


REPORT



## The CDK4/6 inhibitor palbociclib synergizes with irinotecan to promote colorectal cancer cell death under hypoxia

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### ABSTRACT

Hypoxia is an inherent impediment to cancer therapy. Palbociclib, a highly selective inhibitor for CDK4/6, has been tested in numerous clinical trials and has been approved by the FDA. We previously reported that CDK inhibitors can destabilize HIF1 $\alpha$  regardless of the presence of hypoxia and can sensitize tumor cells to TRAIL through dual blockade of CDK1 and GSK-3 $\beta$ . To translate this knowledge into a cancer therapeutic strategy, we investigated the therapeutic effects and molecular mechanisms of CDK inhibition against colon cancer cells under normoxia and hypoxia. We found that palbociclib sensitizes colon cancer cells to hypoxia-induced apoptotic resistance via deregulation of HIF-1 $\alpha$  accumulation. In addition to inhibition of cell proliferation, we observed that palbociclib promotes colon cancer cell death regardless of the presence of hypoxia at a comparatively high concentration via regulating ERK/GSK-3 $\beta$  signaling and GSK-3 $\beta$  expression. Furthermore, palbociclib synergized with irinotecan in a variety of colon cancer cell lines with various molecular subtypes via deregulating irinotecan-induced Rb phosphorylation and reducing HIF-1 $\alpha$  accumulation under normoxia or hypoxia. Collectively, our findings provide a novel combination therapy strategy against hypoxic colon cancer cells that may be further translated in the clinic.

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### Introduction

Colorectal cancer (CRC) is one of the most common cancers and one of the leading causes of cancer mortality worldwide. Although the therapeutic regimens of cytotoxic drugs, FOLFOX and FOLFIRI have improved overall and progression-free survival of advanced CRC, the therapeutic responses vary individually. There is a need to develop additional molecularly-targeted therapies for advanced CRC.

Various biologic roles of CDK4/6 have been identified. Besides regulating cell cycle progression through the pRb/E2F pathway and the G1-S checkpoint,<sup>1</sup> non-cell-cycle effects of CDK4/6 include direct activation of vascular endothelial growth factor (VEGF) transcription, promotion of angiogenesis and nuclear factor (NF)- $\kappa$ B activation via the p65 transcription factor.<sup>2–4</sup> PD0332991 (palbociclib) is a highly selective small molecular inhibitor of CDK4 and 6. Palbociclib was approved by the FDA in 2015 in combination with letrozole as initial hormone- plus CDK-targeted therapy for post-menopausal women with ER(+)/Her2(–) advanced breast cancer.<sup>5</sup> The therapeutic effects of palbociclib have been demonstrated in numerous clinical trials for breast cancer, NSCLC, GBM, lymphoma, and leukemia, in combination with 5-FU and oxaliplatin in solid malignancies (NCT01522989) or with cetuximab in head and neck cancer.<sup>6,7</sup> A combined pharmacological inhibition of MEK and CDK4/6 led to substantial synergy in KRAS-mutated NSCLC *in vivo*.<sup>8</sup> Synergy of palbociclib with a MEK inhibitor

has also been demonstrated in KRAS mutant colon cancer *in vivo*.<sup>9,10</sup> Vemurafenib-resistant tumors remain sensitive to palbociclib suggesting that initial treatment with vemurafenib followed by palbociclib with or without mTOR inhibitors might provide an approach to overcome recurrence of vemurafenib-resistant, metastatic tumors.<sup>11</sup> In brief, these studies indicate combinatorial administration of a CDK4/6 inhibitor with other chemo- or targeted-therapies could provide a promising treatment against various malignancies.

Hypoxia is an inherent impediment to tumor treatment in most solid tumors, and contributes to a poor prognosis as well as resistance to chemo/radiotherapy. HIF-1 $\alpha$  is a transcription factor that mediates the adaptive response to oxygen deprivation, is involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism and invasion by hypoxic tumor cells.<sup>12</sup> Therefore, targeting HIF-1 $\alpha$  provides a rational target for anti-cancer therapy and may combat drug resistance. However, the regulatory mechanisms and effects of HIF-1 $\alpha$  are complex and involve multiple pathways in hypoxic tumor cells. For example, it was recently reported that CDKs regulate lysosomal degradation of HIF-1 $\alpha$  to promote cell cycle progression.<sup>13</sup> PI3K/AKT-mTOR and GSK-3 $\beta$  signals regulate HIF-1 $\alpha$  in an oxygen-independent manner.<sup>14</sup> ERK1/2 proteins are involved in the regulation of HIF-1 $\alpha$  synthesis and transcriptional activation via phosphorylation of the co-activator CBP/p300.<sup>15</sup>

