

# Mechanistic Investigation of Bone Marrow Suppression Associated with Palbociclib and its Differentiation from Cytotoxic Chemotherapies

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## Abstract

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

**Experimental Design:** Reversible hematologic effects and bone marrow hypocellularity have been identified in toxicology studies in rats and dogs after palbociclib treatment. To understand the mechanism by which the hematologic toxicity occurs, and to further differentiate it from the myelotoxicity caused by cytotoxic chemotherapeutic agents, an *in vitro* assay using human bone marrow mononuclear cells (hBMNC) 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. In 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 antiestrogen, 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 antiestrogen treatment with additive effects in combination and remained arrested in the presence of antiestrogen.

**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. *Clin Cancer Res*; 22(8); 2000–8. ©2015 AACR.

## 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, whereas 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 sev-

eral 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), gastrointestinal 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 G<sub>1</sub>–S cell-cycle transition and avoids other CDK targets that may induce apoptosis in quiescent cells (8). Palbociclib was recently approved by the 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 (3 weeks on, 1 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 antiestrogens (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<sup>+</sup>/Her2<sup>−</sup> breast cancer, hematologic 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,

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

Palbociclib is a highly effective cyclin-dependent kinase 4/6 inhibitor approved for metastatic breast cancer. Although 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 with 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.

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 antiestrogens (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.

### *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 per 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. After 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, embedded in paraffin, sectioned to slides, stained with hematoxylin and eosin and examined by a board-certified veterinary pathologist.

### *In vitro* cell culture

Human bone marrow mononuclear cells (hBMNC: a heterogeneous population that includes hematopoietic lineage cells such as lymphocytes, monocytes, stem cells, and progenitor cells) and CD34<sup>+</sup> human bone marrow hematopoietic stem cells were primary cells purchased from Lonza. The cells were cultured in the hematopoietic progenitor growth (HPGM) medium (Lonza) supplemented with 10% FBS and in the presence of the following cytokines (R&D Systems): 25 ng/mL stem cell factor (SCF), 3 U/mL erythropoietin (EPO), 10 ng/mL G-CSF, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 15 ng/mL thrombopoietin (TPO), 10 ng/mL IL3, 10 ng/mL IL6, and 25 ng/mL Flt3 ligand, in a 37°C 5% CO<sub>2</sub> and 98% humidity incubator. Human peripheral blood mononuclear cells (hPBMC) 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 ATCC and cultured in RPMI media supplemented with 10% FBS, in a 37°C 5% CO<sub>2</sub> and 98% humidity incubator.

### *In vitro* testing of lineage-specific effects

The CD34<sup>+</sup> hematopoietic stem cells were stimulated with the following cytokines for 4 days to induce lineage-specific differentiation: SCF, EPO, and IL3 for erythroid lineage; SCF, G-CSF, GM-CSF, IL3, and Flt3 ligand for myeloid lineage; SCF, TPO, and IL3 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 (hPBMC) or 5 days (hBMNC) of continuous exposure, cell viability was measured using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega). 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). 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) according to the manufacturer's protocol. Briefly, cells were pulse treated with 10 μmol/L 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, and 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). In the quantitative assay, cells were preincubated with the senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -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 1 $\times$  cell lysis buffer, followed by centrifugation at 14,000 rpm for 10 minutes at 4°C. An aliquot of 50  $\mu$ L 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 multiplate reader at excitation 360 nm and emission 465 nm. The data were 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  $\gamma$ -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 30 minutes at 4°C in the dark. The percent of cells stained positive for  $\gamma$ -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 were added to the wells, and the cells were resuspended 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  $\mu$ mol/L with a 1- to 3-fold serial dilution, in the presence or absence of fulvestrant (300 nmol/L in hBMNCs or 6 nmol/L 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 were changed to fresh media (test article free) in the second plate or media containing fulvestrant in the third plate. After an additional 5 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.

#### **Statistical analysis**

Data values are expressed as mean  $\pm$  SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett 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.

