in malignant melanoma cells, but not in normal melanocytes (22). Because the effects of anti-estrogen and CDK4/6 inhibition on breast cancer cell converge on the cyclin D1 pathway, an additive or synergistic effect on the induction of cellular senescence would be expected (9). In contrast, since the bone marrow hematopoietic cells are devoid of estrogen receptor alpha (Supplementary Figure S4), anti-estrogens have no appreciable effects on these cells.

The reversibility of palbociclib combined with fulvestrant effects in human bone marrow and MCF-7 cells were investigated. These in vitro reversibility studies were designed to replicate the clinical regimen where the anti-estrogen agent is administered continuously while palbociclib has a treatment free period at the end of each cycle (10). Human bone marrow cells resumed cell proliferation following removal of palbociclib in the presence or absence of fulvestrant. However, MCF-7 cancer cells exhibited partial reversibility in the absence of fulvestrant, and
minimal to no recovery when fulvestrant was present during the palbociclib-free phase. The results from the reversibility experiment can be explained by the mechanistic differences discussed previously, in that bone marrow cells only undergo temporary cell cycle arrest when treated with palbociclib and anti-estrogen combination, while breast cancer cells are susceptible to a non-reversible additive senescent effect from the same combined treatment. In addition, fulvestrant alone has an antiproliferative effect in MCF-7 cells due to the cell line’s dependence on the estrogen receptor, thus keeping the breast cancer cells suppressed during the palbociclib-free period.

**Implication for clinical safety management**

In summary, the data presented provides an understanding of the mechanism of bone marrow suppression and neutropenia associated with palbociclib treatment in the clinic. Results gathered from this work supports the differentiation between palbociclib-induced neutropenia and that caused by cytotoxic chemotherapeutic agents, both in mechanism and in the degree and timing of reversibility of the effects. Because of the lack of DNA damage response following palbociclib treatment in normal bone marrow proliferating cells, there may be a lower risk of secondary hematological cancers, which are a known risk of DNA-damaging chemotherapy (23). Furthermore, the mechanistic differences between palbociclib effects in normal human bone marrow versus MCF-7 cells support the current clinical dosing schedule in breast cancer patients by demonstrating that the short duration of the palbociclib-free period between cycles provides the bone marrow cells enough time to recover without impacting efficacy against tumor cells.
Abbreviations

ANC: absolute neutrophil count; ANOVA: analysis of variance; ATP: adenosine triphosphate; CDK: cyclin-dependent kinase; DMSO: dimethyl sulfoxide; Dox: doxorubicin; EdU: 5-ethynyl-2’-deoxyuridine; EPO: erythropoietin; FBS: fetal bovine serum; Fulv: fulvestrant; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; hBMNCs: human bone marrow mononuclear cells; HPGM: hematopoietic progenitor growth media; IL-3: interleukin 3; IL-6: interleukin 6; PBS: phosphate buffered saline; RPMI: Roswell Park Memorial Institute media; SA-β-Gal: senescence-associated β-galactosidase; SCF: stem cell factor; SD: standard deviation; TPO: thrombopoietin.

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REFERENCES


Figure Legends

Figure 1. Absolute neutrophil count from a 9-month dog study treated with palbociclib. Palbociclib was administered daily to 6 dogs per gender per group at 0.6 and 3 mg/kg/day for 10 cycles each consisting of 3 weeks of daily dosing and 1 week treatment free. Hematology samples were collected at predose, end of each dosing/treatment-free cycle for cycle 1, 4, 7, and 10, as well as week 7 and 12 of the recovery phase. Data represent mean values of ANC from 12 animals per group during treatment phase and 4 animals per group during recovery phase. Error bars represent standard error.

Figure 2. Evaluation of anti-proliferative, apoptotic, DNA damage response, and cellular senescence effects of palbociclib and cytotoxic chemotherapeutic agents in human bone marrow mononuclear cells. hBMNCs were treated with palbociclib, doxorubicin, and paclitaxel at specified concentrations. The effects on A) cell proliferation, B) apoptosis, C) DNA damage response, and D) cellular senescence were measured after five days of test article exposure. Data represent mean values of EdU incorporation, caspase 3/7 activity, γ-H2AX expression, or SA-β-Galactosidase activity, normalized to vehicle control. Error bars represent standard deviation of triplicate measurements. * p<0.05, ** p<0.01, one way ANOVA with Dunnett’s test.