We recently reported that a family of nucleoside analogs (sangivamycin-like molecules, SLMs), act as inhibitors of CDKs, can sensitize tumor cells to TRAIL mediated cell death *in vivo* through dual blockade of CDK1 and GSK-3 $\beta$  and destabilize HIF-1 $\alpha$  regardless of VHL or p53 mutation status or the presence of hypoxia.<sup>16,17</sup> To translate this knowledge into a novel cancer therapeutic strategy, we investigated the anti-tumor effects and mechanisms of CDK inhibition in CRC with or without chemotherapy under hypoxia. We investigated the therapeutic effect of the CDK4/6 inhibitor palbociclib in combination with chemotherapies used to treat CRC to explore a potentially novel and effective treatment against advanced disease. Our data suggest that palbociclib, a highly selective inhibitor of CDK4/6, is a potent cytotoxic agent via deregulation of the accumulation of HIF-1 $\alpha$  under either normoxia or hypoxia while simultaneously reducing the constitutive expression of GSK-3 $\beta$  under hypoxia. We found that the CDK4/6 inhibitor palbociclib can effectively synergize with CPT11 (irinotecan) against CRC under hypoxia. Furthermore, addition of palbociclib to CPT11 deregulates CPT11-induced CDK6 and Rb phosphorylation that could account for palbociclib synergy with CPT11 against CRC under hypoxia. Collectively, these results indicate that palbociclib synergizes with conventional therapy that could be further tested in the clinic against advanced CRC.

## Materials and methods

### Chemicals and reagents

Palbociclib was purchased from MedKoo Biosciences (Research Triangle Park, NC), and was solubilized in PBS at a storage concentration of 10 mM. 5-fluorouracil, oxaliplatin and irinotecan solutions were obtained from Hospira (Lake Forest, IL). Penicillin/Streptomycin, DMEM, McCoy's 5A, PBS and trypsin were purchased from Cellgro (Manassas, VA). Fetal bovine serum was obtained from Genimi Bio-products (Broderick, CA).

The following antibodies were used: GSK-3 $\beta$ , phospho-GSK-3 $\beta$  (Ser9), caspase-3, cleaved-caspase-3, PARP, cleaved-PARP, AKT, phospho-AKT (Ser473), Rb, phospho-Rb (Ser780), ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA); phospho-CDK6 and CDK6 (Santa Cruz Biotechnology, Santa Cruz, CA); HIF-1 $\alpha$  and CDK1 (BD PharMingen, San Diego, CA).

### Cell lines and culture conditions

The human colorectal cancer cell lines HT-29, RKO, DLD-1 and SW-480 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). HCT-116 was generously provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). All cell lines were cultured in their ATCC-recommended media supplemented with 10% (v/v) fetal bovine serum with or without chemotherapy agents at 37°C within a 95% humidified atmosphere containing 5% carbon dioxide in an incubator. Hypoxia experiments were performed at 0.5–1%

O<sub>2</sub> using the INVIVO2 hypoxia workstation (TOUCAN Technologies).

### Western blotting analysis

Cells were collected and lysed using protein lysis buffer. Total protein was collected and quantified using the Bio-Rad Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated in SDS-PAGE gels (NuPage Bis-Tris Gels, Invitrogen, Carlsbad, CA) using the XCell system (Invitrogen). The separated proteins were transferred to PVDF membranes (Millipore) using a transfer apparatus (Bio-Rad). After blocking with 10% (w/v) nonfat-milk in phosphate-buffered saline (PBS), the blots were incubated with primary antibodies at 4°C overnight, and subsequently incubated with the corresponding fluorescent secondary antibodies, and the bands were visualized using Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

### Cell viability assay and cell cycle profile

Cells were seeded in 96-well dark plates at a density of 2000–6000 cells per well and incubated overnight to allow proper attachment. Subsequently the media were replaced using media with or without chemotherapeutic agents and treatment was continued for 48 hours. CellTiterGlo bioluminescence agent (Promega Corporation, Madison, WI) was used to quantify cell viability according to the manufacturer's instruction.

Cells were collected at the indicated time points after the corresponding treatment and fixed with ice-cold 70% ethanol at 4°C overnight. After washing twice with PBS, the fixed cells were re-suspended using 1% (w/v) bovine serum albumin in PBS. Then the prepared cells were stained with propidium iodide (Sigma) at a final concentration of 0.1 mg/ml for 15 minutes and DNA content was measured using an Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA).

### Dose-response assessment and synergy assessment

Half-maximal inhibitory concentration (IC<sub>50</sub>) value is a critical index of the dose-response curve. In this study, it was used to assess single drug inhibition in cell viability experiments after treatment of 48 hours *in vitro*. Prism statistical software (GraphPad Software, La Jolla, CA) was used to calculate the IC<sub>50</sub> values and to plot dose-response curves.

The combinatorial inhibitory effects of multiple drugs against CRC *in vitro* were assessed using a quantitative analysis of dose-response relationships as described previously<sup>18</sup>: multiples of the IC<sub>50</sub> of each drug were used in non-constant ratio combinations and compared against the predicted effects for each drug alone. Combining effects of multiple doses was assessed by combination index (CI)—quantitative measurements based on the mass-action law of the degree of drug interaction that allows determination of synergism and antagonism for a given end point of the effect measurement based on Chou-Talalay analysis.<sup>19</sup> If the sum of the 2 fractional terms is equal to 1, an additive effect is indicated. If the CI value is smaller than 1, synergism is indicated, and if the CI value is greater than 1, antagonism is indicated. CI < 0.5 indicates very

strong synergy. Compusyn software was used to calculate combination index values.

### Statistical analysis

All data are shown as the average  $\pm$  standard deviation (Mean  $\pm$  SD) and each experiment was repeated at least 3 times independently. Measurements were analyzed by the Student's t-test (2-tailed, paired or unpaired) using SPSS software. A P-value of less than 0.05 was considered to be statistically significant.