#### **Hematologic toxicity assessment in dogs after 9-month treatment with palbociclib**

There were palbociclib-related decreases in all lineages (leukocytes, erythrocytes, and thrombocytes), with the greatest effect observed in neutrophils. Absolute neutrophil counts (ANC) were decreased dose dependently for samples following each cycle, down to 0.61 $\times$  and 0.29 $\times$  of pretest values for animals administered 0.6 and 3.0 mg/kg/day palbociclib, respectively (Fig. 1). Samples from the end of the 1-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 pretest levels. The ANC nadir appeared to plateau by the fourth and seventh cycle in animals administered 0.6 and 3.0 mg/kg/day palbociclib, respectively. ANC completely returned to pretest levels by day 50 of the recovery period at both doses (Fig. 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 of 8 animals administered 3.0 mg/kg/day palbociclib and involved all hematopoietic cell lineages. Compared with vehicle control, there was no morphologic evidence of increased hematopoietic cell death (Supplementary Fig. S1). The bone marrow was normal for all dogs on recovery day 85.

#### **Evaluation of lineage-specific effects after treatment with palbociclib in CD34<sup>+</sup> hematopoietic stem cells**

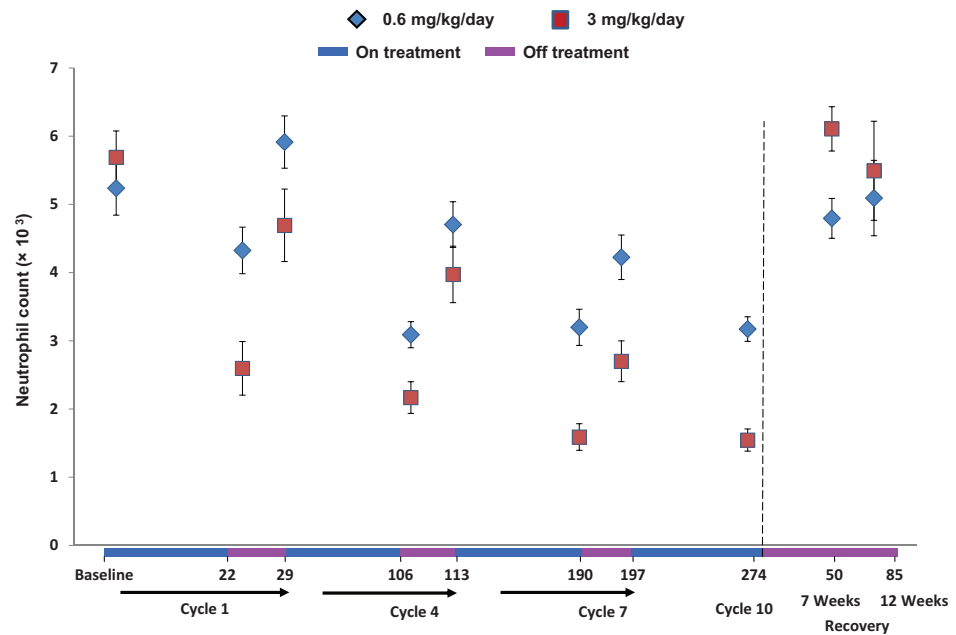
The CD34<sup>+</sup> hematopoietic stem cells were stimulated with various cytokines to allow them to differentiate into erythroid, myeloid, or megakaryocyte-specific lineages, or a mixture of all lineages. The effect of palbociclib in each cell lineage was assessed and compared with all-lineage-differentiated 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 antiproliferative effects of palbociclib (Supplementary Fig. 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 (Fig. 2A). A 25% to 80% reduction in EdU incorporation was observed with palbociclib at 0.1 to 1  $\mu$ mol/L. Upon further cell-cycle analysis using PI DNA content staining, treatment of hBMNCs with palbociclib caused a concentration-dependent increase in G<sub>1</sub> phase, and decreases in the S and G<sub>2</sub>-M phases, consistent with G<sub>1</sub> cell-cycle arrest (Supplementary Table S2). Palbociclib treatment at concentrations up to 1  $\mu$ mol/L caused no induction of apoptosis, DNA damage response, or cellular senescence, as measured by caspase-3/7 activity,  $\gamma$ -H2AX

**Figure 1.**

ANC 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 SE.



phosphorylation, or SA- $\beta$ -Gal activity, respectively (Fig. 2B–D). While the cytotoxic chemotherapeutic agents, paclitaxel and doxorubicin, also caused concentration-dependent inhibition of cell proliferation (Fig. 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 with clinical exposure (Fig. 2B). Both agents also caused concentration-dependent induction of DNA damage (Fig. 2C), but minimal to no effect on cellular senescence as measured by SA- $\beta$ -Gal activity (Fig. 2D).

