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Original Article

AZD7648, a DNA-PKcs inhibitor, overcomes radioresistance in Hep3B xenografts and cells under tumor hypoxia

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Abstract: Radiation therapy is one of the most commonly used cancer treatments. However, it has important concerns such as damage to normal tissues around cancers and radioresistance. To overcome these problems, combination therapy using radiosensitizer and radiotherapy will be a good alternative. The present study investigated the effects of AZD7648 on overcoming radioresistance as well as radiosensitizing in Hep3B xenografts and cells. The results showed that AZD7648 enhanced ionizing radiation (IR)-induced tumor growth not only in radiosensitive but also radioresistant tumors. In particular, the combination of AZD7648 with radiation reduced the expression of hypoxia induce factor-1α (HIF-1α) in radioresistant tumors. In vitro studies, AZD7648 plus IR increased IR-induced G2/M arrest and regulated cell cycle checkpoints such as cyclinB1, p-cdc2 in normoxia but not in hypoxia. AZD7648 induced more radiation-mediated ROS than radiation only under normoxia, but these ROS were not altered by AZD7648 under hypoxia. Interestingly, AZD7648 downregulated HIF-1α expression level under $CoCl_2$-treated hypoxic condition but not in normoxic condition. In conclusion, AZD7648 synergistically increased radiosensitivity through accumulating IR-induced G2/M arrest and further improved radioresistance via regulation of HIF-1α. The present data suggest that AZD7648 may be a strong radiosensitizer in radioresistant as well as radiosensitive cancers.

Keywords: AZD7648, cancer, HIF-1α, hypoxia, ionizing radiation, radioresistance, radiosensitizer, xenograft mouse

Introduction

Cancer is the second leading cause of death worldwide, with increasing incidence and mortality, and more than 50% of cancer patients are currently receiving radiation therapy. Radioresistance has become a major problem in radiation therapy due to various factors and has not been resolved to date. Radioresistance is induced by DNA damage repair, cell cycle arrest, oncogene, tumor suppressor alterations, changes in the tumor microenvironment (TME), autophagy, the generation of cancer stem cells, and tumor metabolism [1]. Hypoxia, in particular, is a major cause of radioresistance among changes in the TME. The tumors have hypoxic conditions, and radiation therapy-mediated changes in TME induce regions of cycling hypoxia [2]. When early tumors grow, the rapid tumor cell proliferation is not consistent with the growth of surrounding blood vessels that supply nutrients and oxygen to the cells inside the tumor [3]. Thus, persistent hypoxia occurs in tumors and consequently activates angiogenic signals; furthermore, these blood vessels become abnormal in their function and structural morphology. Abnormal tumor vessels negatively impact the immune system and the transport of chemotherapy and oxygen [4, 5]. Therefore, these studies prove that tumor hypoxia can induce resistance to chemotherapeutics and radiotherapy. Prolyl hydroxylases (PHD) regulated by O_2, Fe^{2+}, α-ketoglutarate, and ascorbic acid controls the hydroxylation of HIF-1α [6]. Under normoxic conditions, PHD recognizes oxygen and hydroxylates

hypoxia induce factor-1α (HIF-1α) under sufficient oxygen. Von Hippel-Lindau (VHL) recognizes the hydroxyl group of HIF-1α bound by PHD2 and initiates ubiquitination and proteasome degradation of HIF-1α [7]. Conversely, the tumor microenvironment becomes hypoxic, and oxygen that can use PHD2 is insufficient; therefore, the hydroxylation of HIF-1α is inhibited, and then HIF-1α accumulates. Moreover, HIF-1α that was accumulated under hypoxia enters the nucleus by forming HIF-1β and HIF-1α heterodimers. The heterodimeric HIF-1α:HIF-1β upregulates transcription for a variety of factors that generate radiation resistance, such as vascular endothelial growth factor (VEGF) in the hypoxia-responsive elements (HRE) [8-10]. Therefore, to overcome radioresistance caused by hypoxia, research on radiosensitizers that can reduce the resistance and make tumors more sensitive to radiation is needed.

AZD7648 is a representative of an oral DNA-dependent protein kinase (DNA-PK) inhibitor made by AstraZeneca and has been studied as a radiosensitizer and chemosensitizer [11]. AZD7648 consistently inhibits tumor growth in xenograft and patient-derived xenograft (PDX) models as an efficient radiation and doxorubicin sensitizer, a combination that causes more DNA damage to the tumor and induces tumor cell death. AZD7648, when used in combination with the poly (ADP-ribose) polymerase inhibitor olaparib, causes ATM-deficient cells to increase genomic instability, inhibiting growth and inducing apoptosis [12]. A study on syngeneic mice reported that AZD7648 combined with radiation reduced T-cell and PD-1 expression, increased NK-cell granzyme B expression, and elevated type I IFN signaling. Studies on the relationship between DNA-PKcs and hypoxia exist but are incomplete [13]. HIF-1 recruits DNA-PK to HRE in hypoxic breast cancer cells, and DNA-PK activation regulates cellular oxygen detection and oxygen signaling pathways by hypoxia [14, 15]. There are still few studies on the relationship between DNAPKcs and hypoxia. AZD7648 has been established as a radiosensitizer; however, there are no studies related to hypoxia.

Accordingly, this study confirmed two points: 1) whether AZD7648 alleviated radioresistance, focusing on the regulation of HIF-1α, and 2) whether AZD7648 enhanced the radiation response at lower concentrations.

Materials and methods

Drug

AZD7648 (7-Methyl-2-[(7-methyl[1,2,4]triazolo[1,5-a]pyridin-6-yl)amino]-9-(tetrahydro-2H-pyran-4-yl)-7,9-dihydro-8H-purin-8-one) were purchased from LEAP Chem (Hong Kong, China). AZD7648 is an oral DNA-PKcs inhibitor produced by AstraZeneca and has been studied as a radiation and chemosensitizer. AZD-7648 induces apoptosis and exhibits antitumor activity, with a molecular weight of 380.40, and molecular formula CC1=CC2=NC=NN2C=C1NC3=NC=C(N(C)C(N4C5CCOCC5)=O)C4=N3 [11]. AZD7648 was dissolved at 5 mM in DMSO and stored at 4°C.

