Combination Therapy of CDK4/CDK6 Inhibitor Palbociclib and Irradiation in non-Malign and Malign Cells

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Research article

Keywords: palbociclib, Ibrance, kinase inhibitor, ionizing radiation, CDK4/CDK6, cell survival, cell cycle, migration, radio-resistance, radio-sensitivity

Posted Date: May 27th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-29326/v1>

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Abstract

Background: The CDK 4/6 kinase inhibitor palbociclib is approved for first line treatment of metastatic breast cancer in combination with hormonal therapy and investigated within clinical trials for melanoma. In metastatic disease frequently palliative radiation treatment is necessary. Due to recent findings of radiosensitizing effects of palbociclib in HNSCC and HCC, a possible influence of palbociclib on radiosensitivity was studied in malign and non-malign cells.

Methods: Different tumor cells and primary fibroblasts were treated with palbociclib and ionizing radiation (IR) in 2D and 3D cell cultures. Clonogenic assays were performed to study the sensitizing effect of palbociclib on irradiation induced cytotoxicity. Apoptosis, necrosis and cell cycle distribution was analyzed by flow cytometry. Cell migration was studied with scratch assays.

Results: The effect of irradiation on skin cancer cells could be slightly decreased by palbociclib in four out of eight cells comparing 2 Gy vs. 2 Gy + 1 $\mu\text{mol/L}$ in the 2D cell culture model. The 3D cell culture model detected differences in cell death more sensitive than the 2D model. Necrosis was increased in primary fibroblasts (0.2 $\mu\text{mol/L}$ + IR vs. IR; $p = 0.0041$), whereas apoptosis was decreased in skin cancer cells ICNI (0.2 $\mu\text{mol/L}$ + IR vs. IR with $p = 0.0005$ // 2 $\mu\text{mol/L}$ + IR vs. IR with $p = 0.0072$). Overall palbociclib attenuated radiation induced cell death in skin and breast cancer cells. In malign cells combination therapy seems to rise senescence in clonogenic assays, whereas healthy controls showed more cell death. Palbociclib did not influence cell cycle distribution in the fibroblasts but led to an accumulation of the tumor cells in G0/G1 cell cycle phase. Additionally, palbociclib decreased migration behavior of the malign and non-malign cells, but without influence on e-cadherin expression during inhibitor treatment.

Conclusions: Palbociclib increases the radiosensitivity of primary fibroblasts. In cancer cells palbociclib seems to be radioprotective. This effect in cancer cells is probably based on the accumulation of cells in the less radiosensitive G0/G1 cell cycle phase. Further studies are needed to assess whether a combined therapy of palbociclib and radiation is appropriate.

1. Introduction / Background

Targeted therapy has replaced standard chemotherapy in several of different tumor entities. This latest available therapy uses specific cancer characteristics (e.g. mutations) to block or inhibit them and deprive the tumor of its survival advantage. Genes or proteins, ensuring the malign cells to proliferate and survive, can harbor these mutations and therefore hold as a permanent proliferation signal, a mechanism to block apoptosis or supply other survival advantages declared as “hallmarks of cancer” by Hanahan and Weinberg 2000. Nowadays, there are 37 FDA-approved kinase inhibitors (KI) available and over 250 inhibitors are currently studied in clinical trials (Wu *et al.* 2015). Even though cancer patients respond quite well to kinase inhibitors initially, disease progression and formation of metastases occur too often.

Kinase inhibitors can influence effectiveness of irradiation by increasing or decreasing cellular responses. Central nervous system (CNS), spinal and other symptomatic metastases require radiation therapy. However, kinase inhibitors like e.g. the V600E-mutation-specific BRAf-inhibitor vemurafenib can act as radiosensitizer and cause serious side effects when using the kinase inhibitor simultaneously with irradiation, whereas dabrafenib causes radiosensitization to a much smaller degree (Hecht *et al.* 2015; Hecht, Meier *et al.* 2018). Recent data for combination of stereotactic body irradiation and BRAf-inhibitor treatment shows that it is advisable to pause inhibitor treatment during radiotherapy (Kroeze *et al.* 2019; Hermann, Christiansen 2019). Therefore, the need arises to study interactions between kinase inhibitors and irradiation to answer the question if a simultaneous treatment is advisable.

From 2015 to 2017 around 607,960 new breast cancer cases were diagnosed (American Cancer Society, Inc., Surveillance Research) and 13 % of them were treated with palbociclib (Kish *et al.* 2018). Approximately 30 % of all early-stage breast cancer patients developed advanced or metastatic disease, which often leads to a radiation therapy (O'Shaughnessy 2005). Since 2015, palbociclib is approved by the FDA as a treatment option for ER⁺, HER2⁻ advanced or metastatic breast cancer. It binds potently and highly selectively to the cell cycle regulation proteins Cyclin-dependent kinase 4 (CDK4) and Cyclin-dependent kinase 6 (CDK6). Therefore, the phosphorylation of the Retinoblastoma protein Rb is blocked and the cell cycle arrested. The tumor cells are forced in a G1-arrest (Saab *et al.* 2006). Cells rely on proteins like CDK4 to drive the cell cycle. It was tested in various clinical trials for advanced and metastatic melanoma. Since the activity of CDK4 is increased in melanomas mainly due to loss of functional p16^{INK4A} as a result of gene deletion, promoter methylation or genetic mutation (The Cancer Genome Atlas Network. Cell 2015), we have focused on working on melanoma cells in addition to breast cancer cells (Martin *et al.* 2018). Former studies showed that palbociclib increases radiosensitivity in HPV-negative head and neck squamous cell carcinoma (HNSCC) (Göttgens *et al.* 2019) and hepatocellular carcinoma (HCC) (Huang *et al.* 2018). Several findings suggest that palbociclib delay the DNA repair via ATM and p53 pathways, which impede handling with radiation (Hashizume *et al.* 2016; Fernández-Aroca *et al.* 2019). The clinical relevance of addressing the question if palbociclib influences the effect of irradiation at cellular level rises when patients under KI therapy progress or metastases and the need for an additional radiation therapy occurs. Thus, it is necessary to study whether the kinase inhibitor modifies the irradiation effect. We used different tumor and normal tissue cells and commercial cell lines to study a possible influence of a combination of palbociclib therapy with irradiation.