As a comparison with 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 (Fig. 3A) and minimal induction (paclitaxel only) of apoptosis as measured by caspase-3/7 activation (Fig. 3B). However, in contrast to hBMNCs, palbociclib and cytotoxic chemotherapeutic treatments resulted in significant concentration-dependent induction of cellular senescence measured by SA- $\beta$ -Gal activity (Fig. 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 after 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 (Fig. 4). The hBMNCs treated with up to 1  $\mu$ mol/L of palbociclib showed similar levels of cell growth after media change as the vehicle control, indicative of full reversibility of cell-cycle arrest. In 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

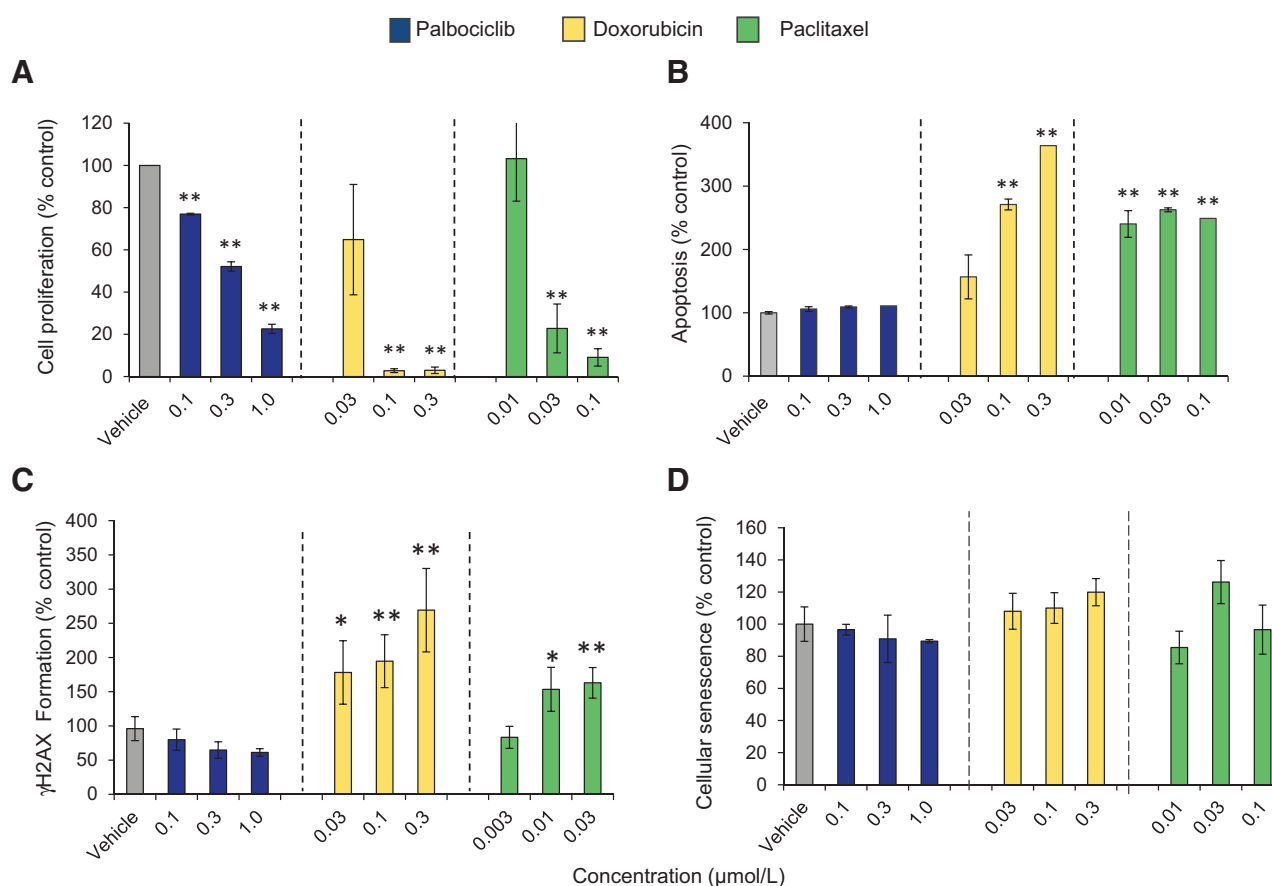
Palbociclib in combination with antiestrogens is approved (in combination with letrozole) or being tested in phase III clinical trials (in combination with fulvestrant) for the treatment of breast cancer. Therefore, we investigated the effect of combining palbociclib and fulvestrant on hBMNCs, contrasted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

