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In vitro radiosensitizing effect of targeting PI4K IIIα using anti-HCV agent

PI4K Ⅲα 억제를 통한 *in vitro* 방사선 감작 효과:

C형 간염 항바이러스제를 이용한 약물 재창출 모델

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ABSTRACT

In vitro radiosensitizing effect of targeting PI4K IIIα using anti-HCV agent

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Introduction: Phosphatidylinositol 4-kinase (PI4K) catalyzes the phosphorylation of phosphoinositide to produce PI4-phosphate (PI4P), a common and essential substrate of both the Phospholipase C (PLC)/Protein Kinase C (PKC) and PI3K/Akt pathways. Inhibition of PI4K simultaneously inactivates these PI4P-dependent pathways. In this study, we tried to identify that which isotype of PI4K may affect a radiosensitivity using RNA interference (RNAi) and to investigate whether anti-hepatitis C viral (HCV) agents, some of which have been shown to inhibit PI4K IIIα activity, could be repositioned as an anti-cancer agent and/or a radiosensitizer in human glioblastoma, breast, and hepatocellular carcinoma.

Methods: A panel of human cancer cell lines including U251 malignant glioma cells, BT474 breast cancer cells, and HepG2 hepatocellular carcinoma cells were used. RNAi was used to specific inhibition of each isotype of PI4K and clonogenic assay was performed to assess the radiosensitizing effect of each isotype. To select an anti-HCV agent for pharmacologic inhibition of PI4K, IC50s of nine commercial antiviral agents were determined. Specific

inhibitory effect on PI4K isotype was determined by in vitro kinase assay. Radiosensitizing effect of the selected anti-HCV agents was tested by clonogenic assay in vitro. Immunoblotting and immunocytochemistry were performed to identify the mechanism of radiosensitization.

Results: A clonogenic assay showed that inhibition of PI4K IIIα using siRNA resulted in a significant increase in radiation-induced death of both U251 cells and BT474 cells. In contrast, inhibition of the other PI4K isotypes did not affect radiosensitivity. These results suggest that PI4K IIIα plays an important role in modulation of the response of malignant glioma cells and breast cancer cells to radiation.

For pharmacologic inhibition of PI4K IIIα, simeprevir was selected based on the results of IC_{50} assays, and its inhibition of PI4K IIIα activity was confirmed in *in vitro* kinase assay. Combination of simeprevir treatment and radiation significantly attenuated expression of p-PKC and p-Akt and increased radiation-induced cell death in tested cell lines. Pretreatment with simeprevir prolonged γH2AX foci and downregulation of p-DNA-PKcs, indicating impairment of nonhomologous end-joining repair. Cells pretreated with simeprevir exhibited mixed modes of cell death, including apoptosis and autophagy. Invasion, migration, and vascular mimicry of tumor cells were markedly inhibited by simeprevir.

Conclusions: Targeting PI4K IIIα using anti-HCV agent is a viable approach to enhance the therapeutic efficacy of radiotherapy in various human cancers, such as glioma, breast, and hepatocellular carcinoma.

Keywords: PI4K, Radiotherapy, anti-hepatitis C viral agent, drug repositioning

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CONTENTS

Abstract i

Contents iii

List of figures iv

List of abbreviations v

Introduction 1

Materials and methods 3

Results 9

Discussion 25

References 30

Abstract in Korean 35

LIST OF FIGURES

Figure 1. Specific inhibition of PI4K IIIα using RNAi increases the radiosensitivity of the U251 cells (A) and BT474 cells (B)..... 10

Figure 2. Specific inhibition of PI4K IIα, IIβ, or IIIβ using RNAi was not associated with increased radiosensitivity in BT474 cells .. 11

Figure 3. The half maximal inhibitory concentration (IC_{50}) screening of various HCV direct acting antiviral agents..... 13

Figure 4. IC_{50} (A) and inhibitory effect on PI4K IIIα activity of simeprevir (B)..... 14

Figure 5. Pharmacologic inhibition of PI4K IIIα using simeprevir increases the radiosensitivity in U251 cells (A), BT474 cells (B), and HepG2 cells (C). 16

Figure 6. Simeprevir inhibits DNA damage repair following irradiation. 18

Figure 7. Modes of cell death..... 21

Figure 8. Effects of simeprevir on invasion, migration, and vascular mimicry..... 23

Figure 9. Cytotoxicity of simeprevir against normal cells..... 24

LIST OF ABBREVIATIONS

PI, phosphoinositide; PI4P, phosphatidylinositol 4-phostate; PI4K, phosphatidylinositol 4-kinase; PKC, protein kinase C; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein kinase; siRNA, small-interfering RNA; HCV, hepatitis C virus; SER, sensitizer enhancement ratio; SF, Surviving faction; PTEN, phosphatase and tensin homolog; INPPB4, inositol polyphosphate-4-phosphatase type II

INTRODUCTION

Dynamic changes in phosphoinositide (PI) levels in the cell membrane play an important role in physiologic control (1). Various kinases, phosphatases, and phospholipases mediate the regulation of PI levels, influencing cell proliferation, receptor signaling, cytoskeletal rearrangements, and cell motility. PI-4-kinase (PI4K) phosphorylates PI to produce phosphatidylinositol 4-phosphate (PI4P). Sequential phosphorylation of PI4P is a key step in the production of the common and essential phospholipid substrate for both the phospholipase C (PLC)/protein kinase C (PKC) and PI3K/Akt pathways (2).