## Results

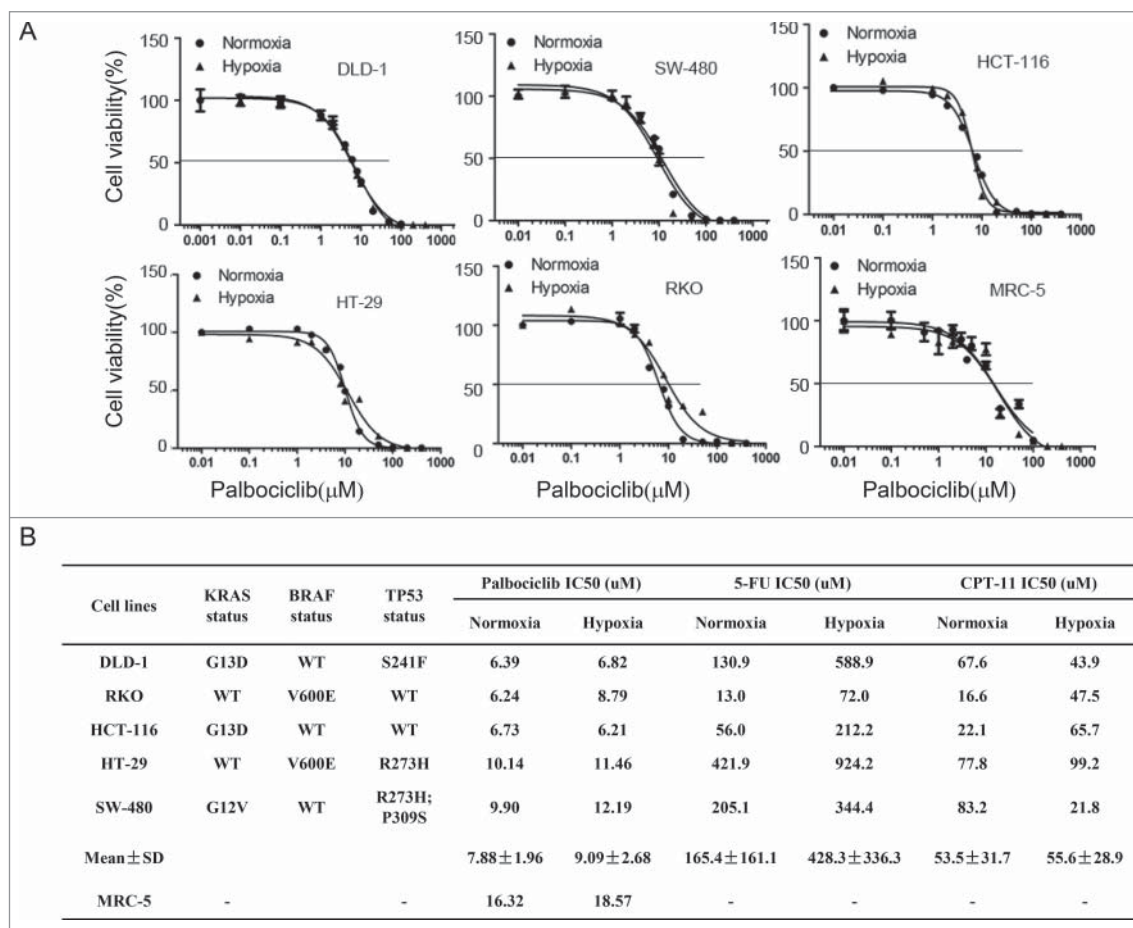
### Palbociclib sensitizes colorectal cancer (CRC) cells to hypoxia-induced drug resistance

Five-fluorouracil (5-FU) and irinotecan (CPT11) are conventional chemotherapies used to treat patients with CRC. We assessed the sensitivity of CRC to palbociclib, irinotecan (CPT11) and 5-FU in CRC cell lines with various molecular genetic subtypes under normoxic as well as hypoxic conditions (Fig. 1). The data show that hypoxia induces resistance in 5 different CRC cell lines to 5-FU (Fig 1B,  $165.4 \pm 161.1 \mu\text{M}$  for the IC<sub>50</sub> value under normoxia for the entire cell line panel

versus  $428.3 \pm 336.3 \mu\text{M}$  for the IC<sub>50</sub> value under hypoxia, again for the entire panel). By contrast to 5-FU, hypoxia has minimal impact on the sensitivity of 5 different CRC cell lines to palbociclib (Fig 1A and B,  $7.88 \pm 1.96 \mu\text{M}$  for the IC<sub>50</sub> values under normoxia for the entire panel vs.  $9.09 \pm 2.68 \mu\text{M}$  for the IC<sub>50</sub> values under hypoxia, again for the entire panel) and irinotecan (Fig 1C,  $53.5 \pm 31.7 \mu\text{M}$  for the IC<sub>50</sub> value under normoxia vs.  $55.6 \pm 28.9 \mu\text{M}$  for the IC<sub>50</sub> values under hypoxia). We previously reported that hypoxia induces apoptotic resistance to 5-FU in CRC cells lacking p53.<sup>16</sup> Irinotecan belongs to the family of camptothecins (CPTs) analogs, which have a topoisomerase I (Top I) inhibitory activity, and it has been reported that hypoxia-induced accumulation of carboxylesterase increases tumor sensitivity to irinotecan (CPT-11).<sup>20</sup> Additionally irinotecan reduces tumor angiogenesis and tumor growth via inhibiting HIF-1 $\alpha$  protein accumulation or translation in hypoxic glioma cells.<sup>21</sup> However, the effect and mechanism of palbociclib is not clear against CRC under hypoxia.