2. Material And Methods:

2.1 Cell culture and inhibitor

Human skin fibroblasts SBLF7 and SBLF9 derived from different healthy donors, melanoma cells ICNI, RERO and ANST derived from different patients, as well as breast cancer cell lines MDA-MB-231 (TNBC) and MCF-7 (ER⁺, PR⁺) were used. MDA-MB-231 and MCF-7 were purchased by CLS cell lines service (Eppelheim, Germany). Primary human melanoma cells (from primary tumors) were collected in the

Department of Dermatology of the University Hospital of Erlangen following approval by the institutional review board. Single cell suspensions were generated by digesting tissue samples with collagenase (Sigma Aldrich, München, Germany), hyaluronidase (Sigma Aldrich, München, Germany), and DNase (Roche, Mannheim, Germany) (Walter & Heinzerling 2018) (Ethic approval no. 204 17 BC). The primary human fibroblasts SBLF7 and SBLF9 were isolated via skin biopsy of the cutis and subcutis after local anesthesia as described previously (Hecht, Harrer *et al.* 2018). Briefly, each biopsy was dissected in small pieces, placed in tissue culture flasks and each covered with a drop of F-12 medium (Gibco, Waltham, USA) supplemented with 40 % fetal bovine serum (FBS) (Merck, Darmstadt, Germany). After the skin pieces had attached to the culture flasks and the first fibroblasts had grown out, they were covered with F-12 medium supplemented with 12 % FBS, 2 % non-essential amino acids (NEA) (Merck, Darmstadt, Germany) and 1 % penicillin/streptomycin (Gibco, Waltham, USA). When the primary cells were approximately 80 % confluent they were detached with 0.5 % Trypsin (Gibco, Waltham, USA) and further cultured in the medium mentioned above. For continuous cell culture, fibroblasts were cultured in F-12 medium, supplemented with 15 % FBS, 2 % NEA and 1 % penicillin/streptomycin. Melanoma cells were cultured in RPMI-1640 (Sigma Aldrich, München, Germany), supplemented with 20 % FBS (Merck, Darmstadt, Germany), 1 % NEA (Merck, Darmstadt, Germany), 1 % Pyruvate-solution (Gibco, Waltham, USA), 1 % L-Glutamine (Merck, Darmstadt, Germany), 1 % HEPES (Merck, Darmstadt, Germany) and 0.05 % Gentamicin (Merck, Darmstadt, Germany). Breast cancer cell lines were cultured in DMEM (PAN biotech, Aidenbach, Germany), supplemented with 10 % FBS and 1 % penicillin/streptomycin. All cells were incubated at 37 °C in a humidified 5 % CO₂ atmosphere. Cells were cultured 50 passages maximum. Palbociclib isethionate (MW 573.7 g/mol) (Selleck Chemicals LLC, Huston, USA) was prepared as stock solution in aqua bidest and stored at – 80 °C with a concentration of 1 mmol/L. The drug was diluted for experiments in dimethyl sulfoxide (DMSO) (Roth, Karlsruhe, Germany). Required aliquots were freshly thawed prior to each experiment.

2.2 FACS

2.2.1 Apoptosis and Necrosis

A single cell suspension was prepared by detaching the cells from cell culture flask by washing with PBS (Sigma Aldrich, St. Louis, USA) and incubation with Trypsin/EDTA (Gibco, Waltham, USA). Cells were seeded in an appropriate concentration to reach a confluence of 50 % up to 80 % in 24 h up to 72 h. To reduce analytical interference and avoid an artificial increase of possible effects of our treatment through stimulation of cell proliferation (Colzani *et al.* 2009; Kramer *et al.* 2005; Mannello & Tonti 2007), medium was exchanged for the experiments by serum-reduced cell culture medium (2 % FBS) including 10 µL of various palbociclib concentrations (1 - 10 µmol/L). We diluted palbociclib in a certain manner so that we always had to add 10 µL of dilution per 1 µmol/L and 2 µmol/L and 10 µL of pure DMSO (Roth, Karlsruhe, Germany) to the control. For 10 µmol/L palbociclib 50 µL of stock solution (1 mmol/L) were added to the cells. Finally we had a DMSO concentration of less than or equal to 1 %. We compared this

DMSO concentration with controls and found no effect, which is similar to other findings, where Costa *et al.* (2017) tested the effect of DMSO on primary cells like peripheral blood mononuclear cells (PBMC). Cells were incubated in presence of the inhibitor for 48 h at 37 °C. Additionally, half of the cells were irradiated with 2 Gy ionizing radiation (IR) by an ISOVOLT Titan X-ray generator (GE, Ahrensburg, Germany) 3 hours after addition of inhibitor. Supernatant and treated cells were harvested by trypsinization. Cells were stained with Annexin V-APC (BD, Heidelberg, Germany) and 7-amino-actinomycin D (BD, Heidelberg, Germany) for 30 minutes on ice to analyze apoptotic and necrotic cells using flow cytometry (Cytotflex, Beckman Coulter, Brea, USA) (Burdak-Rothkamm *et al.* 2005).

