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Original Article

Synergistic radiosensitizing effect of BR101801, a specific DNA-dependent protein kinase inhibitor, in various human solid cancer cells and xenografts

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Abstract: DNA-dependent protein kinase (DNA-PK), an essential component of the non-homologous end-joining (NHEJ) repair pathway, plays an important role in DNA damage repair (DDR). Therefore, DNA-PK inhibition is a promising approach for overcoming radiotherapy or chemotherapy resistance in cancers. In this study, we demonstrated that BR101801, a potent DNA-PK inhibitor, acted as an effective radiosensitizer in various human solid cancer cells and an *in vivo* xenograft model. Overall, BR101801 strongly elevated ionizing radiation (IR)-induced genomic instability via induction of cell cycle G_2/M arrest, autophagic cell death, and impairment of DDR pathway in human solid cancer cells. Interestingly, BR101801 inhibited not only phosphorylation of DNA-PK catalytic subunit in NHEJ factors but also BRCA2 protein level in homologous recombination (HR) factors. In addition, combination BR101801 and IR suppressed tumor growth compared with IR alone by reducing phosphorylation of DNA-PK in human solid cancer xenografts. Our findings suggested that BR101801 is a selective DNA-PK inhibitor with a synergistic radiosensitizing effect in human solid cancers, providing evidence for clinical applications.

Keywords: DNA-PK inhibitor, Ionizing radiation, radiosensitizer, DNA damage repair, solid cancer

Introduction

In most organisms, the DNA damage repair (DDR) mechanism must be properly regulated to maintain genomic stability. If DDR responses are uncontrolled, there is a risk of cancer, changing the cell from a normal cell to a cancer cell, thus inducing cancer progression and poor therapeutic efficacy [1]. In particular, regulation of the DDR pathway is frequently essential in effective anticancer therapy; induction of DNA damage-related synthetic lethality and DNA repair can overcome therapeutic resistance in cancer cells [2, 3].

The main pathways for repair of DNA double strand breaks (DSBs) are homologous recombination (HR) and non-homologous end-joining (NHEJ). HR uses a DNA template to repair breaks and is limited to the S and G_2 phases of the cell cycle. In contrast, NHEJ repairs DSBs without a DNA template and is utilized in all phases of the cell cycle. Moreover, NHEJ processes repair over 80% of DSBs induced by ionizing radiation (IR) in cancer cells [4]. NHEJ repair of IR-induced DSBs mainly requires DNA-dependent protein kinase (DNA-PK), a complex that consists of the DNA-PK catalytic subunit (DNA-PKcs) and the Ku heterodimers (Ku70 and Ku86) [5]. DNA-PK, one of many DNA repair proteins, induces resistance to anticancer therapy, including radiotherapy and chemotherapy [6]. Thus, DNA-PK inhibitors are a promising option for overcoming cancer radioresistance or chemoresistance.

Several DNA-PK inhibitors have been investigated for cancer therapy and have been demonstrated to sensitize cells to IR and other DSB-inducing agents [2, 7, 8]. Small molecule DNA-PK inhibitors, such as NU7441, LY209-4002, and KU-0060648 show an inhibitory effect against phosphatidylinositol 3-kinase related kinase (PIKK) family members [9]. In

various solid tumors, NU7441 enhanced IR and doxorubicin by inhibiting DNA-PK activity and delaying DSB repair *in vitro* [10, 11]. Furthermore, selective DNA-PK inhibitors, including VX-984 and M3814, sensitized IR and liposomal doxorubicin *in vitro* and *in vivo* and are currently undergoing clinical trials [5, 12]. More recently, Fok *et al*. reported that AZD7648, a novel and selective DNA-PK inhibitor, acted as an anticancer agent and a sensitizer in combination with IR, a topoisomerase II inhibitor, or a PARP inhibitor. A number of anticancer studies have shown DNA-PK inhibitors to be promising and important anticancer therapeutic agents. However, a number of DNA-PKcs inhibitors have frequently shown poor solubility and pharmacokinetics, which has limited clinical applications [13-16]. Thus, studying and developing new compounds that can overcome these drawbacks is warranted [8].

In this study, we show that BR101801, a specific DNA-PK inhibitor, potentiates the radiosensitive effect on cancer therapy *in vitro* and *in vivo*, focusing on possible DDR mechanisms caused by IR. Our findings may strengthen the rationale that BR101801 combined with radiotherapy shows therapeutic potential as a novel regimen for clinical use in solid cancers.

Materials and methods

Drug

BR101801 was obtained from Boryung Pharmaceutical (Ansan, Gyeonggi-do, Republic of Korea). BR101801 was dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 10 mM, stored at -20°C for *in vitro* analysis, and prepared in Polyethylene glycol 400 (PEG 400)/Distilled water (DW)/DMSO (5.5:4:0.5 v/v/v) for oral administration *in vivo.*

Cell cultures

HCT116, HT-29, H460, A549, MCF-7, and MDA-MB-231 cells were acquired from the Korean Cell Line Bank (Seoul, South Korea). Cell lines were cultured in the medium recommended for these cells [HCT116, HT29, H460, and MCF-7: Roswell Park Memorial Institute 1640 (RPMI-1640), 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin; A549 and MDA-MB-231: Dulbecco's Modified Eagle Medium (DMEM), 10% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin]. All cell lines were maintained in an atmosphere of 5% CO_2 at 37°C.

Irradiation

For *in vitro* experiments, cells were irradiated using a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Chalk River, Ontario, Canada) at a dose rate of 2.24 Gy/min. Xenograft mice were irradiated with a ^{60}Co γ-ray source (Gammabam 100-80, Atomic Energy of Canada, Chalk River, Ontario, Canada) at a dose rate of 1.10 Gy/min. Xenografts from the IR-treated groups were irradiated at a local cancer region covering a 0.5 cm bolus, and normal, non-cancerous tissues were shielded using lead.

Colony forming assay

Six cancer cell lines were seeded by a designated plating cell number (300-3600) into 60 mm culture plates overnight. Cells were treated with a serial dilution of BR101801 at 0, 0.625, 1.25, 2.5, 5, and 10 μM to determine the optimal concentration of BR101801 and pretreated with 1 μM BR101801 for 24 h prior to irradiation to evaluate the radiosensitizing effect. After 24 h of irradiation, all sample media were substituted with fresh media and cultured for 8-21 days in 5% CO_2 at 37°C. Colonies were fixed with 100% methanol and then stained with 0.4% crystal violet. Colony averages were determined in triplicate in at least three independent experiments. Clonogenic cell survival rate was calculated according to the results.