Combining palbociclib (up to 1  $\mu$ mol/L) and fulvestrant (300 nmol/L) did not alter palbociclib-induced cell arrest of hBMNCs. Furthermore, after a 5-day treatment-free recovery period, hBMNCs regained their proliferation ability to a comparable level as the vehicle control whether fulvestrant (300 nmol/L) was present or absent in the culture media during the recovery phase (Fig. 5A). In 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 nmol/L), 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 nmol/L) remained present during the recovery phase (Fig. 5B).

#### Effects on cellular senescence after 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- $\beta$ -Gal activity was observed following treatment of hBMNCs with palbociclib (100–300 nmol/L) or fulvestrant (30 nmol/L) alone, or in combination (Fig. 6). In contrast, treatment of MCF-7 cells with palbociclib (100–300 nmol/L) or fulvestrant (6 nmol/L) caused significant





**Figure 2.** Evaluation of antiproliferative, apoptotic, DNA damage response, and cellular senescence effects of palbociclib and cytotoxic chemotherapeutic agents in hBMNCs. hBMNCs were treated with palbociclib, doxorubicin, and paclitaxel at specified concentrations. The effects on cell proliferation (A), apoptosis (B), DNA damage response (C), and cellular senescence (D) were measured after 5 days of test article exposure. Data represent mean values of EdU incorporation, caspase-3/7 activity,  $\gamma$ -H2AX expression, or SA- $\beta$ -Gal activity, normalized to vehicle control. Error bars represent SD of triplicate measurements. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , one-way ANOVA with Dunnett test.

increases in SA- $\beta$ -Gal activity over vehicle control, with additive effects observed when combining the two agents (Fig. 6).