2.2.2 Cell cycle

Seeding and treatment procedure was identical to apoptosis and necrosis analysis. After harvesting, cells were fixed in 10 mL of 70 % ethanol (Roth, Karlsruhe, Germany) and 1 mL of serum-reduced cell culture medium for a minimum of 12 hours at + 4 °C and then stained with Hoechst 333258 (Invitrogen, Eugene, USA) for 60 minutes on ice. Cells were analyzed in the Cytotflex flow cytometer. In general, cells need around 24 hours to go through cell cycle. To clearly identify any changes in cell cycle distribution, like G0/G1 or G2/M arrest, treatment of 48 h could be advisable. To test whether 24 h or 48 h of treatment should be done, we tested three cell lines previously (data shown in supplements Figure S1) and performed all experiments later on with 48 h of treatment.

2.3 3-dimensional cell culture and FACS analysis

For 3D-analysis, 5000 cells per 40 µL drop of standard medium (SBLF7: 15 % FBS, ICNI: 20 % FBS, MDA-MB-231, MCF-7: 10 % FBS), including different concentrations of palbociclib (0.002 – 2 µmol/L), were seeded in each well of Perfecta3D hanging-drop plate (3D Biomatrix, An Arbor, USA). After 3 h of incubation (37 °C, 5 % CO₂) defined plates were irradiated with 2 Gy IR. Furthermore, plates were incubated for 4 days to let the cells form spheroids. On day 4 after treatment three samples containing 32 spheroids per condition (control, inhibitor, irradiation, inhibitor + irradiation) were collected. Spheroids were settled down and supernatant was removed. Spheroids were washed twice with PBS. After removing PBS, cells of spheroids were detached with 0.5 % Trypsin/EDTA. Trypsinization was stopped by adding serum-containing medium. Cells were centrifuged at 300 x g and supernatant was discarded. After resuspending the cells in the remaining medium staining was performed by adding 200 µL cold Ringer solution (Fresenius Kabi, Bad Homburg, Germany) and 10 µL of Annexin-7AAD-Mix (1:1) and incubated on ice for 30 minutes. Cells were washed, resuspended in cold Ringer solution and analyzed in the Cytotflex cytometer.

2.4 Scratch assay

The migration assay was used to evaluate the migratory ability of breast cancer cell lines MDA-MB-231 and MCF-7. As a control healthy donor skin fibroblasts (SBLF9) were used. Cells were seeded and incubated to reach a confluence of about 90 % at 37 °C. To minimize the effect of scratch closing through proliferation we starved our cells for 24 hours with 2 %-FBS-medium and exchanged the medium in the beginning of each experiment with fresh 2 %-FBS-medium again. The monolayers in each cavity were then scratched with 10 µL-pipet tip. Cells were treated with none or 10 µmol/L of the inhibitor palbociclib and were irradiated with either 0 Gy or 2 Gy. To record the migration, images of the same area of the cell layer were repeatedly acquired with a microscope (Zeiss Primo Vert, Oberkochen, Germany) at 100x magnification over different periods of time, but always at 24 h and 48 h. The remaining area of the cell scratch was computed by an image analyzing software (Biomass, MSAB, Erlangen, Germany). A smoothing algorithm filtered the image and set the area at point 0 to 100 % and calculated the remaining areas (Wichmann *et al.* 2015).

2.5 Colony forming assay

Regularly splitted cells were seeded in 6-well-plates with a density of 100 – 2000 cells per well. Cells were treated with different concentrations of inhibitor and irradiated after 3 h with 0, 2 or 4 Gy. After another incubation phase of 24 h medium was exchanged by fresh standard medium without any drug and the inhibitor was washed out. Plates were incubated for 10 to 14 days until colonies of minimum 50 cells were developed. Colonies were stained with methylene blue (#66725, Sigma Aldrich, München, Germany) for 30 minutes at room temperature and counted when dry.

2.6 Immunofluorescence microscopy

Immunofluorescence was performed as reported before (Endt *et al.* 2011). E-cadherin protein was stained using an anti-E-cadherin antibody (1:200, #61082 / BD, Heidelberg, Germany) and detected by a goat anti-Mouse-Alexa 488 secondary antibody (A11001, Life Technologies, Carlsbad, USA). Nuclei were stained by DAPI (10236276001 / Sigma Aldrich, St. Louis, USA). Images were acquired with a fluorescence microscope (Zeiss Axio Plan 2, Oberkochen, Germany) at 400x magnification and analyzed with Biomass Software (MSAB, Erlangen, Germany).

2.7 Statistics

Statistical analysis was performed using GraphPad Prism 8 software. All experiments were carried out with n = 3 or n = 4 (as declared in associated figure description). Data is presented as mean ± SD. Differences in mean values between multiple groups were analyzed using one/two-tailed Mann-Whitney-U test. P-value ≤ 0.05 was determined as significant.