Western blot analysis

Cell lysates were prepared in cold radioimmunoprecipitation assay (RIPA) buffer, supplemented with phosphatase and protease inhibitor cocktails (GenDEPOT, Barker, TX, USA). Protein quantity was determined by Bradford protein assay. Proteins were separated using SDS/PAGE and transferred to polyvinylidene fluoride membranes (PVDF). The membranes were blocked with bovine serum albumin (BSA) 5% (v/v) in PBS with 0.1% Tween 20, incubated with the indicated antibodies (1:1,000) and the secondary antibodies (1:5,000 or 1:10,000), and then subsequently developed with the ECL western blotting substrate using the ImageQuant LAS-4000 mini.

Cell cycle analysis

Various solid cancer cell lines were pretreated with 1 μM BR101801 for 24 h, irradiated, and incubated further for 24 h. Cells were harvested and fixed in 1 mL of 70% cold ethanol and incubated in a -20°C freezer overnight. After incubation, cells were washed and then centrifuged at 2,000 rpm for 5 min in cold condition, and the cell pellets were resuspended in 500 μl of BD PI/RNase staining buffer (BD Bioscience, San Diago, CA, USA). The cell cycle distribution of 10,000 cells was analyzed using flow cytometry.

Immunocytochemistry assay

Immunocytochemistry was conducted to define the nuclear distribution of γ-H2AX in individual cells. After exposure to 1 μM BR101801 for 24 h, cells were irradiated at 2 or 4 Gy and incubated for a further 1 or 24 h before harvest. Cells were fixed with 3% paraformaldehyde and then washed with PBS 2 times. Then they were permeabilized with 0.5% Triton X-100 in PBS, blocked with 4% FBS in PBS, suspended overnight in blocking buffer containing primary antibody against γ-H2AX, and then incubated for 1 h with FITC-labeled goat anti-mouse IgG. Nuclei were counterstained with DAPI (1 mg/mL). Coverslips were mounted with a fluorescence mounting medium. The slides were examined using a confocal laser scanning microscope.

Tumor xenograft studies

BALB/c nude mice at 4 weeks of age, were purchased from Orient Bio Inc. (Gapyeong, Gyeonggi-do, Korea) and acclimated for 1 week prior to experimentation (n=5). Mice received subcutaneous injections in the right thigh containing 3×10^6 MDA-MB-231 cells. When the tumor attained a volume of approximately 120 mm^3, the mice were randomly divided into four groups; control, BR101801 (50 mg/kg), IR at a fraction dose of 2 Gy ×5 times, and BR101801 (50 mg/kg) + IR at a fraction dose of 2 Gy ×5 times. BR101801 was administered orally once a day, from grouping to the ending point. When the mean tumor volume in the control group reached approximately 150 mm^3, mice were irradiated with a Co-60 γ-ray irradiator. Mice were euthanized by CO_2 inhalation, and the tumors on the right thigh of mice were extracted when the average tumor volume in the control group was 2,000 mm^3. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Radiological and Medical Sciences.

Immunohistochemistry staining

Immediately after sacrifice, the tumor tissues were fixed, embedded, and sliced into 3 μm-thick sections for immunohistochemistry. Sample sections were deparaffinized with xylene and sequentially rehydrated with an ethyl alcohol series and distilled water. Then, they were washed with tris-buffered saline (50 mM, pH 7.6) containing 0.1% Tween 20 (TBST), and antigen retrieval was performed by autoclaving the sections in sodium citrate buffer (10 mM, pH 6.0) for 30 min. When washing with TBST, the sections were blocked with hydrogen peroxidase solution, depending on visualization conditions. The slides were incubated with the p-DNA-PKcs antibodies (Abcam, Cambridge, UK, 1:200) at 4°C overnight. After washing in TBST, the sections were incubated with EnVision + System-HRP Labelled Polymer anti-Rabbit (DAKO) at room temperature for 20 min. Then, after washing four times in TBST, the immunoreactivity was measured with a mixture of Universal DAB H_2O_2 and Universal DAB Chromogen (UltraView DAB Detection Kit) for 3-6 min. Samples were counterstained with Mayer's hematoxylin.

Statistical analysis

Statistical analyses were performed using SigmaPlot (ver. 14.0; Systat Software, USA). Data were represented as the means ± standard error of the mean (SEM). Student's *t*-test was used depending on the normal distribution of the data. One-way ANOVA analysis of variance was used to examine significant differences between groups. A *P*-value of <0.05 was considered statistically significant.

Results

BR101801 enhances radiation response in various human solid cancer cell lines

To determine the optimal BR101801 concentration, we administered various doses of BR101801 (0, 0.625, 1.25, 2.5, 5, 10 μM) for 48 h in colorectal cancer cell lines (HCT116

and HT-29), lung cancer cell lines (H460 and A549), and breast cancer cell lines (MCF7 and MDA-MB-231), and we then performed a colony forming assay. Among the tested BR101801 doses, 1.25 μM in HCT116 and HT-29 cells and 2.5 μM in H460, A549, MDA-MB-231, and MCF7 cells showed an inhibitory concentration of 20% (Data not shown). When all experimental cell line conditions were considered, 1 μM of BR101801 was comprehensively selected as the optimal concentration. We then evaluated the radiosensitizing effect of BR101801 in various human solid cancer cells. All human cancer cell lines were treated with 1 μM of BR101801 for 24 h, followed by IR, and then further incubation for 24 h. IR-treated groups were irradiated with different doses (0-8 Gy) using a C-137 γ-ray irradiator. As shown in **Figure 1**, the combination of BR101801 and IR significantly induced clonogenic cell death, compared with IR alone, in all human solid cancer cell lines. Our findings indicated that BR101801 radiosensitized various human cancer cell lines.

BR101801 inhibits IR-induced autophosphorylation of DNA-PKcs at Ser2056, but not in a heterodimer of Ku70/86

To assess whether BR101801 acted as a DNA-PK inhibitor in various human solid cancer cells, cells were treated with 1 μM of BR101801 for 0.5 h before IR, and then irradiated at different doses (2 Gy in radiosensitive cells such as HCT116 and H460, 4 Gy in radioresistant cells including HT-29, A549, and MDA-MB-231). At 2 h after irradiation, the expression levels of phosphorylated DNA-PKcs, Ku70, and Ku86 were examined by western blot. As expected, BR101801 combined with IR inhibited radiation-induced DNA-PKcs phosphorylation in radiosensitive and radioresistant cell lines, but did not regulate Ku70 and Ku86 protein levels (**Figure 2**). These results indicated that BR101801 was a potent and selective DNA-PK inhibitor in human solid cancer cells.