Antibody

Anti-phospho-DNA-dependent protein kinase, catalytic subunit (p-DNA-PKcs, ab18192), anti-VHL (ab140989), horseradish peroxidase (HRP) linked goat anti-rabbit IgG antibodies (ab97-057) and HRP-linked goat anti-mouse IgG antibodies (ab6789) were purchased from Abcam (Cambridge, UK). Anti-HIF-1α (sc-13515), anti-VEGF (sc-7269), and anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, sc-257-78) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-DNA-PKcs (4602s), anti-cyclinB1 (4138s), anti-p-cdc2 (9111s), Chk1 (2360s), and p-Chk1 (2348s) were provided by Cell Signaling Technology (CST) (Danvers, Massachusetts, USA).

Cell line and cell culture

Hep3B human hepatocellular carcinoma cell line obtained from the Korean Cell Line Bank (Cat. no. 88064, Seoul, South Korea) and was cultured in DMEM (Welgene, Republic of Korea) containing 10% FBS (Welgene, Republic of Korea), 1% penicillin-streptomycin (Welgene, Republic of Korea) and grown in a 37°C incubator with 5% CO_2.

Irradiation

A ^{60}Co γ-ray source (Gammabam 100-80, Atomic Energy of Canada, Chalk River, Canada) was used for xenograft mice at a dose rate of

0.98 Gy/min. The tumor of xenograft mice was covered with a 0.5 cm bolus and irradiated locally. For in vitro studies, the cells were irradiated using a ^{137}Cs γ-ray source (Biobeam 8000, Gamma-Service Medical GmbH, Leipzig, Germany) at a dose of 1.35 Gy/min.

Tumor xenograft study

Balb/c-nu/nu nude male mice (Orient Bio Inc., Seongnam-si, Gyeonggi-do, South Korea) were used in the study. We were provided with 4-week-old mice, and the mice acclimatized for a week. After the mice were acclimatized, they were injected with 3×10^6 Hep3B cells subcutaneously in the right thigh. The day after Hep3B cell transplantation, the mice were randomly divided into the vehicle and AZD7648 groups. A concentration of 50 mg/kg AZD7648 was administered orally once a day. When the average tumor volume of the vehicle group reached approximately 180 mm^3, mice were randomized into 6 groups: CON (control), AZD (AZD7648), IR (Ionizing radiation), AZD+IR, rIR (resistance IR), and AZD+rIR (resistance AZD+IR), and then IR and AZD+IR group were irradiated with a fraction dose of 2 Gy × 5 times. rIR and AZD+rIR mice were irradiated with the single 8 Gy dose when the average tumor volume of the rIR group reached approximately 300 mm^3. The tumor size of mice was measured twice a week, and the experiment was ended when the average tumor volume of the control group was 2,000 mm^3. After ending, mice were euthanized by carbon dioxide (CO_2) inhalation, and then tumor in mice was extracted.

Immunohistochemistry (IHC)

Tissue paraffin sections (4 μm) were de-paraffinized and attached to coated slides with the block number recorded. Peroxide blocking was performed for 10 min, and protein-blocking serum was applied to the slides for 1 h. Primary antibodies such as HIF-1α (1:100), VHL (1:150), and VEGF (1:200) were diluted according to the manufacturer's protocol and reacted on slides overnight at 4°C. Slides were incubated with secondary antibody for 30 min at room temperature, and DAB staining was performed for 5 min while checked by microscopy. Hematoxylin was used for counterstaining, and histological scores for HIF-1α, VHL, and VEGF expression were measured by Image J (version 1.53a, Bethesda, MD, USA) as a percentage of the positive staining area of the total tissue area.

Water-soluble tetrazolium (WST-1) assay

Cells were treated with a concentration gradient (0, 0.025, 0.1, 0.5, 1, and 5 μM) of AZD7648 for 24 h. After the treatment, the cell culture medium was added 20 μM of WST-1 reagent (EZ-3000, DoGenBio, Seoul, Republic of Korea) and incubated for 2 h. Cell viability was measured by spectrophotometer with a wavelength of 450 nm and then analyzed as a percentage of absorbance relative to controls.

Colony forming assay

To assess cell toxicity by AZD7648, 300-1200 cells were seeded into cell culture plates and treated with 0-5 μM AZD7648 for 24 h. For radiosensitivity testing, 300-2400 cells were treated with 0.01, 0.05, 0.1 μM AZD7648 for 24 h, followed by irradiation, and then further incubated for 24 h. At 24 h after irradiation, the cell medium was replaced with a new one. The colonies generated for approximately 10 days were stained with 0.4% crystal violet. The plating efficiency (PE) means the ratio of cells that grew into colonies to cells seeded under specific conditions. The survival fraction was calculated as follows: survival fraction = colonies counted/(cells seeded × PE/100).

Cell cycle analysis (flow cytometry)

Hep3B cells were pre-treated with AZD7648 (0.1 μM) 24 h before irradiation and harvested. The cells were immediately fixed with cold ethanol, stained with BD PI/RNase staining buffer (Cat. no. 550825, BD Pharmingen), and analyzed using CytoFLEX flow cytometer (Beckman Coulter, Brea, California, USA). Each sample was measured based on 10,000 cells, and data analysis was performed using CytExpert software (version 2.3, Beckman Coulter).

Measurement of intracellular ROS (flow cytometry)

The analysis of ROS levels was performed using cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Cat. no. D399, Thermo Fisher, USA). Hep3B cells were initially cultured and maintained in a 37°C incubator with 5% CO_2. After overnight incubation, the cells were treated with 0.1 μM of AZD7648 and subsequently supplemented with $CoCl_2$ at 18 h after AZD7648 treatment. After the $CoCl_2$ treatment, the medium was supplemented with 10

μM of H_2DCFDA and incubated for 30 min at 37°C under 5% CO_2 conditions. The cells were then harvested using trypsin, and the fluorescence intensity of FITC-ROS was measured using a CytoFLEX flow cytometer.

Western blot

The cells were lysed with RIPA buffer, and cell proteins were separated each size through the sodium dodecyl sulfate-polyacrylamide gel electrophoresis step. The proteins on the gel were transferred to 0.45 and 0.2 μM pore polyvinylidene fluoride; it was incubated with a blocking buffer (Cat. no. 12010020, Biorad) for the blocking step. The first antibody was incubated overnight at 4°C, and the second antibody worked for 1 h. The proteins on the membrane were revealed with Enhanced Chemiluminescence western blotting substrate using the Chemi fluoro imager (CAS-400SM, Davinch-K, Seoul, South Korea), and signal intensity of bands was measured.