### Palbociclib promotes cell death of CRC regardless of the presence of hypoxia

It was previously demonstrated that palbociclib could arrest the transition of the G1-S phase of the cell cycle at a concentration



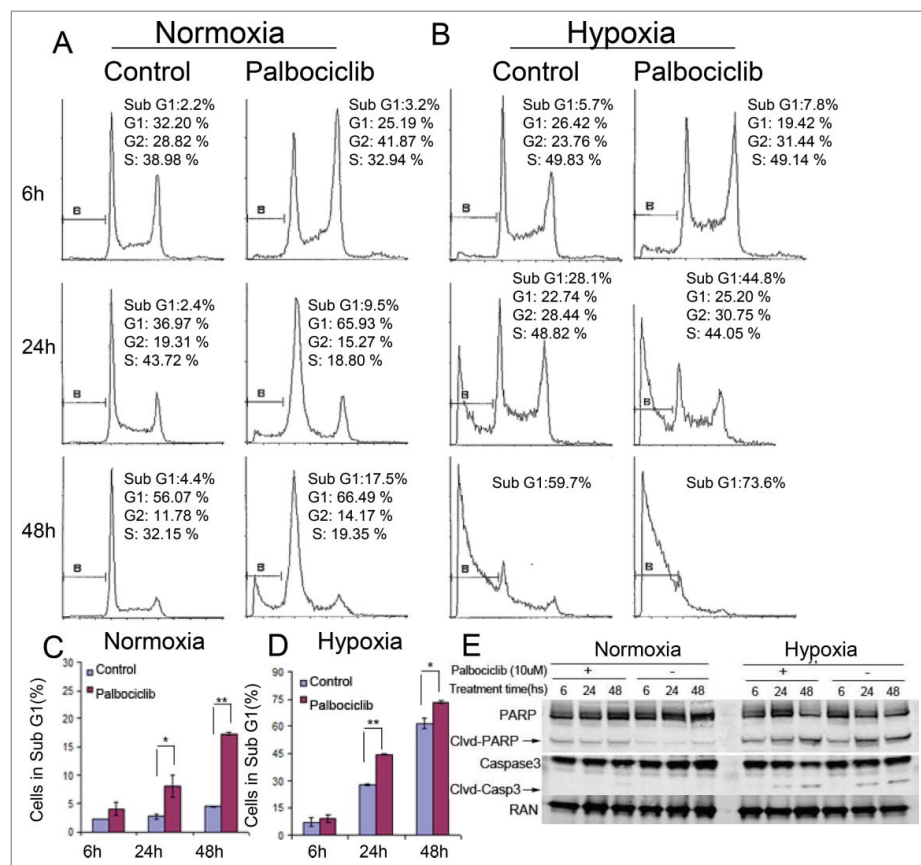
**Figure 1.** Hypoxia has minimal impact on colorectal cancer (CRC) cell viability after palbociclib or irinotecan treatment, as compared with 5-FU. (A) Dose-response curves of palbociclib against a panel of 5 human colon cancer cell lines under either normoxia or hypoxia, after treatment of 48 hours. (B) Tables of IC<sub>50</sub> values with a panel of 5 human colon cancer cell lines with various molecular genetic subtypes under normoxia and hypoxia after treatment of 48 hours. Cell viability was detected with CellTiter-Glo assay. Prism statistical software was used to calculate IC<sub>50</sub> values and plot dose-response curve. Each experiment was repeated 4 times.

range from 0.08  $\mu\text{M}$  to 10  $\mu\text{M}$  in Colo-205 human colon cancer cells under normoxia.<sup>22</sup> Besides its anti-proliferative effect in a variety of malignancies, palbociclib inhibits the epithelial-mesenchymal transition (EMT) and metastasis in breast cancer cells via downregulation of the c-Jun/COX-2 pathway at approximately 10  $\mu\text{M}$ .<sup>23</sup> High concentrations of palbociclib were recently used to allow for tissue perfusion, and a significant increase in the percentage of cleaved-caspase 3-positive cells was observed in human HCC samples. Moreover, palbociclib-treated mice showed a remarkable reduction in tumor volume, indicating that palbociclib treatment can be effective against liver tumors *in vivo*.<sup>24</sup> These findings suggest that the anti-tumor effects and mechanisms of palbociclib could be more complicated than the primary CDK inhibitory activities. To further investigate how palbociclib may affect cell viability in CRC, cell death was evaluated by DNA fragment analysis (sub-G1 analysis). Our data show that palbociclib promotes cell death in CRC cells under either normoxia (Fig 2A and C) or hypoxia (Fig 2B and D) at 10  $\mu\text{M}$ . Immunoblotting detected PARP/cleaved-PARP and Caspase-3/cleaved-caspase-3 and confirmed that palbociclib promoted CRC cell apoptosis under either normoxic or hypoxic conditions (Fig 2E). In addition, hypoxia might delay the G1-phase arrest observed with palbociclib under hypoxic vs. normoxic conditions (Fig 2B and A). Interestingly, palbociclib increased the percentage of CRC cells

in the G2-phase at 10  $\mu\text{M}$  under either normoxia (Fig 2A) or hypoxia (Fig 2B) after an early period of treatment.