BR101801 mediates IR-induced G_2/M arrest or accumulation of subG_1 phase population

To investigate the effects of BR101801 on IR-induced cell cycle progression, we analyzed the cell cycle phase distribution using flow cytometry. The cells were pretreated with 1 μM of BR101801 for 24 h, followed by IR, and then fixation for 24 h after IR. BR101801 combined with IR clearly increased the proportion of subG_1 phase compared to control and IR alone in HCT116 and H460 cells (**Figure 3A**), whereas BR101801 + IR strongly increased the proportion of G_2/M phase cells compared to IR alone in HT-29, A549, and MDA-MB-231 cells (**Figure 3B**). Thus, we demonstrated that BR101801 prevented cell cycle progression by significantly increasing IR-induced G_2/M phase in radioresistant cells and tending to be augment subG_1 phase in radiosensitive cells.

BR101801 prolongs the repair of IR-induced DNA damage

The effects of BR101801 on the repair of IR-induced DSBs were evaluated by the formation of γ-H2AX foci, the DNA damage marker, in HCT116, H460, HT-29, and MDA-MB-231 cell lines by immunocytochemistry assay. At 1 h after IR, BR101801 + IR exhibited a significantly increased number of γ-H2AX foci, compared with IR alone. Additionally, BR101801 combined with IR consistently retained the γ-H2AX foci for 24 h after IR in all cell lines (**Figure 4**). These results showed that BR101801 not only promoted DNA damage by IR, but also postponed the repair of IR-induced DSBs.

BR101801 combined with IR regulates cell cycle arrest, autophagic death, and HR repair-related proteins

We further examined whether BR101801 affected IR-related mechanistic markers in HCT116, H460, HT-29, and MDA-MB-231 cell lines by western blot. First, in cell cycle-related mechanisms, BR101801 combined with IR significantly increased the expression of G_2/M checkpoint regulators, including cyclin B1 and phosphorylated cdc2 (Tyr15), compared with IR alone in all cell lines (**Figure 5**). Furthermore, we found that treatment with BR101801 + IR caused the conversion of LC3A to LC3B, which is a specific marker of autophagic death in HT-29 and MDA-MB-231 (**Figure 5B**), but this did not occur in HCT116 and H460 cells (**Figure 5A**). Interestingly, BR101801, a potent DNA-PK inhibitor of NHEJ repair, inhibited BRCA2, which is a critical factor in the HR repair pathway in HCT116, H460, HT-29, and MDA-MB-231 cells (**Figure 5**). Together, these data indicated that BR101801 enhanced the radiation response