3. Results

3.1 Apoptosis and Necrosis (Annexin V, 7-AAD)

Kinase inhibitors can influence effectiveness of irradiation by increasing or decreasing cellular responses. We studied whether palbociclib is able to change cellular mechanisms like cell death or cell cycle. Therefore, apoptosis and necrosis induction by combined treatment of the kinase inhibitor palbociclib (3-dimensional structure shown in Figure 1A) and IR was studied 48 h after treatment. A previous dose-escalation study helped us to figure out the relevant KI concentration. Therefore, cells were treated with 2 nmol/L up to 10 μ mol/L (data shown in supplemental figure S3). Cells responded only with concentrations more than 200 nmol/L in comparison to the control. Melanoma cells of six different patients and skin fibroblasts of two healthy donors were treated with inhibitor, IR or a combination of both. Moreover, breast cancer cell lines MDA-MB-231 (TNBC) and MCF-7 (ER⁺, PR⁺) were studied. Apoptotic and necrotic populations were analyzed in a classical 2D cell culture model as well as in a 3D hanging-drop cell culture system.

3.1.1 2D cell culture

Cells were stained with Annexin V-APC and 7-AAD for apoptosis and necrosis and analyzed by flow cytometry. In the following representative dot plots (Figure 1B) the biggest population of viable cells (Annexin⁻/7-AAD⁻) is clearly seen. During treatment cells are shifting to the apoptotic (Annexin⁺/7-AAD⁻) or the necrotic quadrant (Annexin⁺/7-AAD⁺). We used cells of six different malign melanoma patients (ANST, ARPA, HV18MK, LIWE, ICNI, RERO) with different BRAf status and two breast cancer cell lines (MDA-MB-231, MCF-7). Skin fibroblasts (SBLF7, SBLF9) of healthy donors were used as a control. In all studied tumor cells (2-dimensional) there was no difference between the inhibitor treatment with or without 2 Gy IR. Combined therapy tends to increase apoptotic and necrotic rates in both controls and in malign HV18MK, whereas LIWE and ICNI showed no influence of additional palbociclib treatment (2 Gy + 1 μ M) (Figure 1C). In SBLF7, ANST and HV18MK, the highest inhibitor concentration (10 μ mol/L) leads to an apoptotic population of more than 20 % in contrast to all other cell cultures. Treatment of both breast cancer cell lines lead to dramatic increase of necrosis (55 – 89 %). In general, healthy fibroblasts tended to show a decrease of cell death comparing 2 Gy to 2 Gy + 1 μ mol/L, in contrast to malign cells which tended to show no influence or increase of combination therapy. Since the malign skin cancer cells were all isolated from different patients with diverse mutation profiles, it is plausible that these cells behave differently.

3.1.2 3D cell culture

A 3-dimensional cell culture model was established and used to explore the impact on apoptosis and necrosis of palbociclib and IR. We used healthy donor skin fibroblasts as control, both breast cancer cell lines and one BRAf-wt and one BRAf-V600E-mutated cells (ICNI, Figure 2A). After four days healthy fibroblasts SBLF7, the melanoma cells ICNI (Figure 2B) and breast cancer cell lines MDA-MB-231, MCF-7

and formed stable spheroids with diameters between 200 µm and 600 µm (Figure 2B). BRAf-wt cells ANST did not form spheroids within 96 hours.

ICNI, MDA-MB-231, MCF-7 and SBLF7 were able to form stable spheroids within four days, even under combined treatment of kinase inhibitor and IR (Figure 2B). Necrotic cells increased in healthy skin fibroblasts after 0.2 µmol/L inhibitor and combined 2 Gy IR treatment compared to IR alone ($p = 0.0041$). In contrast, the amount of apoptotic cells was clearly decreased in malign cell culture ICNI by 2 Gy irradiation and combined with 0.2 µmol/L or 2 µmol/L palbociclib ($p = 0.0005$ and $p = 0.0072$) (Figure 2C).

3.2 Colony forming assay (cell survival)

Since radiation can not only trigger apoptosis or necrosis, it also can lead to a senescence in malign and non-malign cells. Therefore, we used a colony forming assay as the gold standard for radiosensitivity to measure cell survival during different treatment conditions. Cells were seeded in 6-well-plates, treated for 24 h and incubated for 10 up to 14 days until colonies emerged. We measured cell survival fraction (sf) for different inhibitor concentrations (0.5 µmol/L, 1 µmol/L palbociclib) and different irradiation schemes (0, 2 or 4 Gy).

We tested three subgroups of different cell types. Healthy tissue fibroblasts (SBLF7, SBLF9), breast cancer cell lines (MDA-MB-231, MCF-7), skin cancer cells with BRAf wt status (ANST, ARPA) and V600E mutation status (HV18MK, LIWE, RERO, ICNI). SBLF7 reacted the strongest with sf of 2 %, 0.4 % and 0.3 %, nonetheless second healthy control SBLF9 showed similar behavior compared to the malign cancer cells. Although, just slight effects of combination therapy were observed for MDA-MB-231, RERO and ICNI, in contrast to stronger effects in all other tested cells. Treating cells with palbociclib additionally leads to significant decrease of cell survival for 0.5 µmol/L and 1 µmol/L ($p = 0.05$).

3.3 Cell cycle (Hoechst)

The kinases CDK4 and CDK6 are responsible to overcome the G1-checkpoint and to reach the S-phase of cell cycle (Figure 4A). Since palbociclib is a CDK4/CDK6 inhibitor we performed a cell cycle analysis to find differences in the distribution of cell cycle phases depending on inhibitor treatment. Furthermore, it is of clinical interest if kinase inhibitors lead to an increase of cells in G2/M phase, which is known to be more radiosensitive and therefore more beneficial during irradiation as a cancer therapy. In general, cells need around 24 hours to go through cell cycle. To clearly identify any changes in cell cycle distribution, like G0/G1 or G2/M arrest, treatment of 48 h could be advisable. To test whether 24 h or 48 h of treatment should be done, we tested three cell lines previously (data shown in supplemental material Figure S1) and performed all experiments later on with 48 h of treatment. Cells were harvested after treatment, and cell DNA was stained by Hoechst 333258 and analyzed by flow cytometry. Representative DNA histograms of

the breast cancer cell line MCF-7 under palbociclib and different irradiation schemes are depicted in Figure 4B.