### Palbociclib destabilizes HIF-1 $\alpha$ and impacts GSK-3 $\beta$ , ERK1/2 and Akt signaling in CRC differentially under hypoxia vs. normoxia

Treatment with a CDK4/6 inhibitor reduces the formation of the CDK4/6-CyclinD1 complex, and, as a result the phosphorylation of Rb is downregulated at the Ser780 and 795 sites so that Rb-positive cells arrest in the G1-phase of the cell cycle.<sup>22</sup> We confirmed the activity of palbociclib as a highly selective inhibitor of CDK4/6. Palbociclib efficiently deregulated the phosphorylation of CDK6 and Rb under either normoxic or hypoxic conditions (Fig 3). However, Hypoxia induced Rb phosphorylation and attenuated palbociclib-induced Rb phosphorylation at a time-dependent manner. We further investigated the anti-tumor mechanisms of palbociclib in CRC by western blotting. Our previous studies showed that CDK inhibitors can destabilize HIF-1 $\alpha$  regardless of the presence of hypoxia and the combinatorial inhibition of GSK-3 $\beta$  and CDK1 kinases could reverse the apoptotic resistance of hypoxic CRC cells.<sup>16,17</sup> We found that palbociclib destabilizes HIF-1 $\alpha$  under either normoxic or hypoxic conditions (Fig 3). Palbociclib reduced GSK-3 $\beta$  expression under hypoxia and activated Akt



**Figure 2.** Effects of Palbociclib on cell death and cell cycle in CRC cell lines. RKO colon cancer cells were treated at the indicated time points with palbociclib (10  $\mu\text{M}$ ) under normoxic (A and C) or hypoxic (B and D) conditions. Representative curves are shown in the upper panels (A and B). Bar diagrams (C and D) represent Sub-G1 distribution within the cell cycle profiles. Data are shown as Mean  $\pm$  SD (n = 3, Student's t-test, 2-tailed, <sup>\*\*\*</sup> represents P < 0.05, <sup>\*\*\*\*</sup> represents P < 0.01) (E) Immunoblotting shows cleavage of apoptosis-related proteins with or without palbociclib under either normoxia (Left panels) or hypoxia (Right panels). Cell death and cell cycle alterations are shown using flow cytometry. Cell death after treatment of 48 hours under hypoxia was significant. Thus there were not enough cells to support cell cycle analysis (B).

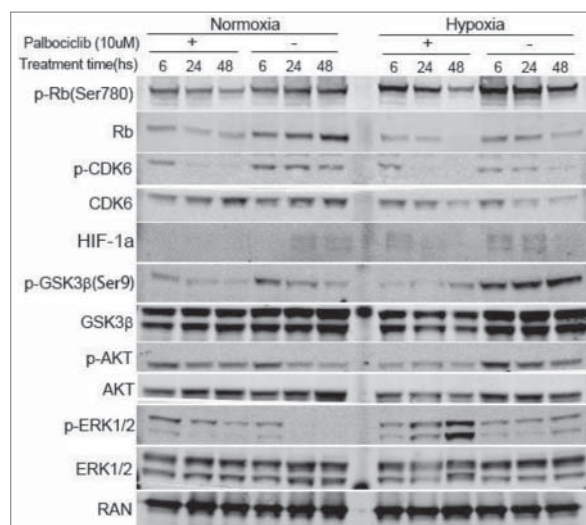
signaling under normoxia (Fig 3), although these roles of palbociclib were not obvious under normoxia for GSK-3 $\beta$  or hypoxia for Akt (Fig 3). Furthermore, palbociclib inactivated inhibitory GSK-3 $\beta$  signaling under normoxia and simultaneously activated ERK1/2 signals in an identical time-dependent manner under hypoxic conditions. Thus, the trends for palbociclib effects on ERK1/2 and GSK-3 $\beta$  were different under hypoxia vs. normoxia (Fig 3).

### Palbociclib synergizes with CPT11 and reduces the viability of CRC cells under hypoxia

There are previous reports combining palbociclib with 5-FU and oxaliplatin in solid malignancies (NCT01522989) or with cetuximab in head and neck cancer under normoxia.<sup>6,7</sup> Our assessment of IC<sub>50</sub> values indicated that the CDK4/6 inhibitor palbociclib reduces the viability of CRC cells regardless of the presence of hypoxia (Fig 1). 5-FU, oxaliplatin and irinotecan are the backbone standard chemotherapy agents used to treat patients with advanced CRC. We assessed the therapeutic effect of palbociclib alone or in combination with these chemotherapy agents under hypoxia *in vitro*. To quantitatively analyze the combinatorial effects of palbociclib with these conventional CRC chemotherapies, we constructed isobolograms using palbociclib, 5-FU, oxaliplatin and irinotecan. The data shows that palbociclib can synergize with a variety of chemotherapy agents *in vitro* under hypoxia (Fig. 4A, B, C and D). Among these chemotherapy agents, CPT11 (irinotecan) showed a highly significant synergy with palbociclib in CRC cell lines at various doses under hypoxia (Fig 4A and B). However, the underlying mechanism of palbociclib synergy with CPT11 is not clear.