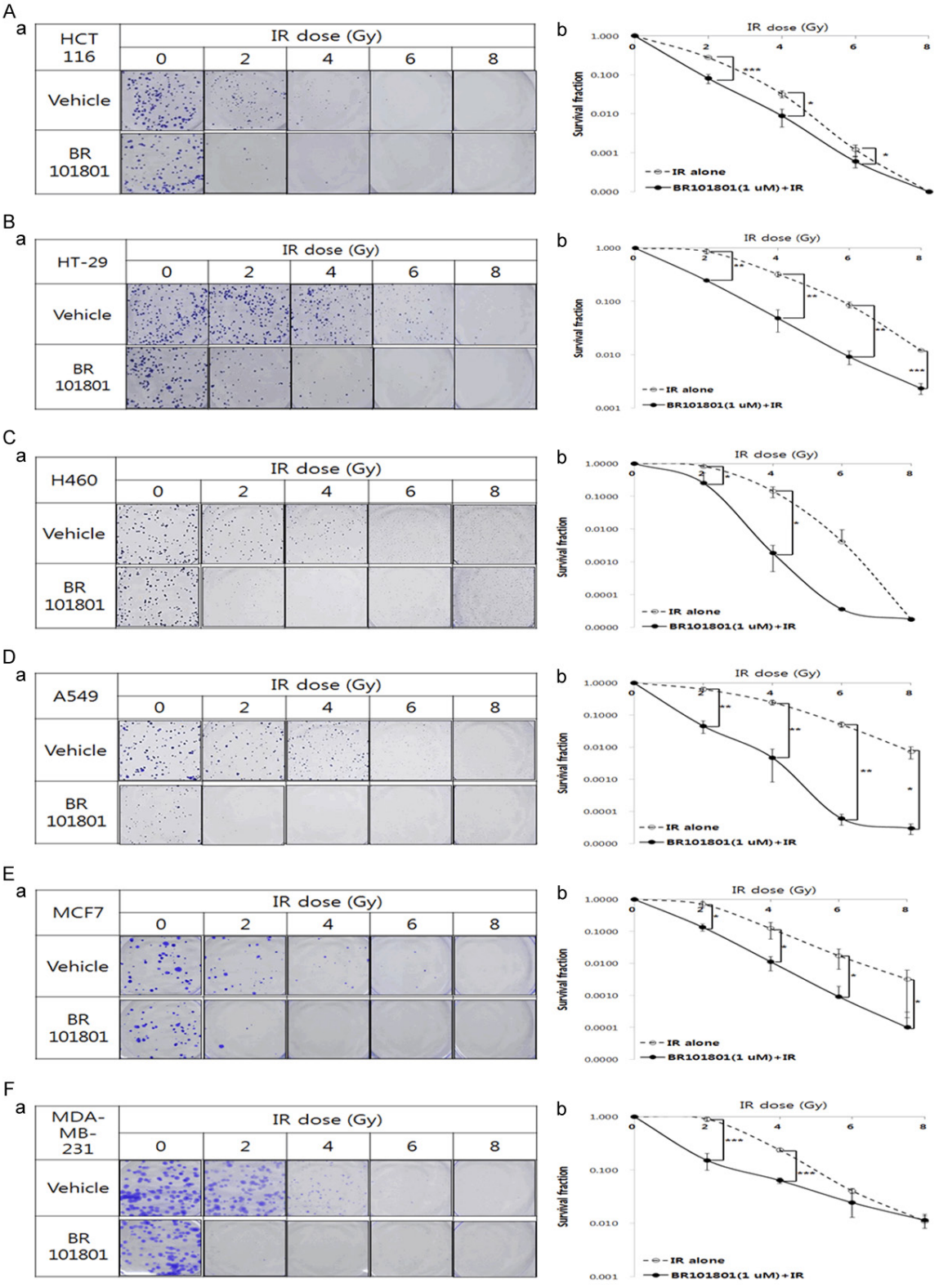

Figure 1. BR101801 promotes radiosensitivity in various human solid cancer cell lines. (A) HCT116, (B) HT-29, (C) H460, (D) A549, (E) MCF-7, and (F) MDA-MB-231 human cancer cell lines were pretreated with 1 μM BR101801 for 24 h, followed by IR with various doses of C-137 γ-ray irradiation. The radiosensitizing effect of BR101801 was measured by a colony forming assay at the end of experimentation. The photographs show (A) colony images, and the graphs indicate (B) survival fraction in all human solid cancer cell lines. Values are shown as mean ± SEM for at least three independent experiments; $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$.

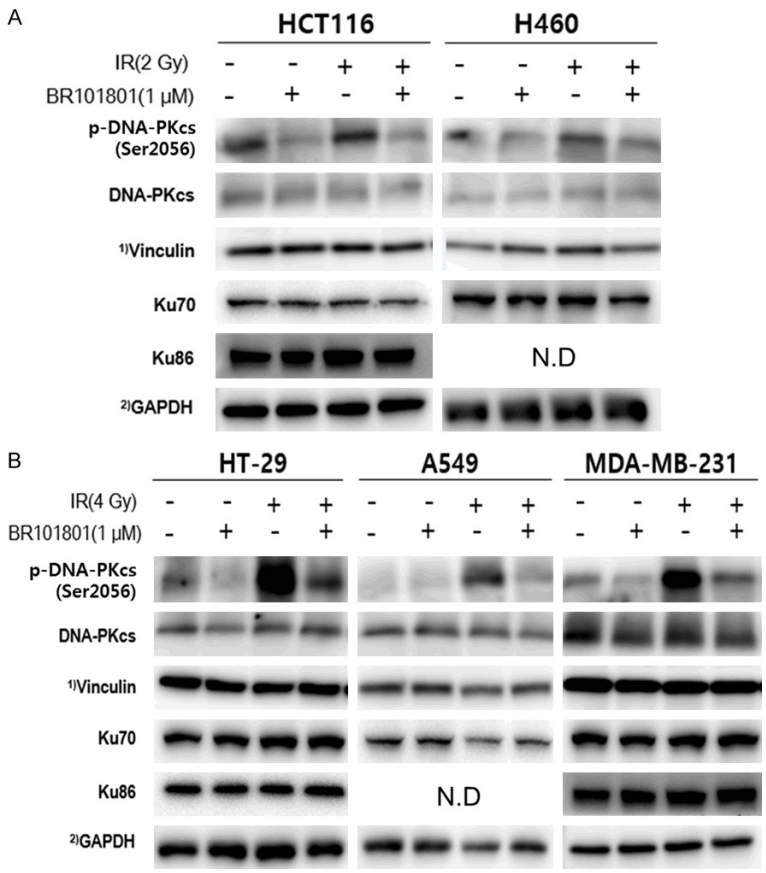

Figure 2. BR101801 suppresses IR-induced DNA-PKcs phosphorylation (Ser2056) in human cancer cell lines. Various human solid cancer cells were treated with 1 μM BR101801 for 30 min and then irradiated with (A) 2 Gy or (B) 4 Gy. At 2 h after irradiation, we analyzed key factors of the NHEJ repair pathway, including phosphorylated DNA-PKcs and a heterodimer of Ku70/86 proteins in HCT116, HT-29, Hep3B, H460, A549, and MDA-MB-231 cells. Phosphorylated DNA-PKcs levels were normalized for vinculin. Ku70 and 86 expressions were normalized for GAPDH. N.D: not detected.

through modulation of cell cycle arrest, autophagic death, and impaired HR repair in human solid cancer cells, especially in radioresistant cells.

BR101801 radiosensitizes MDA-MB-231 cancer xenografts by attenuating DNA-PKcs phosphorylation

According to our *in vitro* results, MDA-MB-231 cells showed the highest radiosensitivity among the various human solid cancer cell lines. Therefore, we validated whether BR101801 enhanced the radiation response *in vivo* by using a MDA-MB-231 xenograft mouse model. MDA-MB-231 cells were injected subcutaneously into the right hind leg of individual mice. As shown in the indicated experimental plan (**Figure 6A**), BR101801 was administered orally once daily (q.d) at 50 mg/kg, and local-regional irradiation was administered according to the fractional irradiation plan (2 Gy, once a day, 5 times) in MDA-MB-231 xenograft mice. The combination of BR101801 and IR enhanced tumor growth inhibition more significantly than IR alone (**Figure 6B**). An interesting finding in our xenograft study was that two of seven xenografts in the BR101801 + IR group were cured or only presented with remaining debris, which indicated a highly synergistic radiosensizing effect of BR101801 (**Figure 6C**). Additionally, we evaluated DNA-PK inhibition of BR101801 *in vivo* by immunohistochemical analysis. The phosphorylated DNA-PK level of IR treatment alone was markedly higher, whereas that of BR10181 + IR was significantly lower than IR alone (**Figure 6D**). Consequently, BR101801 enhanced the radiation response by inhibiting the phosphorylation of DNA-PKcs in MDA-MB-231 xenografts.