Immunocytochemistry (ICC)

Cells were treated with 0.1 μM AZD7648 for 24 h, 500 mM $CoCl_2$ (Cat. no. 232696-5G, Sigma) was added at 18 h after AZD7648 exposure, and then cells were harvested. After fixing with 4% paraformaldehyde and blocking with BSA, the HIF-1α antibody was reacted overnight at 4°C. The cells were incubated with FITC-labeled goat anti-mouse IgG for 1 h, and nuclei were stained with DAPI. Coverslips were mounted and analyzed by fluorescence microscopy (AxioImager.M2, Zeiss, Jena, Germany).

Statistical analysis

The *in vivo* data were expressed as the mean ± standard error of the mean (S.E.M.), and the *in vitro* data were presented as the mean ± standard deviation (S.D). All data were analyzed using one-way ANOVA, followed by Tukey HSD test and Bonferroni (SPSS 23 software, Chicago, Illinois, USA), except for ROS measurement data that analyzed student *t*-test. Statistical significance was established at the level of $P<0.05$.

Ethical approval and consent to participate

All animal studies followed the National Research Council guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korean Institute of Radiological and Medical Sciences (Kirams2021-0052).

Result

AZD7648 synergistically increased radiosensitivity and improved radioresistance in Hep3B xenograft mice

We confirmed whether AZD7648 could enhance radiation response using the Hep3B xenograft mouse model. The individual mice were subcutaneously injected with Hep3B cells into the right thigh, and AZD7648 (50 mg/kg) was administered orally once daily. When the average tumor volume of the vehicle group reached approximately 180 mm^3, mice were randomly grouped into CON, AZD, IR, AZD+IR, rIR, and AZD+rIR. To analyze the effect of AZD7648 according to different radiosensitivity of xenografts, each IR plan was established: radiosensitive xenografts (At grouping day, 2 Gy × 5 times) and radioresistant xenografts (at the mean tumor volume≒300 mm^3, single 8 Gy) (**Figure 1A**). All groups showed no change in body weight compared with CON, indicating no toxicity of AZD7648 and/or IR.

In radiosensitive xenografts, IR and AZD+IR groups significantly suppressed the average tumor volume compared with the CON group but AZD by itself did not affect tumor volume (AZD, 2.15%; IR, 31.35, $P<0.05$; AZD+IR, 65.31%, $P<0.001$) (**Figure 1B**). AZD+IR group strongly inhibited the tumor growth compared with IR group ($P<0.01$) (**Figure 1B**). Extracted tumor weights of xenograft mice were similar tendency to tumor growth inhibition (**Figure 1C** and **1D**). Accordingly, AZD7648 acted synergistic tumor suppression effect together with IR in radiosensitive xenografts. Furthermore, to investigate whether AZD7648 reduced tumor growth in radioresistant xenografts, we established a radioresistant xenograft mouse model that was produced by irradiation after the tumor had overgrown; when the tumor volume reached approximately 300 mm^3, xenograft mice were locally irradiated with single 8 Gy. Compared with the CON group, there was no difference in the tumor growth in the rIR groups; this result showed xenograft mice in the rIR group were radioresistant. However, the AZD+rIR group significantly inhibited tumor growth compared with the rIR group ($P<0.001$) (**Figure 1B**). As

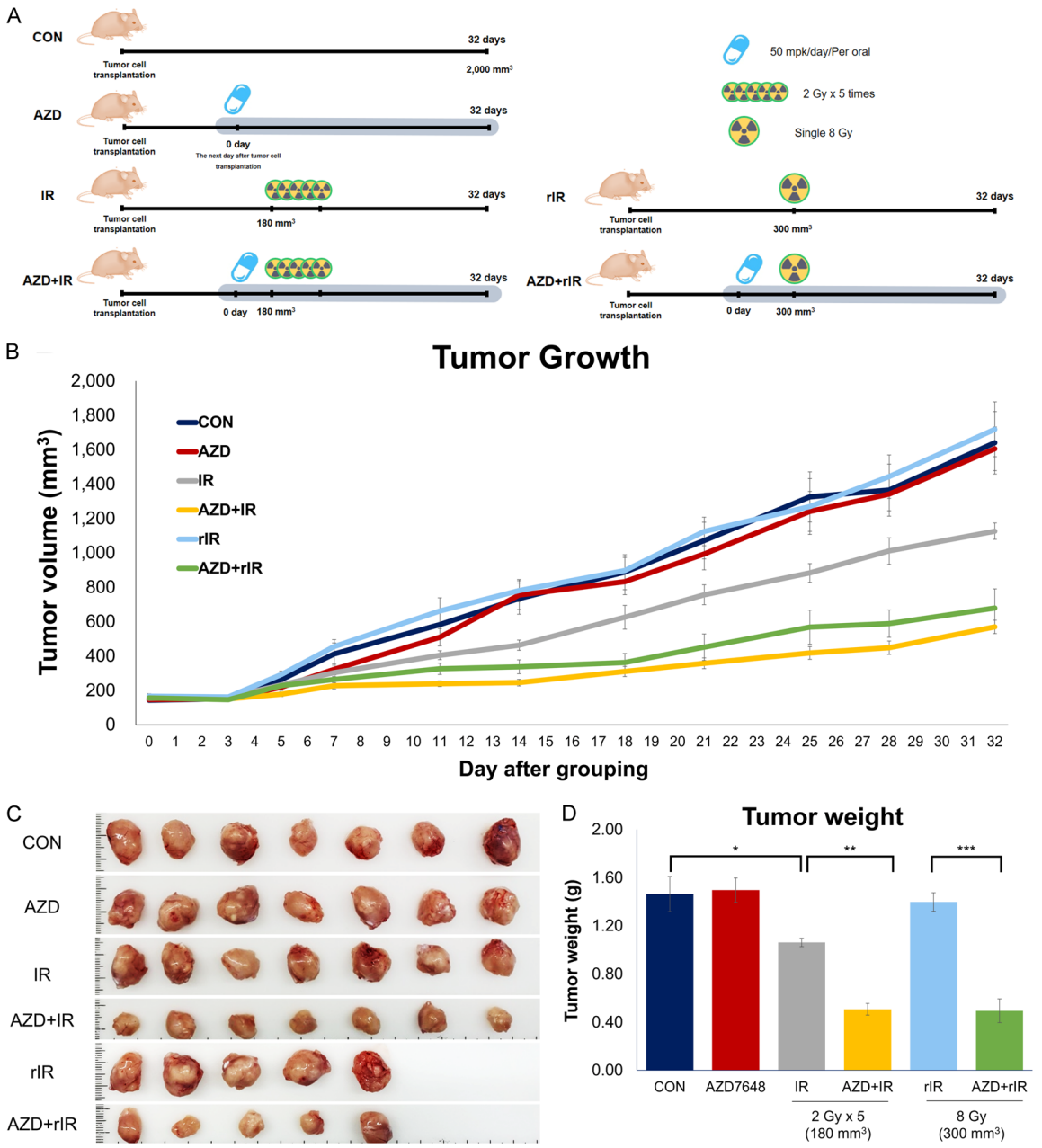


Figure 1. AZD7648 combined with IR inhibited tumor growth. Balb/c-nu/nu mice were inoculated with Hep3B cells (n=5~7). A. Xenograft's study followed this schedule. B. The tumor size of all mice was measured twice a week, and tumor volume was calculated as [(length) × (width)^2] × (π/6). C. The tumors extracted from all mice are listed. D. The graph shows extracted tumor weights. Values represent the mean ± S.E.M of 5-7 mice per group. ($^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001).