To get an adequate overview of cellular response to palbociclib we tested a range from 2 nmol/L – 10 μ mol/L for cell death and from 1 μ mol/L up to 10 μ mol/L in cell cycle (FACS) analysis. The data presented in this section is based on this previous dose-escalation experiment and physiological achievable plasma serum concentration of approximately 0.4 μ mol/L (Tamura *et al.* 2016). Data for cell cycle distribution treating cells with 2 μ mol/L or 10 μ mol/L is shown in the supplemental figure S2. Because response of cells to palbociclib only included changes in G0/G1 or G2/M, which correlate to each other, only data for this cell cycle phases are depicted here (Figure 4C).

No differences were found in our healthy controls (fibroblasts) when adding palbociclib to irradiation treatment. Fibroblasts are in G0/G1 phase at almost 90 % compared to malign cells with around 80 %. In five out of eight malign cell lines (MDA-MB-231, MCF-7 and RERO with $p = 0.05$; ANST and HV18MK with $p = 0.03$) combination treatment significantly (2 Gy + 1 μ mol/L) leads to fewer cells in G2/M phase. ARPA cells show no difference and LIWE show an increase of cell population in G2/M ($p = 0.03$).

3.4 Migration (Scratch assay)

Despite effects on apoptosis and necrosis of palbociclib in malign cell cultures it is interesting to see if there is an influence on migration or invasion. Scratch assays were performed to study the influence of palbociclib on breast cancer cell lines MDA-MB-231 and MCF-7 and healthy donor skin fibroblasts (Figure 5A - 5D). Our previous findings based on cell death FACS analysis led us to decide to run further experiments with a concentration of 10 μ mol/L palbociclib. For cell death analysis we were able to found moderate effects without extreme death rates.

The scratch area using the breast cancer cell line MCF-7 was not closed within 48 h in the untreated control. Irradiation of 2 Gy did influence the migration (5 % vs. 39 % at 24 h, $p = 0.0286$). MDA-MB-231 migrated fastest and the scratch area was closed without treatment or 2 Gy IR alone within 22 h. Combined treatment leads to an inhibition of migration and scratch area was closed after 48 h (Figure 5E), whereas palbociclib monotherapy leads to loss of scratch closing within 48 h significantly (0 % vs. 22 %, $p = 0.0500$). MCF-7 treated with 10 μ mol/L palbociclib distinctly inhibited migration (10 % vs. 72 % at 48 h, $p = 0.0286$) and the closing of the scratch within 48 h was lost (Figure 5F). Comparing 10 μ mol/L to 10 μ mol/L + 2 Gy IR showed no difference (72 % vs. 72 %, $p = 0.8857$). Healthy donor skin fibroblasts migrated slowest and scratch area was not closed within 48 h even without inhibitor or irradiation treatment. Addition of 10 μ mol/L inhibitor leads to a reduction of migration (0 Gy: 76.3 % vs. 106.1 %, $p < 0.001$; 2 Gy: 67.6 % vs. 85.7 %, $p = 0.11$), hardly influenced by irradiation (Figure 5G). To check if the differing migration behavior is driven by varying expression of E-cadherin we used immunofluorescence for further examination.

3.5 Expression of E-cadherin

Different migration behavior in the scratch assays prompted us to study the expression of E-cadherin, a calcium-dependent cell adhesion protein. Breast cancer cell lines MCF-7 and MDA-MB-231 and healthy control cells SBLF9 were used for E-cadherin immunostaining (Figure 6).

Comparing the untreated breast cancer cell lines, MCF-7 (Figure 6A) express E-cadherin more intense than MDA-MB-231 (Figure 6B). E-cadherin is essential for strong adhesion to other cells and matrix components, therefore a higher expression could be an explanation for slower migration like MCF-7 showed in the scratch assay. Untreated primary fibroblasts SBLF9 (Figure 6C) express lowest E-cadherin. Treating cells with either palbociclib, irradiation or a combination of both for 48 h showed no variations in E-cadherin expression (data shown in supplemental figure S4).

4. Discussion

As mentioned before palbociclib is proven to increase radiosensitivity in e.g. HNSCC and HPC (Göttgens *et al.* 2019, Huang *et al.* 2018). Furthermore, findings of Young *et al.* (2014) suggest that the majority of melanoma cell lines are sensitive to CDK4 inhibition, with p16^{INK4A} loss a biomarker of sensitivity. Even under palbociclib treatment, progression and metastases occur and can lead to further therapy of cancer patients like irradiation. To address the question if palbociclib can act as a radiosensitizer in this clinical context we analyzed different cell types (skin cancer, breast cancer) and healthy donor fibroblasts as a control.