### Palbociclib synergy with CPT11 and cell death occurs through a variety of mechanisms under hypoxia

CPT11 (irinotecan), as a cytotoxic chemotherapeutic agent against CRC, is a potent and selective inhibitor of DNA topoisomerase I (Top1). CPT11 can induce DNA double-strand breakage and trigger a DNA-damage response leading to cell death. CPT11 in combination with other chemotherapies can improve therapeutic effects and reduce hematological and digestive toxicity by reducing the treatment dose of single drug in gastric cancer.<sup>25</sup> Our data shows that palbociclib promotes cell death at 10  $\mu$ M in CRC despite the presence of hypoxia (Fig 2). We assessed the effect of palbociclib in combination with CPT11 on cell death *in vitro* by DNA fragment analysis. The results show that palbociclib in combination with CPT11 significantly increases cell death under hypoxia after treatment of 24 hours, as compared with single drug treatment and control (Fig 5A and B). Combined administration of palbociclib with CPT11 significantly increases the ratio of caspase-cleaved protein/total protein after treatment of 24 hours, as compared with respectively single drug administration and control (Fig 5C). Immunoblotting showed that a single administration of CPT11 upregulates the phosphorylation of CDK6 and Rb, whereas addition of palbociclib to CPT11 treatment decreases the phosphorylation level of CDK6 and Rb under hypoxia regardless of whether CPT11 is administered (Fig. 5C). Single agent palbociclib or CPT11-combined with palbociclib



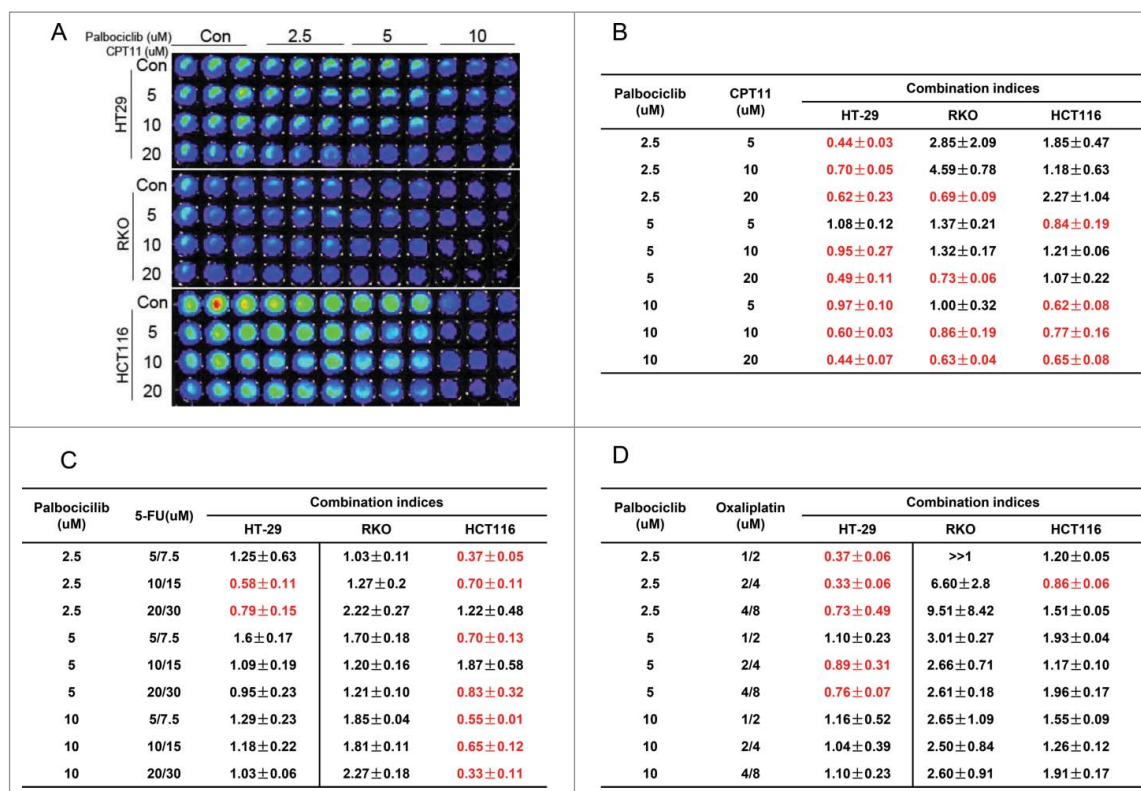
**Figure 3.** Palbociclib therapeutic effects against CRC cells via regulation of various cellular signaling pathways under normoxia and hypoxia. RKO colon cancer cells were treated at the indicated time points with palbociclib (10  $\mu$ M) under normoxia (Left panels) and hypoxia (Right panels). Whole cell lysates were separated in 4–12% SDS-PAGE gels.

significantly reduced HIF-1 $\alpha$  by 24 hours, as compared with single agent CPT11 or control (Fig. 5C). Palbociclib deregulated the phosphorylation of GSK-3 $\beta$  and upregulated the phosphorylation of ERK1/2 in a time-dependent manner regardless of the administration of CPT11 (Fig. 5C).