Discussion

DNA DSBs are more cytotoxic to rapidly dividing cancer cells than to slowly dividing normal cells. The difference in DNA DSBs between cancer cells and normal cells is a good advantage for cancer therapy. In addition, several targeted therapies or combination therapies have been developed to reduce toxicity to normal tissues [17]. Recently, modern radiotherapy techniques, such as stereotactic body radiation therapy, heavy ion therapy, and proton radiotherapy, have been administered to cancer cells in precise doses, while minimizing damage to healthy tissues [18]. Although many cancers are treated with modern IR and chemotherapy targeting DNA DSBs in cancer cells and minimizing toxicity to normal tissues, seri-

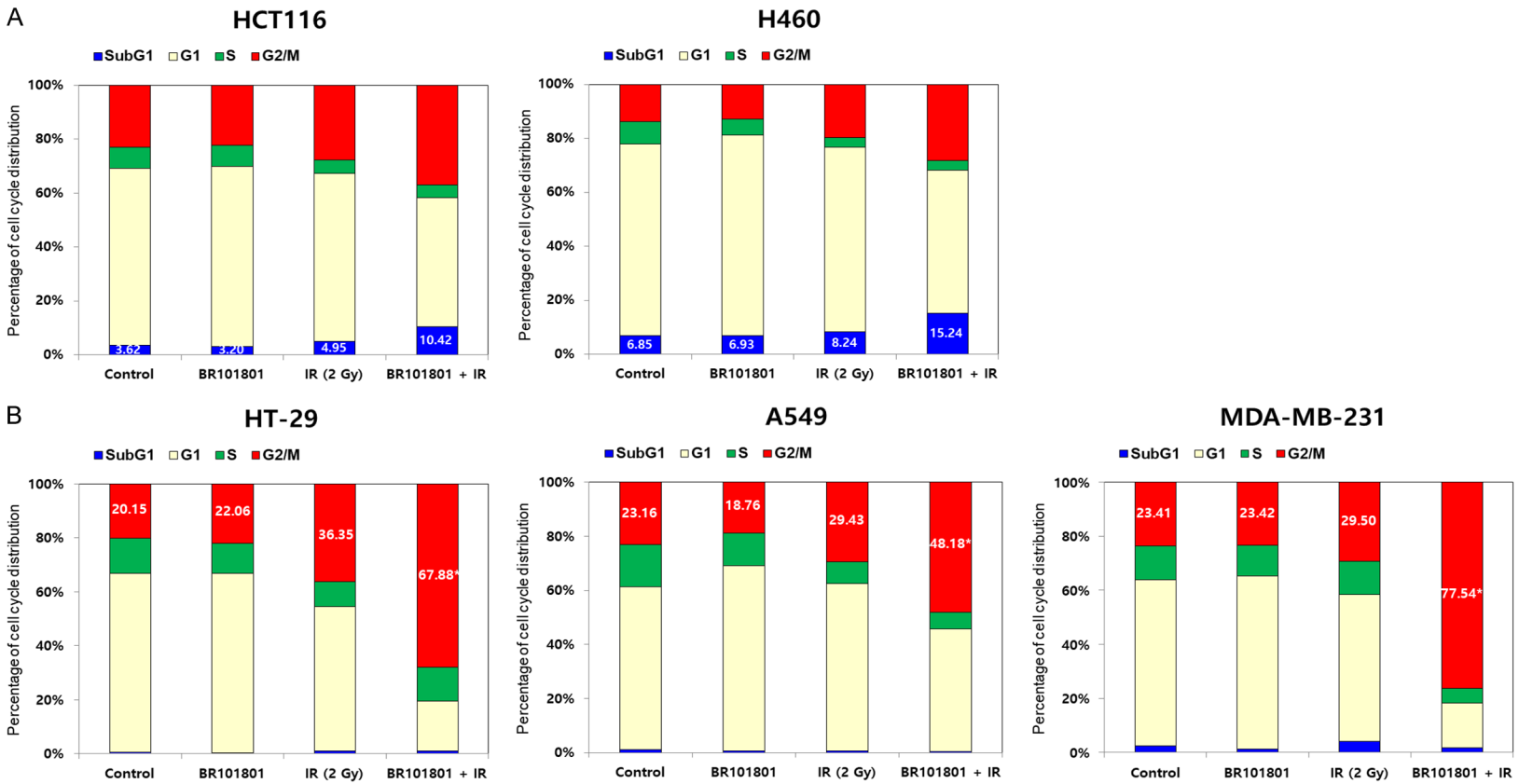

Figure 3. BR101801 inhibits cell cycle progression via IR-induced G2/M arrest or attenuated sub G1 phase. To investigate whether BR101801 affected cell cycle progression, human cancer cells were treated with 1 μM BR101801 for 24 h prior to irradiation [(A) 2 Gy or (B) 4 Gy]. At 24 h after irradiation, cells were fixed with 70% methanol over 30 min, stained with PI, and analyzed by flow cytometry. The cell cycle distribution was analyzed quantitatively. Values represent the mean of three experiments ± SEM; $^{*}P<0.05$ vs IR.