Figure 3. Evaluation of anti-proliferative, apoptotic and cellular senescence effects of palbociclib and cytotoxic chemotherapeutic agents in MCF-7 cells. MCF-7 was treated with palbociclib, doxorubicin, or paclitaxel at specified concentrations. The effects on A) cell proliferation, B) apoptosis, and C) cellular senescence were measured after 7 days of compound exposure. Data
represent mean values calculated as % of vehicle control for EdU incorporation, caspase 3/7 activity, or SA-β-Galactosidase activity. Error bars represent standard deviation of triplicate measurements. * p < 0.05, ** p < 0.01, one way ANOVA with Dunnett’s test.

Figure 4. Assessment of the reversibility of effects of palbociclib and cytotoxic chemotherapeutic agents in human bone marrow mononuclear cells. HBMNCs were treated with palbociclib, doxorubicin, and paclitaxel. Cell viability was measured at the end of dosing on day 5 and at the end of recovery on day 9. Data represents mean luminescence difference between day 9 and day 5, calculated as % of vehicle control. Error bars represent standard deviation of triplicate measurements.

Figure 5. Assessment of the reversibility of effects of palbociclib in combination with fulvestrant in hBMNCx and MCF-7 cells. Palbociclib was tested at specified concentrations in combination with 300nM, or 6nM of fulvestrant in A) hBMNCs or B) MCF-7 cells, respectively, for 7 consecutive days. Following 7 days of compound treatment, cell viability was assessed via ATP content in one plate, while media was changed to test article-free media or media containing fulvestrant in the other plates. Following an additional five days of culture, relative cell viability was measured from the recovery plates. Data represents the difference in mean luminescence between the end of recovery phase and the end of dosing phase. Error bars represent standard deviation of triplicate measurements.
Figure 6. Evaluation of cellular senescence effects of palbociclib, fulvestrant alone or in combination in hBMNCs and MCF-7 cells. A) hBMNCs and B) MCF-7 were treated with palbociclib at 100nM or 300nM, fulvestrant at 30nM, or the combination of the two agents for 7 consecutive days. Cellular senescence was measured using the cellular senescence assay, in which the SA-β-galactosidase activity was normalized to total protein concentration. Data represent mean value calculated as fold of vehicle control. Error bars represent standard deviation of triplicate measurements. ** p<0.01, one way ANOVA with Dunnett’s test.
Neutrophil Count ($\times 10^3$)

Baseline 22 29 106 113 190 197 Cycle 10 7 Weeks Recovery 274 50 85

Cycle 1 4 7

On-treatment

Off-treatment

0.6 mg/kg/day

3 mg/kg/day

Figure 1
Figure 2

A. Cell Proliferation (% Control) for different treatments: Vehicle, Palbociclib (0.1, 0.3, 1.0), Doxorubicin, Paclitaxel. Palbociclib shows increased proliferation compared to other treatments.

B. Apoptosis (% Control) for different treatments: Vehicle, Palbociclib (0.1, 0.3, 1.0), Doxorubicin, Paclitaxel. Doxorubicin shows the highest apoptosis, followed by Paclitaxel and Palbociclib.

C. γ-H2AX Formation (% Control) for different treatments: Vehicle, Palbociclib (0.03, 0.1, 0.3), Doxorubicin, Paclitaxel. Paclitaxel shows the highest γ-H2AX formation compared to other treatments.

D. Cellular Senescence (% Control) for different treatments: Vehicle, Palbociclib (0.003, 0.01, 0.03), Doxorubicin, Paclitaxel. Doxorubicin shows the highest cellular senescence, followed by Paclitaxel and Palbociclib.

*p<0.05, **p<0.01, ANOVA
Figure 4

Cell Growth Post-treatment (% Control)

Concentration (μM)

- Palbociclib
- Doxorubicin
- Paclitaxel
Figure 5

A. Human Bone Marrow Cells

Cell Growth Post Media Change (% Vehicle)

Recovery with Blank Media  
Recovery with Fulvestrant

Vehicle  FUL 300nM  0.1  0.3  1.0

Palbociclib (μM)

B. MCF7 Cells

Cell Growth Post Media Change (% Vehicle)

Vehicle  FUL 6nM  0.1  0.3  1.0

Palbociclib (μM)

*  **  ***  ****  *****
Figure 6

The figure shows the normalized β-Gal staining (fold-change over vehicle control) for Bone Marrow Cells and MCF7 Cells under different treatments.

- **Vehicle**: No significant change.
- **Palbociclib 100nM**: Mild increase.
- **Palbociclib 300nM**: Significant increase.
- **Fulvestrant 30nM**: More significant increase.
- **Palbociclib 100nM + Fulvestrant**: Even more significant increase.
- **Palbociclib 300nM + Fulvestrant**: Most significant increase.

*Significance levels marked with ** indicate p < 0.01.*
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