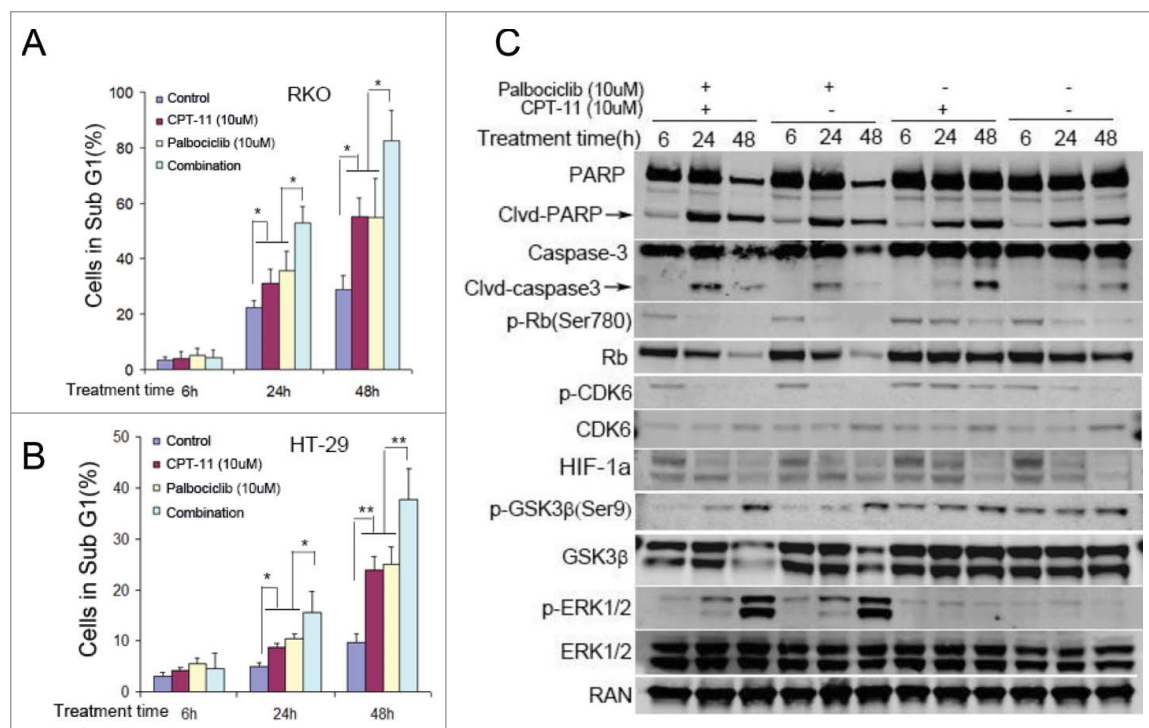
## Discussion

Our previous study indicated that CDK1 stabilizes HIF-1 $\alpha$  to promote tumor growth,<sup>17</sup> whereas inhibition of CDK1 increases the apoptotic sensitivity of hypoxic cells via deregulating HIF-1 $\alpha$ .<sup>16</sup> Our present data indicates that hypoxia has little impact on the sensitivity of CRC cells to palbociclib (Fig. 1). We found that palbociclib destabilizes HIF-1 $\alpha$  in colon cancer cells under either normoxic or hypoxic conditions (Fig. 3), suggesting that palbociclib may sensitize CRC cells to hypoxia-induced apoptotic resistance (Fig. 1). 5-FU is the backbone of chemotherapy against CRC. It has been reported that hypoxia induces 5-FU resistance in gastrointestinal cancer, whereas this role of hypoxia may be reversed via inhibition of a HIF-1 $\alpha$  signal regulating glycolysis.<sup>26</sup> Our previous study reported that hypoxia induces apoptotic resistance to 5-FU in CRC cells lacking p53.<sup>16</sup> We confirmed that hypoxia rapidly induces 5-FU resistance in diverse CRC cell lines (Fig 1). Irinotecan is also part of the backbone chemotherapy against advanced CRC, and hypoxia-induced accumulation of carboxylesterase increases tumor sensitivity to irinotecan (CPT-11).<sup>20</sup> CPT11 can reduce angiogenesis and tumor growth via inhibiting HIF-1 $\alpha$  protein accumulation or translation in hypoxic glioma cells.<sup>21</sup> We found that CPT11 exerts its therapeutic effect in CRC cells regardless of the presence of hypoxia (Fig. 1). Collectively, hypoxia-induced drug resistance represents a formidable issue in CRC therapy, and our findings shed light on a potential therapeutic strategy against hypoxic tumor cells.

High concentrations of palbociclib have been used to allow for tissue perfusion, where a significant increase in cleaved-caspase



**Figure 4.** Synergy assessment of palbociclib in combination with a variety of chemotherapeutic agents in CRC cell lines under hypoxia. HT-29, RKO and HCT-116 CRC cell lines were treated with the indicated concentrations of palbociclib in combination with CPT11 (A and B), 5-FU (C) or Oxaliplatin (D) for 48 hours in various non-constant ratios/doses. (A) Representative fluorescent images of palbociclib in combination with CPT11 in a panel of 3 CRC cell lines are shown. Combination index (CI) < 1 and > 1 respectively indicate synergism, additivity or antagonism. CI < 0.5 indicates very strong synergy. Red colored numbers highlight synergy at the indicated concentrations.