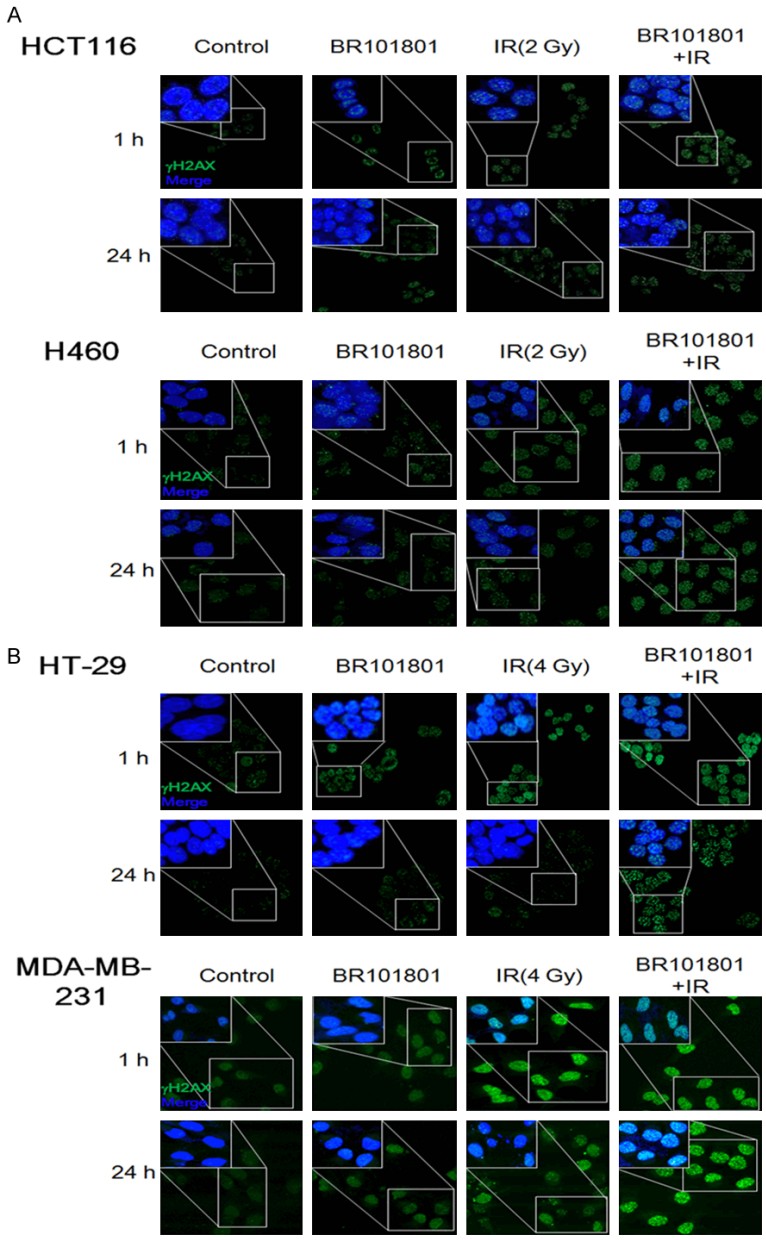

Figure 4. BR101801 causes prolongation of IR-induced DNA damage. To confirm DNA damage response by BR101801 and/or IR, we examined a number of γ-H2AX foci, a marker of DNA damage, using immunocytochemistry staining. HCT116, H460, HT-29, and MDA-MB-231 cells were grown on chambered slides, followed by 1 μM BR101801 treatment for 24 h, and then irradiated with (A) 2 Gy or (B) 4 Gy. Cells were stained for anti-γ-H2AX and DAPI at 1 and 24 h after irradiation. Representative images show the merged fluorescence photomicrographs of cells stained with γ-H2AX (green) and counterstained for the nucleus with DAPI (blue).

ous therapeutic resistance issues still remain. Thus, targeting DDR pathways to cause tumor sensitivity could be a potential cancer therapy. Particularly, the inhibition of NHEJ is one of the clinically promising strategies for DDR-targeted combination therapy in cancer [4].

BR101801 is currently undergoing a first-in-human clinical trial as an inhibitor of PI3K and DNA-PK against non-Hodgkin lymphomas in the United States of America and the Republic of Korea (Clinical trial identifier: NCT04018248). The present study demonstrated that BR101801 was a first-in-class triple inhibitor of PI3K-γ/δ and DNA-PK in various solid cancers. First, a patent registration from the Republic of Korea (No. PCT/KR2016/005798) showed that BR101801 inhibited four PI3K isotypes; α, β, γ, and δ, significantly selective of PI3K-γ/δ. Furthermore, BR101801 as triple inhibitor of PI3K-γ/δ and DNA-PK has completed a patent registration in the United States of America, Australia, Japan, Russia, Singapore, Republic of South Africa, Republic of China, and European Patent Office such as United Kingdom, France, and Germany. In our study, we focused on the DNA-PK inhibitory effect of BR101801 in various solid cancers. BR101801 reduced bleomycin-induced DNA-PKcs phosphorylation of A549 lung cancer cells in a dose-dependent manner (Figure S1). The critical action of the NHEJ repair process is mediated by the DNA-PKcs and the Ku70/86 heterodimer. When DSBs occur, the Ku70/86 heterodimer recognizes them quickly, recruits DNA-PKcs, and then DNA-PKcs is autophosphorylated at Ser2056 [19]. DNA-PKcs or Ku70/86 deficiency reduces lifespan and body size of mice and highly sensitizes cells to drugs and IR [20-22]. We confirmed that BR101801 inhibited IR-induced phosphorylation of DNA-PKcs, but not that of the Ku70/86 heterodimer (**Figure 2**). Interestingly, BR101801 decreased the expression of

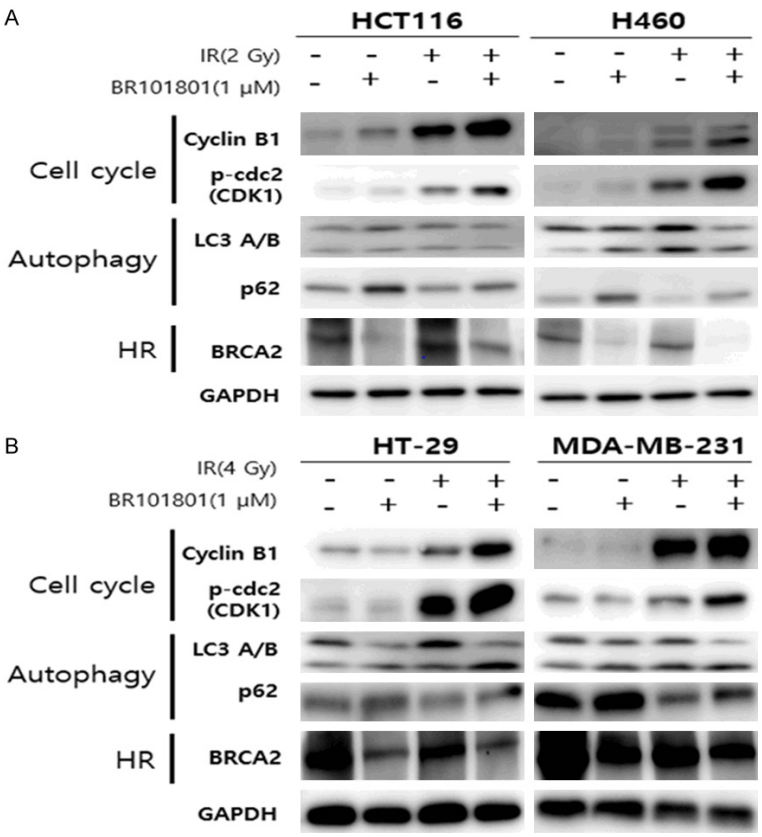

Figure 5. Combined treatment with BR101801 and IR modulates IR-related mechanistic markers. HCT116, H460, HT-29, and MDA-MB-231 cell lines were treated with 1 μM BR101801 for 24 h and then irradiated with (A) 2 Gy or (B) 4 Gy. At 24 h after irradiation, the expression of G2/M checkpoint regulators (cyclin B1 and *p*-cdc2), autophagic markers (LC3A/B and p62), and a key factor in HR repair (BRCA2) was measured by western blot. The indicated antibodies were normalized for GAPDH.