## Discussion

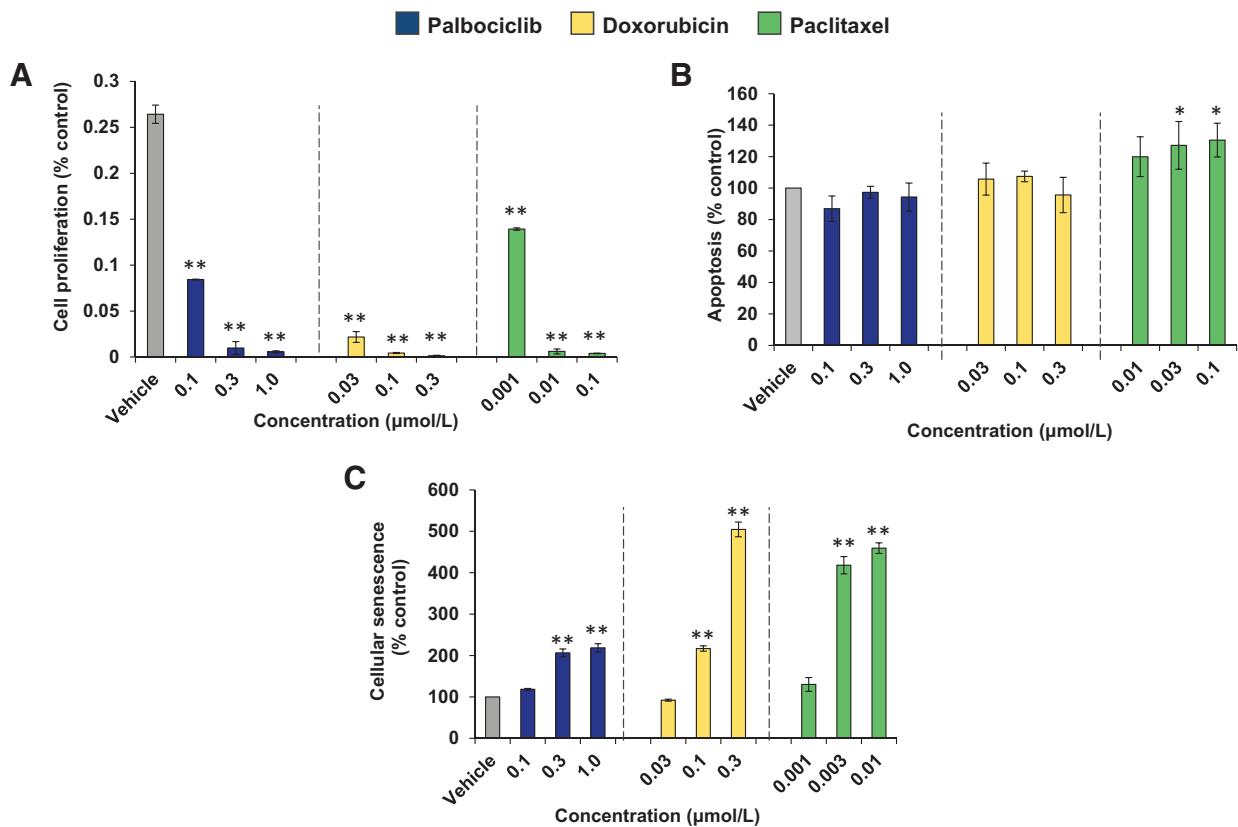
### Mechanism of bone marrow toxicity

Hematologic 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  $\mu$ mol/L supporting that the mechanism is through bone marrow suppression (Supplementary Fig. S3).

The mechanism of bone marrow suppression resulting from chemotherapy treatment can be multifaceted, 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 with 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 with 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 3-week on/1-week off regimen, which can progress to neutropenia at suprapharmacologic 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 1-week treatment-free period for each cycle. The ANC nadir never fell below 1,500 neutrophils/ $\mu$ L for any dose group, and did not decrease overtime, suggesting a plateauing of the nadir. Palbociclib