First, we tested a range of increasing palbociclib concentrations from 2 nmol/L up to 10 µmol/L in our cell death FACS protocol. This allowed us to cover a range from a minor response (using 1-2 µmol/L) to a strong response (10 µmol/L) of cells treated with KI w/o IR. Based on our dose escalation study we run further experiments with 1 µmol/L – 10 µmol/L because no effects were detected with lower concentrations. For more sensitive clonogenic assays we used 0.5 µmol/L – 1 µmol/L, which is close to the serum plasma concentration of 0.4 µmol/L (Tamura *et al.* 2016). The effect of irradiation on melanoma cells could be slightly decreased by the CDK 4/6 kinase inhibitor palbociclib in four out of eight cells comparing 2 Gy vs. 2 Gy + 1 µmol/L. Decreasing apoptosis and necrosis rates indicated a radioprotective effect by palbociclib in cancer cells, whereas healthy fibroblasts seem to be more sensitive to combined treatment of both inhibitor and IR than IR alone. That suggests a smaller effect on the tumor than on non-neoplastic tissue in patients, which could lead to increased side effects. Finally, FACS analysis of apoptosis and necrosis in ten different cell lines representing three different subgroups (healthy tissue, breast cancer, skin cancer) seem to be distinctly. The leading cause for these differences could be due to the fact that the six tested skin cancer cells representing cells from different patient biopsies. Different mutation profiles of these patients could lead to such diverse behavior.

Furthermore, we established a 3D hanging drop system in our lab and generated cancer spheroids of different cell lines to test them also in FACS analysis for cell death. A 3-dimensional cell culture system is

considered to be less artificial because of a more tissue-like architecture and therefore zones of varying proliferation and nutrition supply (Edmondson *et al.* 2014). Finally, our 3D cell culture model was able to confirm the results of the 2D examination and detected differences in cell death more sensitive than the 2D model. Necrotic cells increased in healthy skin fibroblasts (0.2 $\mu\text{mol/L}$ + 2 Gy IR treatment vs. IR; $p = 0.0041$). In contrast, the amount of apoptotic cells was clearly decreased in malign cell culture ICNI comparing 2 Gy to 0.2 $\mu\text{mol/L}$ + 2 Gy and 2 $\mu\text{mol/L}$ + 2 Gy ($p = 0.0005$ and $p = 0.0072$).

Besides analyzing apoptosis and necrosis inducing effects of palbociclib and irradiation, it is important to investigate another major consequence of irradiation, senescence. Since the gold standard for cell survival testing is the colony formation test, we have examined cells accordingly. After testing a wider range of palbociclib concentrations, we focused here on a concentration of 0.5 $\mu\text{mol/L}$ and 1 $\mu\text{mol/L}$ to treat cells for 10 to 14 days that are more compatible, in contrast to 48 h-treatment in our FACS analysis (1 – 10 $\mu\text{mol/L}$). All cell lines showed a significant reduction of the survival fraction after 0.5 $\mu\text{mol/L}$ or 1 $\mu\text{mol/L}$ palbociclib treatment ($p = 0.05$) comparing AUC (area under curve). Nevertheless healthy fibroblasts SBLF7, as our control for normal tissue, reacted the strongest to irradiation, kinase inhibitor treatment and the combination of both. These results support the conclusion of our cell death (FACS) analysis that the healthy fibroblasts seem to more sensitive to combination therapy than the malignant cells. However, it also showed up that treating SBLF7 with 0.5 $\mu\text{mol/L}$ + 4 Gy and SBLF9 with 1 $\mu\text{mol/L}$ + 4 Gy seemed to rise the survival fraction in a radioprotecting pattern. Whereas colony forming of ANST (0.5 $\mu\text{mol/L}$ + 4 Gy), RERO (1 $\mu\text{mol/L}$ + 4 Gy) and especially ARPA (1 $\mu\text{mol/L}$ + 4 Gy) decreased evidently indicating a radiosensitizing effect. Our cell death and clonogenic experiments suggests that healthy tissue fibroblasts are triggered more to die via apoptosis and necrosis after treating them with kinase inhibitor and irradiation. In contrast, malign cells showed reduced survival fractions after combination therapy in our colony forming assays, which could indicate an increase of senescence. Because cancer cells often deregulate their cell death pathways to proliferate uncontrolled it is conceivable that they prevent themselves from apoptosis (Reed 1999), developing senescence. Interestingly, Tao *et al.* 2016 showed that malign lung cancer cells treated with palbociclib alone did not change survival fraction in a significant manner. This should lead to more awareness using palbociclib and irradiation together in treating malign cells *in vitro*. Noticeably, we detected almost no supra-additive effect normalizing the combination treatment to a 2-Gy-irradiation alone. Nevertheless, it should also be mentioned that additive effects could lead to side effects in normal tissue during anti-cancer therapy.

Keeping palbociclib in mind as a disruption of the cell cycle by binding to CDK4/6 we analyzed of cell cycle distribution. Combining palbociclib with irradiation showed no response in healthy controls but in seven out of eight malign cells. The cell population in G0/G1 phase was higher in fibroblasts then in cancer cells and changes in G0/G1 correlate contrary with changes in G2/M. In five out of eight malign cells we were able to detect a decrease of cells in G2/M comparing IR and IR + inhibitor ($p \leq 0.05$). In general, G2 and mitosis are most sensitive for radiation, G1 is less and S phase least radiosensitive (Sinclair 1968, Pawlik & Keyomarsi 2004). Fewer cells in the G2/M phase leads to a lower radiation sensitivity and could be an explanation for a reduced response of malignant cells to IR during palbociclib treatment. Interestingly, Xie *et al.* 2019 found a contrary effect compared to our data. They showed an