BRCA2 in solid cancer cells (**Figure 5**). BRCA2 is a key factor in the HR repair process that acts as a Rad51 mediator; however, it is not associated with the NHEJ repair process [23, 24]. Overall, BR101801 is a novel and potent DNA-PK inhibitor in various solid cancers; additionally, it is expected to act directly as a possible inhibitor of HR repair-related BRCA2. Further studies are required confirm expression of various HR repair-related proteins by BR101801.

Recent studies have shown that potent DNA-PK inhibitors, including NU7441 [11], M3814 [5], VX-984 [15], and AZD7648 [4] sensitized cancer cells to IR and topoisomerase II inhibitors *in vitro*. Consistent with previous reports, BR101801 + IR strongly decreased clonogenic survival in various human solid cancer cells, compared to IR alone (**Figure 1**). The reported limitations of DNA-PK inhibitors *in vivo* or in clinical application include poor solubility and pharmacokinetics [5, 8, 14, 15]. In our *in vivo* study, BR101801 + IR inhibited the tumor growth of the MDA-MB-231 xenograft model more significantly than IR alone and attenuated IR-induced phosphorylation of DNA-PKcs (**Figure 6**). Notably, two of seven mice were cured or showed only remaining debris in the BR101801 and IR combination group. In addition, the combination of BR101801 and doxorubicin suppressed the tumor growth in MDA-MB-231 xenograft mice (Figure S2). Our findings demonstrated that BR101801 enhanced not only the radiation response but doxorubicin in human solid cancer cells and xenografts. The mechanism of action of DDR-related radiosensitizers mainly involves prolongation of IR-induced G_2/M arrest and delayed DSB repair by IR [25, 26]. As shown in **Figure 3**, BR101801 + IR blocked IR-induced G_2/M phase in radioresistant cells or tended to be augment the subG_1 phase in radiosensitive cells; BR101801 can play different roles in in radioresistant cells and radiosensitive cells. Moreover, BR101801 combined with doxorubicin increased γ-H2AX protein levels (Figure S3), and the combination of BR101801 and IR postponed the disappearance of IR-induced γ-H2AX foci (**Figure 4**). These results indicated that BR101801 enhanced the DNA damage response and attenuated DSB repair by IR or doxorubicin in human solid cancer cells.

In the past few years, many investigators have been interested in the association between DDR and autophagy. Autophagy plays a dual role in regulating DDR; mild DNA damage can be repaired, whereas severe DNA damage can be cytotoxic [27]. Several researchers reported that the inhibition of DNA-PKcs enhanced radi-

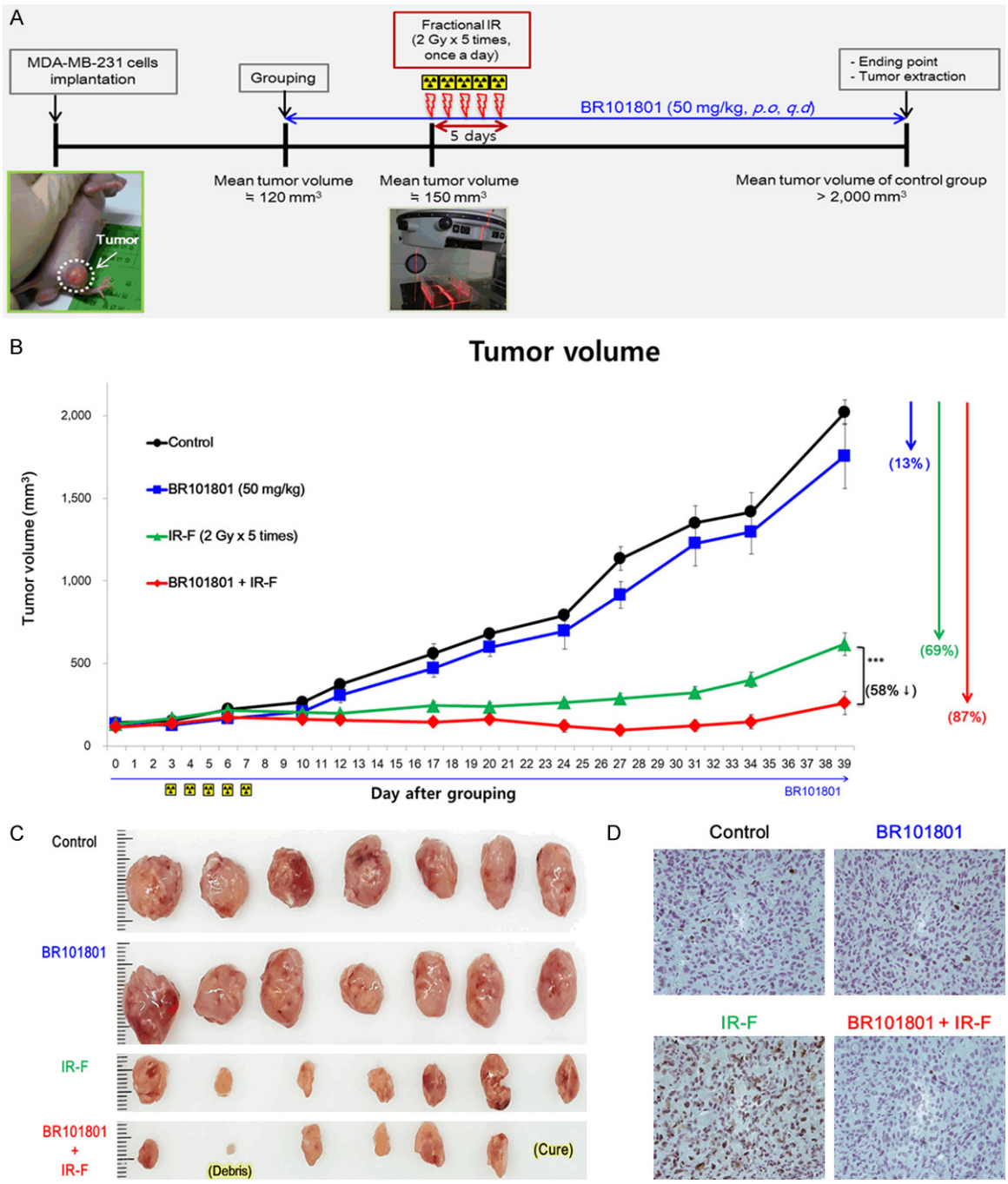

Figure 6. BR101801 enhanced radiosensitivity in MDA-MB-231 xenografts via inhibition of phosphorylated DNA-PKcs. Following the experimental plan (A), BR101801 (50 mg/kg, once daily) was orally administered, and IR groups were treated with fractional IR (2 Gy, once a day, 5 times) for local-regional irradiation using a Co-60 γ-ray irradiator in MDA-MB-231 xenograft mice. The graph represents (B) the tumor volume and tumor growth inhibition in mice bearing MDA-MB-231 cells. Also, the image shows (C) extracted cancers in the control group that reached approximately 2,000 mm^3. (D) The expression level of phosphorylated DNA-PK (brown) of extracted cancers was analyzed by immunohistochemistry. Data are represented as mean of each group ± SEM. Statistically significant differences are shown; $^{***}P<0.001$.