shown in **Figure 1C**, it was confirmed through photographs of extracted tumors. Consistent with the tendency of the tumor volume, tumor weights showed no difference among CON, AZD, and rIR groups; however, the combination group had a 64.66% tumor suppression effect compared with the CON group (P<0.001). Consequently, AZD7648 elevated radiation response in radioresistant xenografts. In summary, these results indicated AZD7648 enhanced radiation response in both radiosensitive and radioresistant xenografts.

AZD7648 elevated radiosensitivity in Hep3B cells without the anti-cancer effect of AZD7648 alone

Using WST-1 and colony-forming assay, we determined the suitable AZD7648 concentra-

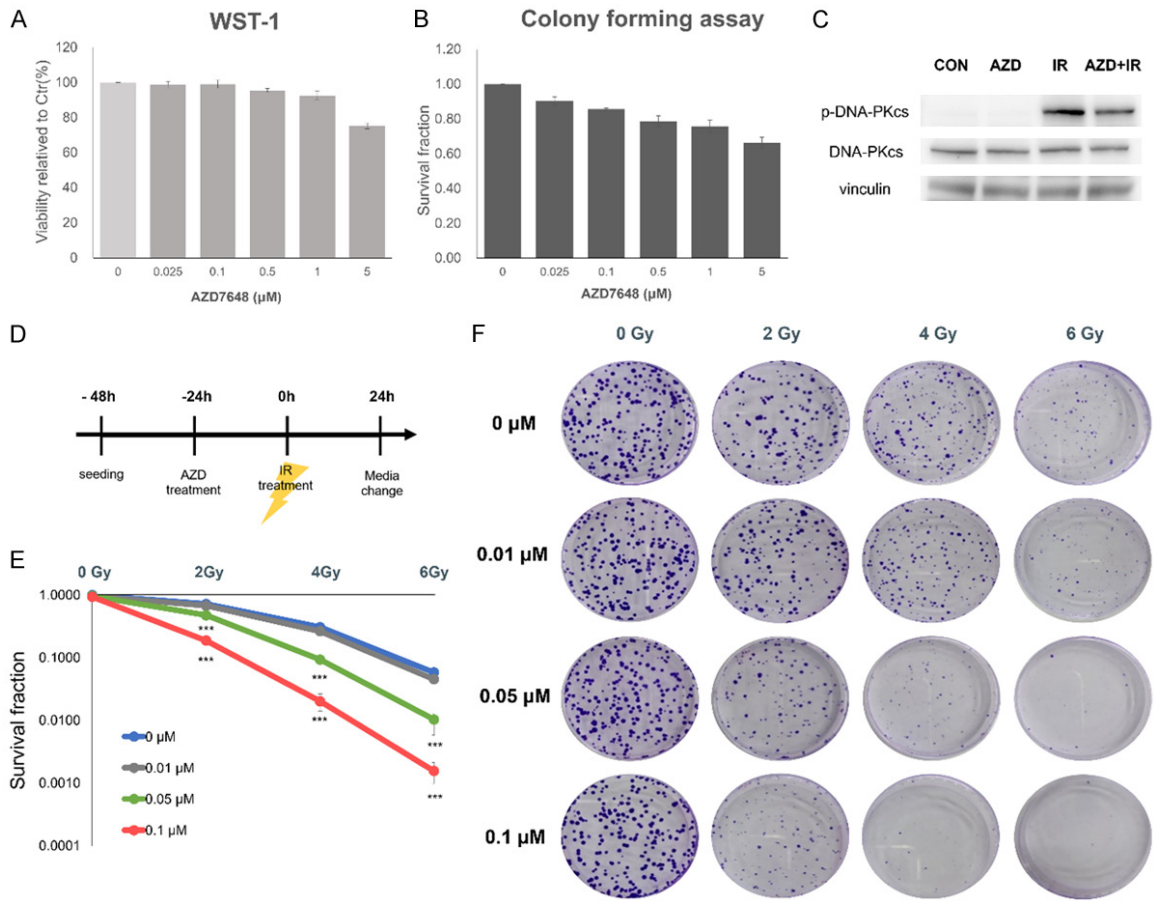


Figure 2. AZD7648 combined with IR inhibited cell proliferation of Hep3B cells in a dose-dependent manner with no cell toxicity. A. Cell viability was measured by WST-1 assay. B. Clonogenic survival fraction with only AZD7648 carried out using colony forming assay was calculated as follows: survival fraction = colonies counted/(cells seeded × PE/100). Values represent the mean ± S.D of three experiments. C. The DNA-PKcs inhibiting ability of AZD7648 was identified. D-F. These data showed the quantification graph of clonogenic survival fraction (***P<0.001 versus IR alone) and representative pictures for colony formation. Values represent the mean ± S.D of four experiments.

tion for *in vitro* experiments and assessed the anti-cancer effect of AZD7648. After Hep3B cells were treated with AZD7648 at 0.025, 0.1, 0.5, 1, and 5 μM for 24 h, we carried out WST-1 and colony-forming assays. Cell viability and clonogenic survival fraction were measured at more than 80% at concentrations up to 5 μM AZD7648 (**Figure 2A** and **2B**). Thus, AZD7648 alone was not cytotoxic in Hep3B cells.

Furthermore, to evaluate the radiosensitizing effect of AZD7648, we treated Hep3B cells with 0.01, 0.05, and 0.1 μM AZD7648 for 24 h, followed by IR (2, 4, and 6 Gy) and then replaced with the culture medium at 24 h after IR. Clonogenic survival fraction significantly reduced in 0.05 and 0.1 μM AZD7648-treated cells following IR as compared with that of cells irradiated without AZD7648 (P<0.001) (**Figure 2D-F**). Moreover, the combination of 0.1 μM AZD7648 and IR reduced IR-induced phospho DNA-PKcs expression (**Figure 2C**). Therefore, we found that AZD7648 sensitized Hep3B cells to radiation in a dose-dependent manner with no cell toxicity.