increase of G2/M phase and as a result more apoptosis, not in melanoma cells, but in nasopharyngeal carcinoma cells. Other findings using intracranial atypical teratoid rhabdoid tumor cell lines showed an inhibition of DNA double-strand break repair and promoted increased tumor cell apoptosis (Hashizume *et al.* 2016). However, it is noticeable that both groups worked with established and therefore more artificial cell lines. For our experiments, we used skin cancer cells, generated of human skin biopsies and harboring a more primary cell status. This radioprotective effect of palbociclib in cancer cells is probably based on the accumulation of cells in the less radiosensitive G0/G1 cell cycle phase. We found that palbociclib decreases the amount of malign cell in G2/M phase significantly. Cancer cells are more forced to cell cycle to progress as mentioned in the “hallmarks of cancer” (Hanahan & Weinberg 2000). Additionally, one knows that melanoma show a higher CDK4 activity because of p16^{INK} loss, an inhibitor of CDK4 (Cancer Genome Atlas N. 2015). When cancer cells are more addicted to a short cell cycle, an inhibition of the G1-checkpoint could lead to a smaller amount of cells in the radiosensitive G2/M phase. This results in a reduced sensitivity to radiation. This could explain why primary malign cells react to a combined therapy of palbociclib and ionizing radiation with an increase of cell death. The question raised if different abilities of our cell lines to use homologous recombination or non-homologous end-joining after irradiation may influence the outcome of combining targeted with radiation therapy. Possibly homologous recombination (HR) deficiency is induced by palbociclib via myc-regulated gene expression (Yi *et al.* 2019). Until now it remain unclear if palbociclib decrease or increase DNA repair but may shift the cells from using HR to NHEJ (non-homologous end joining) (Dean *et al.* 2012). Further characterization of our cell lines with focus an HR pro- or deficiency should be done in the future.

Thinking of possible effects of kinase inhibitors and combination of them with IR rises not just questions of influence on cell death and cell survival. A noticeable proportion of patients are going to be irradiated because of progression or occurring metastases. Metastasis, as a hallmark of cancer (Hanahan, Weinberg 2000), is based on the migration behavior of tumor cells. Hence, we performed scratch assays to address the question if palbociclib has a potential influence on the migration behavior of benign cells and tumor cells. Breast cancer cell lines MDA-MB-231 and MCF-7 migrated differently without kinase inhibitor or IR. This may be due to higher expression of E-cadherin (Ziober *et al.* 2006) on the cell surface of MCF-7 compared to MDA-MB-231. Fastest movement within 24 h was shown by the TNBC cell line MDA-MB-231. Cell migration of MDA-MB-231 was only clearly inhibited when palbociclib was combined with irradiation. Interestingly, migration of MCF-7 (ER⁺, PR⁺) decreased dramatically using palbociclib. Healthy primary skin fibroblasts SBLF9 migrate distinctly slower in the presence of palbociclib. In summary, palbociclib is able to influence the migration of MCF-7 and SBLF9 significantly. Similar findings are shown by Qin *et al.* 2015 who postulates that decreased migration is driven by lower COX2-expression and PGE2 production during palbociclib treatment.

MCF-7 cells express much more E-cadherin compared to MDA-MB-231 cells. That could be an explanation for the low migration of MCF-7 in contrast to MDA-MB-231. Nevertheless, staining healthy skin fibroblasts showed moderate E-cadherin expression, which does not correspond to the observed migration behavior. However, there are findings that the influence of E-cadherin is more recognizable

studying single cell migration, like in transwell assays, than measuring collective migration in scratch assays (Elisha *et al.* 2018).

5. Conclusion

Data of cell death (2D) analysis in healthy fibroblasts showed a slight tendency of decreased apoptosis and necrosis using palbociclib and irradiation simultaneously compared to IR alone. In contrast, four out of eight malign cell lines increased cell death at the same condition. Nevertheless, all cell lines especially the six representing different melanoma patients respond quite divers. However, we were able to determine significant differences in our newly established 3D model showing radiosensitizing effects of palbociclib in fibroblasts and radioprotective effects in malign skin cancer cells. This radioprotective effect of palbociclib in cancer cells is probably based on the accumulation of cells in the less radiosensitive G0/G1 cell cycle phase as mentioned before.

Further colony forming assays implied a higher susceptibility of fibroblasts representing healthy tissue to the combination treatment because of minimal survival fractions. However, plotted curves indicate a radioprotective effect in fibroblasts and radiosensitizing effect in cancer cells. Our cell death and clonogenic experiments suggest that healthy tissue fibroblasts are triggered more to die via apoptosis and necrosis after treating them with kinase inhibitor and irradiation. In contrast, malign cells showed reduced survival fractions after combination therapy in our colony forming assays, which could hint for an increase of senescence.

Finally we studied whether palbociclib is able to influence migration of cells or not, since this KI is approved in a later, progressed or metastasized stage of cancer (FDA approval 2015, Ref ID 4217399). Our data suggests that palbociclib decelerate migration behavior of malign and non-malign cells, even in cell lines with low E-cadherin expression like MDA-MB-231 and non-malign SBLF9 cells. Advanced *in vivo* experiments could help to verify this observation more precisely.

All *in vitro* data presented in this study could give a hint for assuming palbociclib can act as a radiosensitizer causing cell death and more likely senescence, not just in HCC and HNSCC cell, but in malign melanoma cells too. Palbociclib appears to reduce the effect of ionizing radiation *in vitro*. Therefore, further studies are needed to assess whether a combined therapy of palbociclib and radiation is appropriate. Advanced *in vivo* research and highly monitored clinical trials could help to investigate how palbociclib is able to interact with ionizing radiation in healthy and tumor tissue.