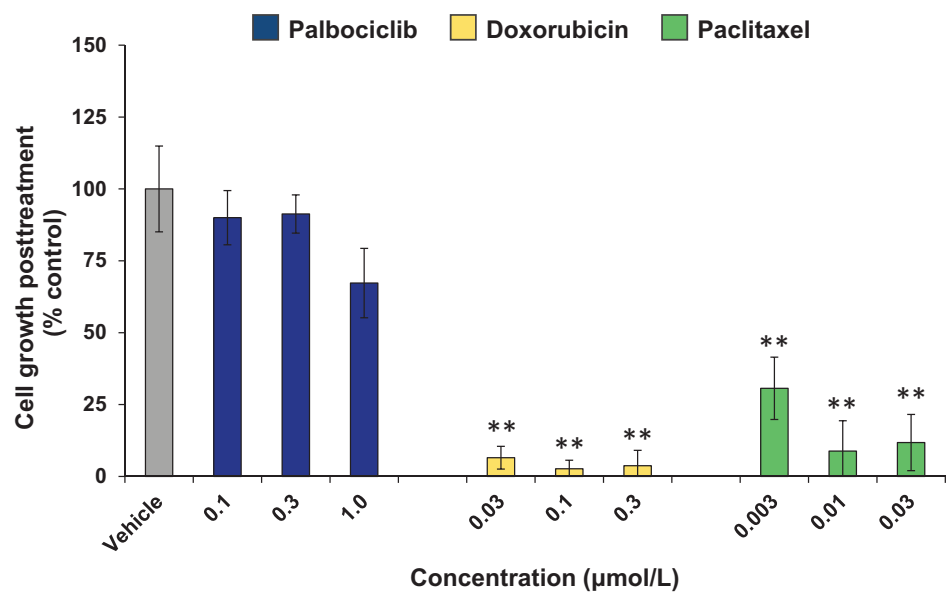


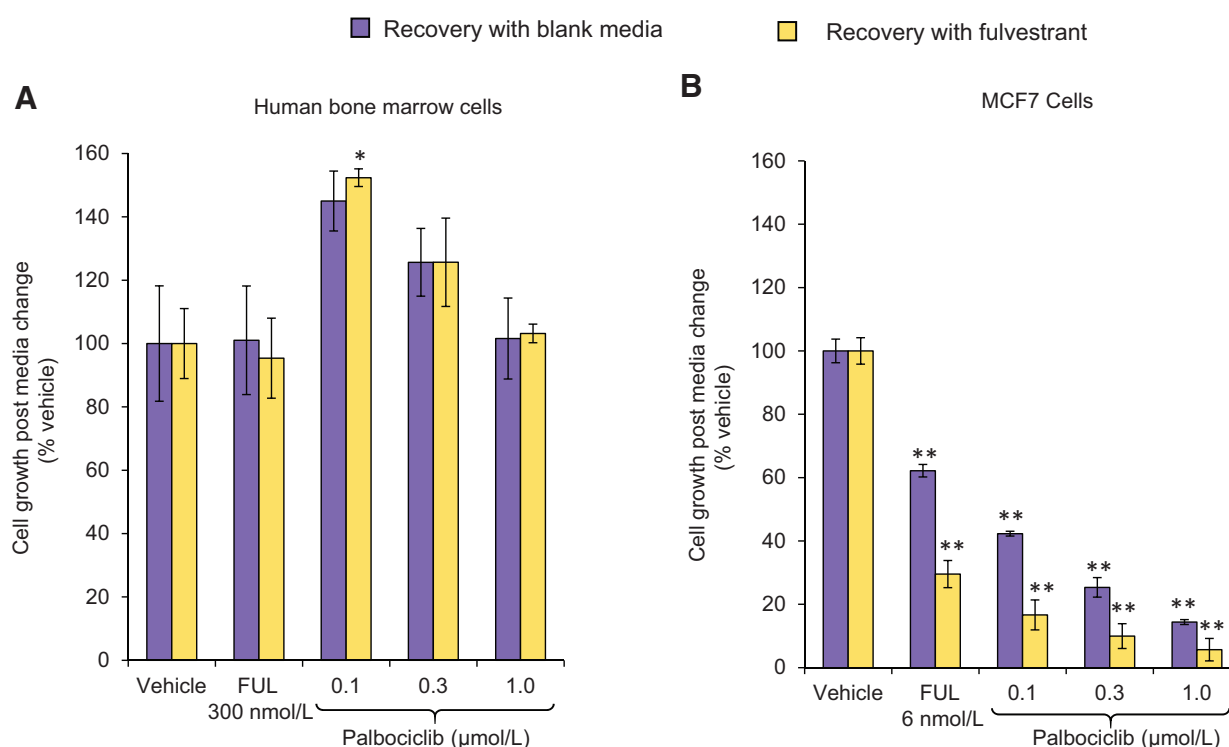
**Figure 3.** Evaluation of antiproliferative, apoptotic, and cellular senescence effects of palbociclib and cytotoxic chemotherapeutic agents in MCF-7 cells. MCF-7 was treated with palbociclib, doxorubicin, or paclitaxel at specified concentrations. The effects on cell proliferation (A), apoptosis (B), and cellular senescence (C) were measured after 7 days of compound exposure. Data represent mean values calculated as the percentage of vehicle control for EdU incorporation, caspase-3/7 activity, or SA-β-Gal activity. Error bars represent SD of triplicate measurements. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , one-way ANOVA with Dunnett test.

treatment at 0.6 mg/kg/day or 3 mg/kg/day achieved unbound AUC<sub>24</sub> of 112 ng/h/mL or 954 ng/h/mL, approximately 0.4- or 3-fold clinical exposure at 125 mg dose every day, respectively. The

preclinical findings in dogs were consistent with clinical observations, as demonstrated using a pharmacokinetic-pharmacodynamic model, where ANC nadir was reached approximately

**Figure 4.** Assessment of the reversibility of effects of palbociclib and cytotoxic chemotherapeutic agents in hBMNCs. 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 represent mean luminescence difference between day 9 and day 5, calculated as the percentage of vehicle control. Error bars represent SD of triplicate measurements.