AZD7648 accumulated IR-induced G2/M arrest under normoxia but not under hypoxia

We investigated whether AZD7648 affected IR-related cell cycle progression under normoxia and hypoxia. Hep3B cells were pre-treated with 0.1 μM AZD7648 for 24 h, 500 mM $CoCl_2$ was added at 18 h after AZD7648 treatment (in hypoxic condition), irradiated at 6 Gy, fixed 24 h after IR, and then analyzed by flow cytometry for cell cycle or western blot for the associated proteins. As shown in **Figure 3A** and **3B**, the

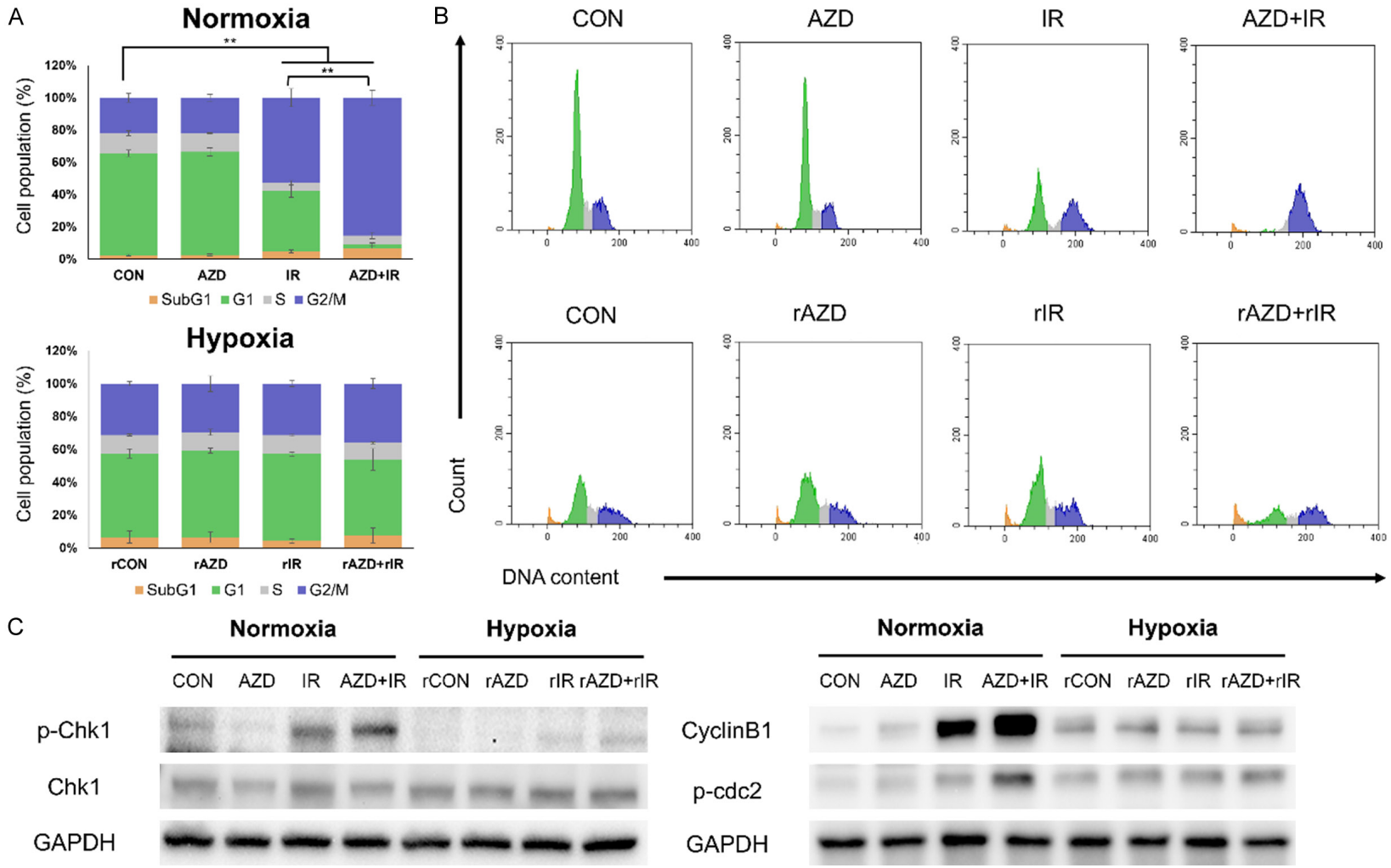


Figure 3. AZD7648 induced G2/M arrest under normoxia, but not at hypoxia. A, B. Graphs and histograms show Hep3B cell cycle changes, specifically G2/M arrest upon AZD7648 and IR treatment (**P<0.01). C. Western blot analysis detected the expression of proteins for cyclinB1, p-cdc2, and p-Chk1, proteins involved in the cell cycle. Values represent the mean ± S.D of three experiments.

combination of AZD7648 and IR strongly increased G2/M arrest compared with IR alone in normoxia (P<0.001), whereas the proportion of G2/M phase did not differ among all groups in the $CoCl_2$-treated hypoxic condition. Additionally, compared with IR, which increased the G2/M phase in normoxia, AZD7648 combined with IR increased expression levels of G2/M phase-related proteins, including cyclin B1 and p-cdc2 in normoxia, but not in hypoxia (**Figure 3C**). Moreover, AZD7648 significantly elevated IR-induced p-Chk1 protein level in normoxia, but not in hypoxia. Our findings demonstrated that AZD7648 plus IR caused IR-induced G2/M arrest more strongly than IR-induced G2/M arrest under normoxia but not under hypoxia.