Abbreviations

7-AAD	7-amino-actinomycin D
APC	Allophycocyanin
ATM	Ataxia telangiectasia mutated
AUC	Area under curve
BSA	Bovine serum albumin
CDK	Cyclin-dependend kinase
CNS	Central nervous system
Co	Control
DAPI	4',6-Diamidin-2-phenylindol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Estrogen receptor
FACS	Fluorescence associated cell sorting
FBS	Fetal bovine serum
FDA	Food and drug administration
HCC	Hepatocellular carcinoma
HNSCC	Head and neck squamous cell carcinoma
HR	Homologous recombination
KI	Kinase inhibitor
IF	Immune fluorescence
IR	Irradiation
NEA	Non-essential amino acid
NHEJ	Non-homologous end joining
PBS	Phosphate-buffered saline
PR	Progesterone receptor
Rb	Retinoblastom-protein
SD	Standard deviation
sf	Survival fraction
TBS	Tris-buffered saline

Declarations

Ethics approval and consent to participate

Ethical approval was obtained in the Department of Dermatology of the University Hospital of Erlangen following approval by the institutional review board (No. 204 17 BC). The patients provided written informed consent.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

TJ performed the FACS analysis of cell survival and cell cycle as well as immune fluorescence staining. PK performed the scratch assays for the breast cancer cell lines and fibroblasts. TJ wrote the manuscript with support from LD, LH and MH. LH provided the primary cells used. RF supervised the project. All authors read and approved the final manuscript.

Acknowledgements

The author would like to thank Doris Mehler and Elisabeth Müller for excellent technical support for the study.

Authors' information (optional)

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Figures

Figure 1

Figure 1

Apoptosis and necrosis induction in healthy cells and tumor cells. Healthy donor skin fibroblasts (SBLF7, SBLF9) and tumor cells (breast cancer, skin cancer) were treated 48 h. Concentrations of palbociclib w/o 2 Gy irradiation were increased from 1 $\mu\text{mol/L}$ up to 10 $\mu\text{mol/L}$. (A) 3-dimensional structure of palbociclib (pubchem) (B) Shifting of ANST cell population in control, 2 Gy IR, 10 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$ + 2 Gy IR sample (representative FACS dot plots). (C) Apoptosis was analysed by Annexin V-APC staining and necrosis by 7-AAD staining using flow cytometry. Each value represents mean $\pm$ SD (n = 4).

Figure 2

Figure 2

3-dimentional cell culture of healthy donor skin fibroblasts and malign cells. (A) Malign melanoma cell culture ICNI forming spheroids; left: directly after seeding 5000 cells in a 40 μ L drop of medium, right: matured spheroid after 4 days at 37 $^{\circ}$ C. (B) Matured 4-days-old spheroids of malign melanoma cell culture ICNI (top) and healthy donor skin fibroblasts SBLF7 (bottom) without treatment (co), 2 μ mol/L palbociclib and 2 μ mol/L palbociclib + 2 Gy IR. (C) Apoptotic and necrotic cells in the spheroids analyzed by Annexin V-APC and 7-AAD staining flow cytometry. Each value represents mean $\pm$ SD (n = 3).

Figure 3

Figure 3

Survival fraction of different non-malign and malign cell lines using different inhibitor concentrations and irradiation schemes. Healthy tissue fibroblasts, breast cancer cell lines and skin cancer cells were treated with either 0.5 $\mu\text{mol/L}$ or 1 $\mu\text{mol/L}$ palbociclib and additional irradiated with 0, 2 or 4 Gy. For comparison, we calculated the “area under the curve” for each treatment in all tested cell lines. All cells responded to palbociclib treatment in a significant manner (co vs. 0.5 μM and co vs. 1 μM ; $p = 0.05$) (Mann-Whitney-U test). Each value represents mean $\pm$ SD ($n = 3$).

Figure 4

Distribution of the cell cycle phases under palbociclib treatment at 0 Gy and 2 Gy. (A) Palbociclib inhibits progression of cell cycle by blocking phosphorylation of RB protein and forcing the cell to stay in G0 / G1 phase. (B) Representative histograms of DNA distribution in breast cancer cell line MCF-7 after 48 h treatment under rising palbociclib concentrations. (C) Changes in cell populations in G0/G1-, (C) S- and G2/M-phase during inhibitor w/o irradiation treatment. Each value represents mean $\pm$ SD (n = 4).

Figure 5

Figure 5

Cell migration analysis in breast cancer cell lines and healthy donor skin fibroblasts. Tumor cell lines (MDA-MB-231, MCF-7) and healthy cells (SBLF9) under palbociclib treatment w/o 2 Gy IR. Representative scratch areas of cell line MCF-7 at 0 h, 24 h and 48 h under (A) control, (B) 10 μmol/L palbociclib, (C) 2 Gy irradiation or (D) 10 μmol/L palbociclib + 2 Gy IR treatment. Decrease of scratch area within 48 h (triplicates) under Palbociclib treatment w/o 2 Gy IR in (E) Breast cancer cell line MCF-7, (F) MDA-MB-231 and (G) healthy donor skin fibroblasts SBLF9. Each value represents mean $\pm$ SD (n = 3).

Figure 6

Figure 6

Expression of E-cadherin in breast cancer (MCF-7, MDA-MB-231) and control (SBLF9) cells. Representative immunofluorescence images (IF) of untreated MDA-MB-231, MCF-7 and SBLF9 cells cultivated on glass slides. Cells were stained for E-cadherin (green, BD 1:200) and nucleus (blue / DAPI, Roche 1 mg/mL). Scale bars 25 μm.

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