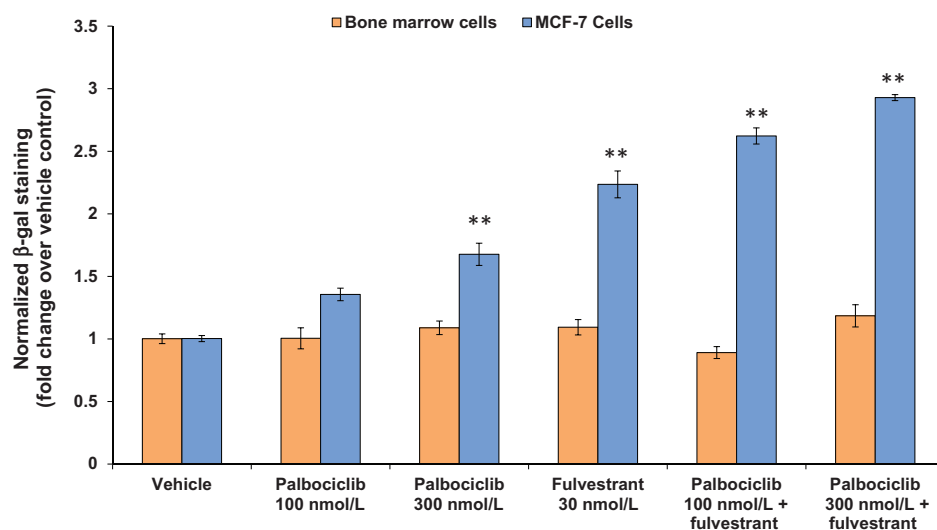


**Figure 5.** Assessment of the reversibility of effects of palbociclib in combination with fulvestrant in hBMNCs and MCF-7 cells. Palbociclib was tested at specified concentrations in combination with 300 nmol/L or 6 nmol/L of fulvestrant (FUL) in hBMNCs (A) or MCF-7 cells (B), respectively, for 7 consecutive days. After 7 days of compound treatment, cell viability was assessed via ATP content in one plate, while media were changed to test article-free media or media containing fulvestrant in the other plates. After an additional five days of culture, relative cell viability was measured from the recovery plates. Data represent the difference in mean luminescence between the end of recovery phase and the end of dosing phase. Error bars represent SD of triplicate measurements.

21 days after palbociclib treatment initiation in which the neutropenia rapidly reversed and was noncumulative (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 G<sub>1</sub>-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. In contrast, cytotoxic chemotherapeutic agents, including paclitaxel and doxorubicin, caused bone marrow toxicity primarily through DNA damage and apoptotic pathways. As hematopoietic stem cells are usually resistant to chemotherapy treatment, bone marrow suppression should be reversible upon treatment cessation. The timing and



**Figure 6.** Evaluation of cellular senescence effects of palbociclib, fulvestrant alone or in combination in hBMNCs and MCF-7 cells. hBMNCs and MCF-7 were treated with palbociclib at 100 nmol/L or 300 nmol/L, fulvestrant at 30 nmol/L, 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-Gal activity was normalized to total protein concentration. Data represent mean value calculated as fold of vehicle control. Error bars represent SD of triplicate measurements. \*\*, *P* < 0.01, one-way ANOVA with Dunnett test.

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 nmol/L of palbociclib resumed cell proliferation to the same degree as vehicle control-treated cells upon treatment withdrawal, consistent with the reversal of G<sub>1</sub>-S arrest. In 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 3 to 4 weeks to return to pretreatment levels (10).

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

Palbociclib in combination with antiestrogen 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). While most human somatic cell types have little to no detectable levels of telomerase activity, 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. In contrast, many tumor cells bypass replicative senescence through different mechanisms. The tumor suppressor gene *p53* and the CDK inhibitors p16<sup>INK4A</sup> and p21<sup>WAF1</sup>

have been reported to play pivotal roles in orchestrating replicative and stress-induced senescence (19, 20). By inhibiting CDKs, p16 activates the G<sub>1</sub>-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; ref. 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 antiestrogen 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, as the bone marrow hematopoietic cells are devoid of ER $\alpha$  (Supplementary Fig. S4), antiestrogens 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 antiestrogen 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 antiestrogen combination, while breast cancer cells are susceptible to a nonreversible 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 provide 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 hematologic 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.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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