AZD7648 accelerated IR induced intracellular ROS, while its effects were not altered under hypoxic conditions

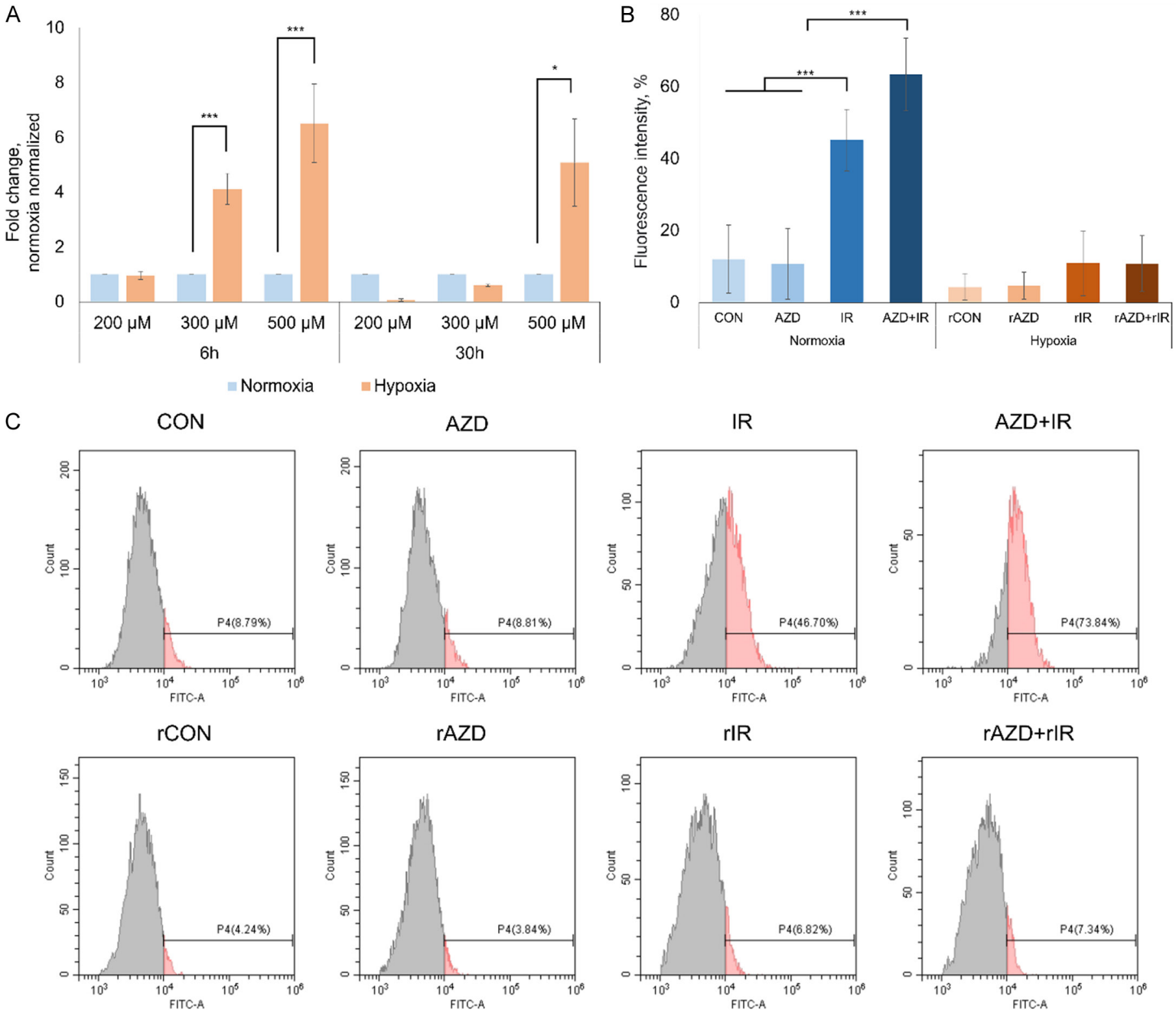
Furthermore, we assessed whether AZD7648 affected intracellular ROS in Hep3B cells under normoxic and hypoxic conditions. First, we determined the suitable hypoxic condition by treatment of various $CoCl_2$ concentrations for 6 and 30 h in Hep3B cells. At 6 h after treatment with $CoCl_2$ at the concentrations 200, 300, and 500 μM, intracellular ROS in Hep3B was increased at 300 and 500 μM $CoCl_2$ compared with the untreated group, but not 200 μM $CoCl_2$ (**Figure 4A**). In addition, at 30 h after treatment of $CoCl_2$, the ROS level of 300 μM $CoCl_2$ treatment group was similar to the untreated group, and that of 500 μM $CoCl_2$ treatment group was increased compared with the untreated group (**Figure 4A**). Therefore, 300 μM $CoCl_2$ in Hep3B cells was selected as the suitable hypoxic condition. Further, we demonstrated that the combined effect of IR and AZD7648 on intracellular ROS in Hep3B cells was compared under normoxic and hypoxic conditions. Under normoxic conditions, IR-induced intracellular ROS and AZD7648 plus IR increased ROS levels compared with CON (CON, 12.07%; AZD, 10.73%; IR, 45.25%, P<0.001; AZD+IR, 63.38%, P<0.001), however, AZD7648 alone remained unchanged. While, the ROS level of the combination AZD7648 and IR was similar to that of IR under hypoxic conditions (CON, 4.28%; AZD, 4.61%; IR, 10.90%; AZD+IR, 10.78%) (**Figure 4B** and **4C**). These results revealed that AZD7648 modulates IR-induced intracellular ROS under normoxic but not in hypoxic conditions.

AZD7648 reduced radioresistance by regulating the HIF-1α pathway in Hep3B xenograft mice

Radioresistance occurs for a variety of reasons in tumor microenvironments, including the immune system and hypoxia. Among these diverse causes, we focused on tumor hypoxia. Therefore, we evaluated the effect of AZD7648 on the regulation of HIF-1α as a hypoxia marker by immunohistological analysis of extracted xenografts in CON, AZD, rIR, and AZD+rIR groups. As shown in **Figure 5A** and **5B**, the HIF-1α and VEGF expression markedly increased in the rIR group compared with in CON group (P<0.05), and the expression of HIF-1α and VEGF in AZD+rIR group was significantly less than in rIR group (P<0.05). On the contrary, the VHL expression level of rIR was the lowest in all groups, and the expression level of AZD+rIR was higher than that of rIR. Therefore, AZD7648 sensitized radioresistant xenografts to radiation response by downregulating HIF-1α and VEGF along with upregulating VHL.

AZD7648 inhibited HIF-1α and PHD expressions in Hep3B cells under $CoCl_2$-induced hypoxia

Through our tumor xenograft study, we found that AZD7648 sensitized radioresistant xenografts to radiation response; furthermore, AZD7648 plus IR reduced IR-induced HIF-1α through upregulation of its degradation-related proteins. However, the effect of AZD7648 under hypoxic conditions was not detected. Thus, using an immunocytochemistry stain, we further examined whether AZD7648 affected HIF-1α and its degradation-related proteins under $CoCl_2$-treated hypoxic conditions. According to these results, the rCON group under $CoCl_2$-treated hypoxic condition had higher HIF-1α (P<0.05) and PHD protein levels than those in normoxia had (**Figure 6A**). AZD group in hypoxia alleviated HIF-1α and PHD protein levels overexpressed by $CoCl_2$ treatment, but not in normoxia (**Figure 6B** and **6C**). These data demonstrated AZD7648 enhanced radiation response in radioresistant hypoxic tumor cells via the reduction of upregulated HIF-1α and PHD proteins.