**Figure 5.** Palbociclib synergizes with CPT11 against CRC cell lines via deregulating CPT-11-induced Rb activation and further reducing HIF-1 $\alpha$  accumulation under hypoxia. RKO (A and C) and HT-29 (B) CRC cell lines were treated with or without palbociclib (10  $\mu$ M) in combination with CPT11 (10  $\mu$ M) at the indicated time points under hypoxia. Bar diagrams (A and B) depict the sub-G1 percentage in the cell cycle profiles. Data are shown as Mean  $\pm$  SD (n = 3, Student's t-test, 2-tailed,  $^{*}$  represents P < 0.05,  $^{***}$  represents P < 0.01). (C) Immunoblotting shows protein levels (as indicated) with or without chemotherapy under either normoxia (Left panels) or hypoxia (Right panels).

3-positive cells was observed in human HCC samples. Single agent palbociclib-treated mice showed a remarkable reduction in tumor volume, indicating that palbociclib treatment can be effective in liver tumors *in vivo*.<sup>24</sup> Our data indicates that palbociclib promotes apoptosis at 10  $\mu$ M regardless of the presence of hypoxia (Fig. 1 and 2). These findings suggest that the therapeutic effect and mechanism of palbociclib is more complex than what is presently understood. Glycogen synthase kinase-3 $\beta$  is an essential serine/threonine kinase in cell survival and NF- $\kappa$ B activation.<sup>27</sup> Inhibition of GSK-3 $\beta$  induces glioma cell death.<sup>28</sup> ERK1/2 upregulating pGSK-3 $\beta$  (Tyr216) mediates basic fibroblast growth factor (FGF)-induced apoptosis in neuroblastoma cells, whereas blockade of GSK-3 $\beta$  and ERK1/2 protects cells from FGF2-induced apoptosis.<sup>29</sup> Atractylenolide I (AT-I), owning the activities of p21activator and CDK2 inhibitor isolated from *Atractylodes macrocephala* rhizome, induces apoptosis and G1-S arrest in melanoma via deregulating pGSK-3 $\beta$  (Ser9), whereas blockade of GSK-3 $\beta$  reverses AT-1-induced apoptosis and G1-S arrest.<sup>30</sup> These findings are consistent with our finding that palbociclib promotes apoptosis in CRC cells, and simultaneously activates ERK1/2 signals and deregulates inhibitory pGSK-3 $\beta$  (Ser9). Moreover, palbociclib reduces GSK-3 $\beta$  expression under hypoxia (Fig. 3), which may explain why more CRC cell death is observed under hypoxia (Fig. 2). In addition, we found that hypoxia induced Rb activation and reduced the inhibition of palbociclib on Rb phosphorylation (Fig 3). Consequently, hypoxia may delay palbociclib-induced G1-phase arrest (Fig. 2). We also found that palbociclib can arrest cells in the G2-phase even prior its noted effect of G1-phase arrest (Fig. 2). This is not consistent with a prior study,<sup>22</sup> and the reason could be one 24-hour time point was evaluated and different cell lines were previously used. P276-00, a cyclin-dependent kinase inhibitor, inhibits HIF-1 $\alpha$  and induces G2/M arrest under hypoxia in prostate cancer cells.<sup>31</sup> Some studies have reported that ERK- or JNK/MAPK activation induces G2/M phase arrest and apoptosis in solid tumors.<sup>32-36</sup> We found that palbociclib destabilizes HIF-1 $\alpha$  and activates ERK1/2 under either normoxic or hypoxic conditions (Fig. 3). These mechanisms could explain the effects of palbociclib on G2/M phase arrest and apoptosis in CRC cells (Fig. 1 and 2).

Numerous studies and clinical trials have indicated that palbociclib synergizes with conventional chemotherapy or targeted agents and improves the efficacy of tumor treatment.<sup>5-11</sup> We analyzed the combinatorial effects of palbociclib with the commonly used chemotherapy agents against CRC, as a prelude to possible translation. Chou-Talalay analysis revealed that palbociclib can synergize with a variety of chemotherapies under hypoxia (Fig. 4). Among these chemotherapy agents, irinotecan showed comprehensive and often potent synergy with palbociclib in CRC cells with diverse molecular genetic sub-types under hypoxia. We found that the combinatorial administration of irinotecan with palbociclib further reduced HIF-1 $\alpha$  in hypoxic CRC cells (Fig. 5). Our finding is consistent with studies indicating that irinotecan synergizes with a variety of chemotherapies via regulating HIF-1 $\alpha$  signaling in hypoxic tumor cells<sup>37,38</sup> Additionally, we found that single agent irinotecan administration increases Rb phosphorylation, whereas addition of palbociclib reduces irinotecan-induced Rb phosphorylation. Thus, palbociclib addition can reverse irinotecan-induced cell-cycle progression in hypoxic CRC cells (Fig. 5).

Our studies have translational significance and suggest possible testing of a CDK4/6 inhibitor in combination with CPT11 (irinotecan) in CRC therapy. The observed effects and mechanistic insights involving HIF-1 $\alpha$ , pRb, GSK-3 $\beta$ , Akt, and ERK1/2 further support the proposed clinical trial rationale. In particular the strategy combining a CDK4/6 inhibitor with irinotecan is expected to target both normoxic as well hypoxic tumor cell populations that may contribute to conventional therapy resistance or tumor relapse. The combination of a CDK4/6 inhibitor plus irinotecan (CPT11) merits further investigation in the clinic for patients with advanced colorectal cancer and potentially other tumor types.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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This work was presented in part at the 2016 annual AACR meeting in New Orleans. W.S.E-D. is an American Cancer Society Research Professor.

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