ation responses via induction of autophagy in malignant glioma cells [26], pancreatic cancer cells [28], and prostate cancer cells [29]. We found that BR101801 + IR increased cleaved LC3, an indicator of autophagy (**Figure 5**), which is in agreement with previous studies [28-

30]. An interesting observation in our study was the demonstrated autophagic effect in radioresistant, but not in radiosensitive cancer cells. The underlying mechanism explaining why BR101801 + IR induced autophagy only in radioresistant cancer cells requires further studies to investigate various important markers of autophagic death.

In summary, we demonstrated that BR10-1801, a specific and selective DNA-PK inhibitor, enhanced radiation response via accumulation of G_2/M phase cells and retardation of DNA repair in human solid cancer cells. Additionally, BR101801 synergistically inhibited tumor growth in an *in vivo* xenograft mouse model, compared with IR alone. Based on our study, we suggest that BR101801, a specific DNA-PK inhibitor, has a synergistic effect with radiotherapy in human solid cancers, thus showing promising clinical potential.

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Disclosure of conflict of interest

None.

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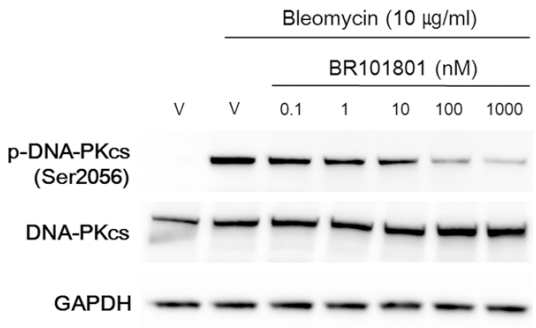

Figure S1. BR101801 inhibited bleomycin-induced DNA-PKcs phosphorylation of A549 lung cancer cells in a dose-dependent manner. A549 lung cancer cells were exposed to various concentrations of BR101801 for 30 min prior to 10 ug/ml bleomycin treatment. At 2 h after bleomycin treatment, cell lysates were analyzed by western blotting. The indicated antibodies were normalized for GAPDH. V: DMSO (Final concentration of DMSO: 0.1%).

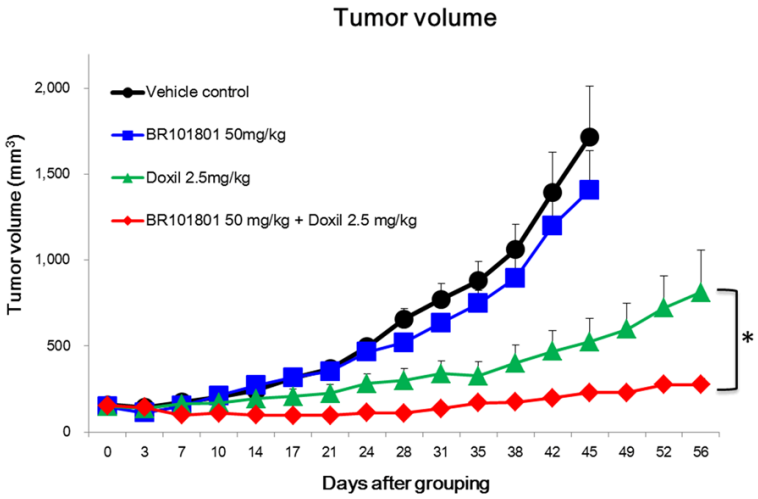

Figure S2. The combination of BR101801 and doxorubicin synergistically inhibit the tumor growth in MDA-MB-231 tumor xenograft model. To evaluate the synergistic effect of BR101801 with Doxorubicin Liposomal (Doxil) on MDA-MB-231 tumor xenografts, BR101801 (50 mg/kg, q.d) was orally administered, and Doxil (2.5 mg/kg, q.w) was administered by intravenous injection to their relevant groups. Values show mean per group ± SEM; $^{*}P<0.05$.

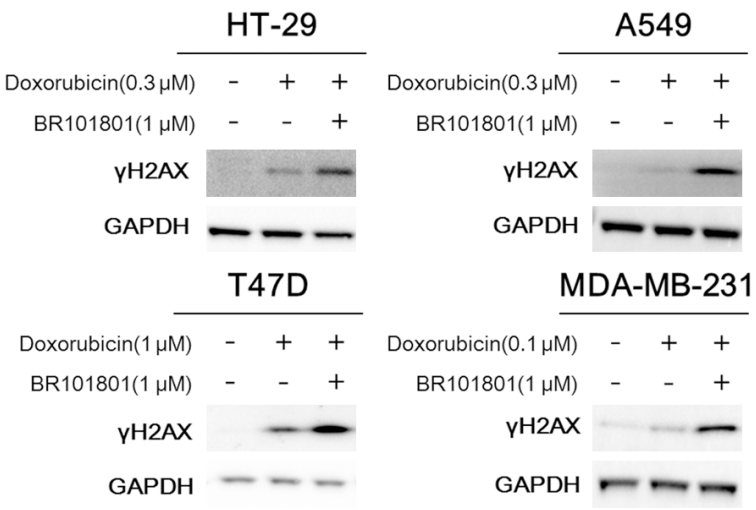

Figure S3. BR101801 combined with doxorubicin enhances DNA damage response by increasing the expression of γ-H2AX protein in various human cancer cell lines, suggesting that BR101801 retained doxorubicin-induced DNA damage. HT-29, A549, T47D, and MDA-MB-231 cell lines were treated with BR101801 and doxorubicin for 24 h. After treatment, γ-H2AX protein level was analyzed by western blot. The γ-H2AX antibodies were normalized for GAPDH.