A
Fold change, normoxia normalized
200 μM
300 μM
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6h
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B
Fluorescence intensity, %
CON
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FITC-A

Figure 4. AZD7648 increased IR-induced ROS under normoxia but not under hypoxia. In both normoxic and hypoxic conditions, intracellular ROS was measured using H_2DCFDA. (A) Variable concentration of $CoCl_2$ induced intracellular ROS at 6 h and 30 h after treatment (*P<0.05, ***P<0.001). (B, C) The combined effect of AZD7648 and IR was compared with other groups (CON, AZD, IR) under both (A) normoxic and (B) hypoxic conditions (***P<0.001 versus CON). Values represent the mean ± S.D of three experiments.

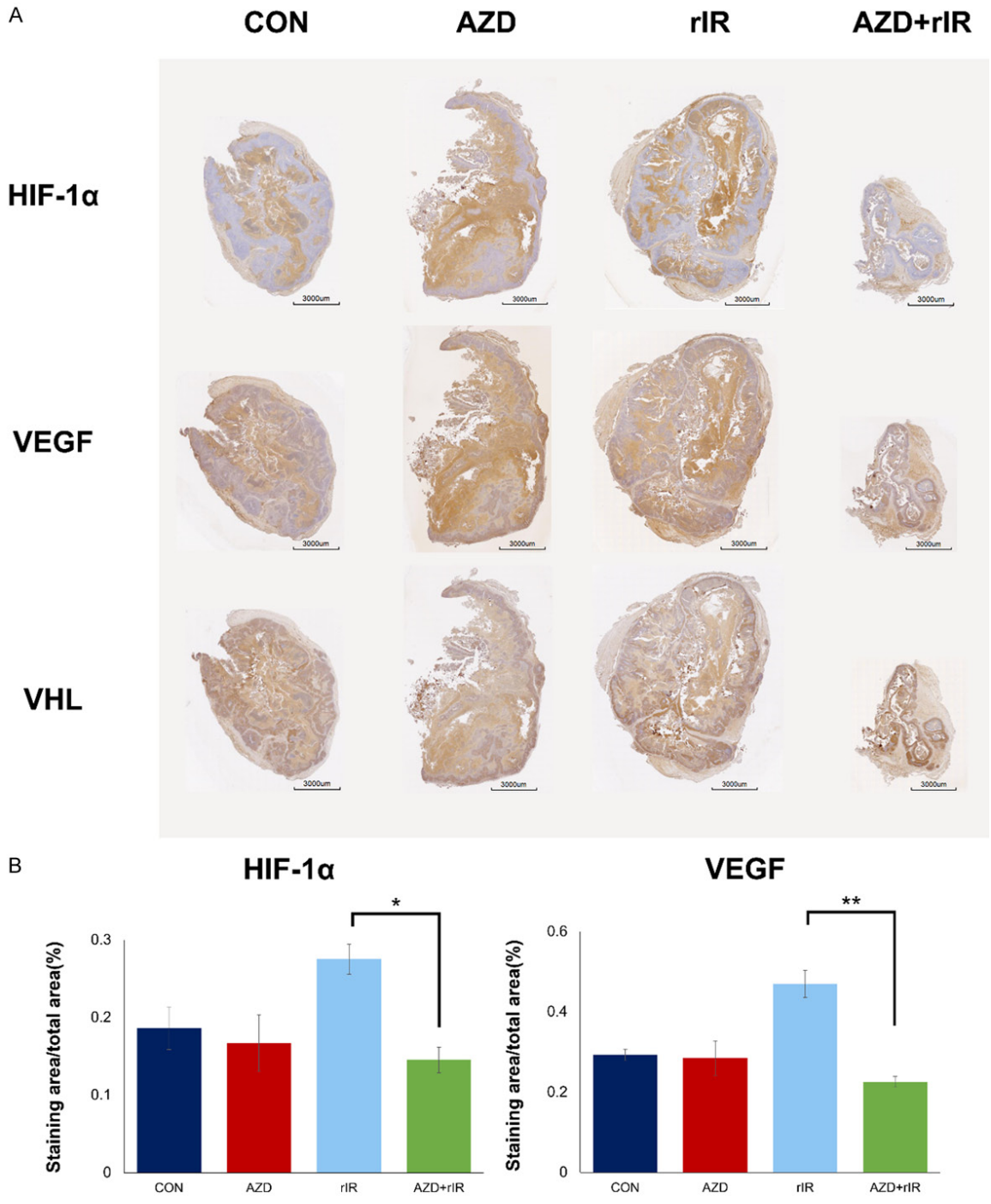

Figure 5. AZD7648 decreased IR-induced HIF-1α and VEGF expressions. To confirm the effect of AZD7648 on the regulation of HIF-1α, (A) IHC staining was performed for HIF-1α, VEGF, and VHL, and (B) Quantified HIF-1α and VEGF using Image J. Quantification was calculated as the staining area divided by the total area of the tissue values represent the mean ± S.E.M of 3 mice per group (*P<0.05, **P<0.01 versus rIR).

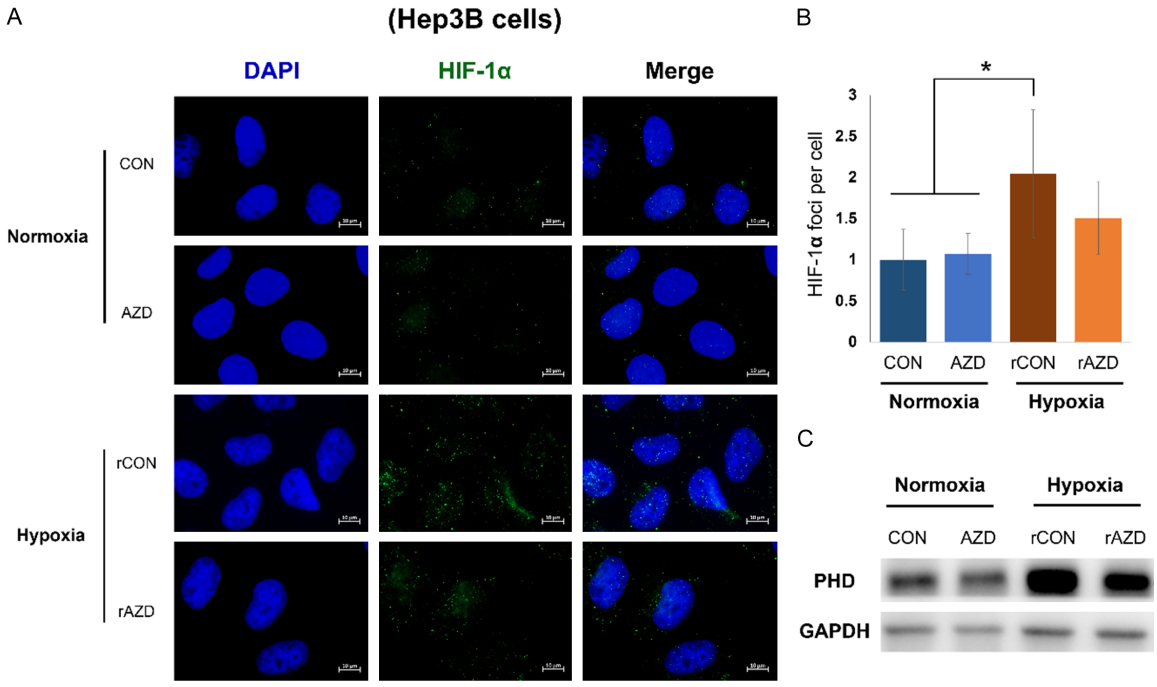

Figure 6. AZD7648 regulated $CoCl_2$-induced HIF-1α in Hep3B cells. To confirm the effect of AZD7648 under hypoxic conditions. (A) HIF-1α expression was examined using immunocytochemical staining, and (B) HIF-1α foci per cell were quantified (*P<0.05). Also, (C) PHD2 expression was detected by western blot analysis. Values represent the means ± S.D.

Discussion

For a long time, cancer has been a leading cause of death in the global population. Many cancer therapies were developed to treat cancers; however, limitations of treatment remain. Among cancer therapies, radiation therapy is an effective cancer treatment and is used to treat most cancers. Radiation therapy basically causes DNA damage to the tumor, leading to cell cycle arrest and death of the tumor [16-18]. When cancer patients are treated by radiation therapy, the limitations include damage to surrounding normal tissue and resistance to radiation [16]. Notably, radiation resistance is caused by various factors, including tumor microenvironment, hypoxia, metabolic changes, and tumor heterogeneity [1, 19, 20]. Among them, hypoxia is a representative cause of radioresistance and plays a broad role in tumor growth. As the tumor grows rapidly, tumor angiogenesis increases to supply adequate blood and nutrients to the tumor, so the tumor microenvironment eventually becomes strongly hypoxic. Actually, many malignancies result from increased HIF-1α which is a marker of hypoxia, ionizing radiation upregulates HIF-1α expression, and HIF-1α activates transcription of growth factors that induce radiation resistance [5]; HIF-1α is an important key in resistance to radiotherapy [21].

A promising clue to enhance the efficacy of radiotherapy is the development and discovery of radiosensitizers that have synergistic anticancer effects or can overcome radioresistance [22, 23]. Recently, a DNA-PK inhibitor, AZD7648, was developed as a novel radiosensitizer and used as a positive control in several studies [11-13]. Fok et al. [12] showed that AZD7648 combined with radiation was a potent radiosensitizer via synergistic tumor growth inhibition and prolongation of G2/M arrest at 72 h after radiation. Consistent with Fok et al., we evaluated that the combination of AZD7648 and IR synergistically reduced tumor cell growth rate in Hep3B xenografts (**Figure 1**) and cells (**Figure 2**) compared with IR alone. Especially, the AZD7648 concentration in this study was lower than in previous studies [12, 13], and there was no body weight loss. Furthermore, the present study newly found that AZD7648 induced accumulation of IR-induced G2/M phase at an early time point (P<0.01) and AZD7648 plus IR increased G2/M checkpoint protein levels, including cyclinB1 and p-cdc2

compared with IR alone (**Figure 3**). In addition, AZD7648 significantly increased IR-induced ROS level (**Figure 4**). To sum it up, our new findings strongly supported that AZD7648 synergistically enhances radiosensitivity in Hep3B xenografts and cells.

Several studies have discussed the relationship between DNA-PK and HIF. HIF recruits DNA-PK to the HRE, and HIF also requires DNA-PK for stabilization [14]. DNA-PK is activated during hypoxic stress, and this stress response favors hypoxic adaptation by accumulating HIF-1α [15]. ROS-dependent TPEN-induced DNA damage is mediated by Chk1 and DNA-PK [24]. There are no studies related to overcoming radioresistance by the DNA-PK inhibitor AZD-7648. To confirm the improvement of radioresistance, a radioresistant tumor xenograft model was employed. Tumor growth in radioresistant xenografts was not inhibited by IR. Interestingly, the combination of AZD7648 and IR significantly inhibited tumor growth in radioresistant xenografts ($P<0.001$) (**Figure 1**). HIF-1α activates transcription by binding to HRE in the nucleus. After its transcription, HIF-1α produces growth factors and other factors that aid tumor growth and induce resistance to tumor treatments [25]. VEGF, a representative tumor growth factor induced by HIF-1α transcription, is responsible for angiogenesis to supply more oxygen and nutrients to the tumor [5]. In normoxia but not in hypoxia, PHD2 hydroxylates HIF-1α using oxygen inside the tumor, and VHL ubiquitinates the hydroxyl group to induce the degradation of HIF-1α [7]. As a result of the tumor xenograft study, AZD7648 plus IR markedly alleviated highly expressed HIF-1α and VEGF proteins by IR ($P<0.05$), whereas AZD7648 combined with IR tended to increase VHL expression compared with IR (**Figure 5**). In addition, AZD7648 decreased HIF-1α and PHD expressions of Hep3B cells under $CoCl_2$-induced hypoxia compared with control (**Figure 6**). These data suggested that AZD7648 improved radioresistance by modulating HIF-1α pathway. Future studies to dissert, the exact mechanism of how AZD7648 modulates HIF-1α-related radioresistant proteins in cancer remains.

In summary, the present study showed that AZD7648 synergistically enhanced radiation response by accumulating IR-induced G2/M arrest in radiosensitive tumors. The most interesting finding we observed was that AZD-7648 improved radioresistance via regulation of the HIF-1α pathway in radioresistant tumors. Consequently, the present data suggest that AZD7648 may be a strong radiosensitizer in radioresistant as well as radiosensitive cancers.

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Disclosure of conflict of interest

None.

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