Sustained activation of PKC by PLC in cancer cells directly or indirectly affects cell migration, invasion, survival, proliferation, and apoptosis (3). Similarly, upregulation of PI3K/Akt signaling plays an important role in most human malignancies. Enhanced PI3K activity results in the activation of Akt, which is involved in modulating cell survival, cell cycle progression, and cell growth. Enhanced PI3K activity is also associated with treatment resistance (4). Moreover, activated PKC and Akt play important regulatory roles in the response to ionizing radiation (5,6). Thus, treatment strategies involving inhibition of either the PLC/PKC or PI3K/Akt pathway in conjunction with radiosensitization have received intensive study. However, few studies have examined PI4K as a cancer treatment target, even though PI4K is responsible for the generation of PI4P, which is a common upstream substrate for both the PLC/PKC and PI3K/Akt pathways.

Four PI4K isotypes have been identified: PI4K type II (α- and β- forms) and type III (α- and β- forms). Each isotype exhibits different characteristics and plays different intracellular roles. PI4K IIα is important for wortmannin activity and epidermal growth factor receptor PI signaling (2,7). PI4K IIβ is

very similar to PI4K IIα and is thought to play an important role in the intracellular trafficking of angiotensin II G protein–coupled receptors. A zebrafish gene-silencing study showed that inhibition of PI4K IIIα leads to disruption of the fibroblast growth factor receptor–stimulated mitogen-activated protein kinase signaling pathway. PI4K IIIβ is involved in vesicular trafficking along the secretory pathway and in the regulation of sphingolipid metabolism (2). Research on the tumorigenic role of each PI4K isotype is still in its infancy, and no studies have demonstrated how PI4K could be utilized in cancer treatment, especially radiotherapy (RT).

PI4K IIIα was identified as a crucial host factor for hepatitis C virus (HCV) replication in a small-interfering RNA (siRNA) screening study (8). A recent report indicated that 4-anilino quinazoline, a new HCV replication inhibitor targeting the viral protein NS5A, depletes PI4P by inhibiting PI4K IIIα directly (9). This effect is similar to that resulting from siRNA silencing of PI4K IIIα. The safety and efficacy of various anti-HCV agents, including protease inhibitors and NS5A inhibitors, were demonstrated in clinical trials (10).

In this study, we assessed the effect of targeting PI4K on radiosensitivity in a diverse array of human cancer cells. We hypothesized that inhibition of PI4K could be achieved by the repositioning of existing anti-HCV agents for radiosensitization.

MATERIALS AND METHODS

1. Cell culture

Human malignant glioma cells (U251), human breast adenocarcinoma cells (BT474), and human hepatocellular carcinoma cells (HepG2) were purchased from the American Type Culture Collection (Rockville, MD, USA). All cells were cultured at 37℃ in a 5% CO_2 humidified chamber. Cells were maintained in either Dulbecco's Modified Eagle Medium (Life Technology Inc., Gaithersburg, MD) supplemented with 10% fetal bovine solution or Roswell Park Memorial Institute 1640 medium (Life Technology Inc.), depending on the cell line.

2. RNA interference

Cells were plated in 6-well culture plates and transfected with PI4K isotype–specific (PI4K IIα L-006770, PI4K IIβ L-006769, PI4K IIIα L-006776, PI4K IIIβ L-006777; GE Dharmacon, Lafayette, CO) or non-specific control siRNA at 100 nM using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) in reduced-serum medium (OPTIMEM, Life Technology), according to the manufacturer's protocol. At 48 hours after transfection, cells were trypsinized, diluted to the appropriate cell density, and plated in petri dishes to allow for colony formation. Immunoblotting using lysates from these cultures was performed to screen for the expression of PI4K isotype protein.

3. Pharmacologic inhibition

The following nine commercial anti-HCV agents were purchased from MedchemExpress (Monmouth Jct, NJ): sofosbuvir (HY-15005), ribavirin (HY-B0434), simeprevir (HY-10241), daclatasvir (HY-10466), telaprevir

(HY-10235), boceprevir (HY-10237), ledipasvir (HY-15602), asunaprevir (HY-14434), and danoprevir (HY-10238). The agents were diluted in dimethylsulfoxide (DMSO). For determination of the half-maximal inhibitory concentration (IC_{50}), 500 cells were plated into each well of a 6-well plate. Cells were treated with varying doses of the above-mentioned drugs and incubated for 2 weeks. After verifying colony formation, colonies were fixed, stained, and counted in the same way using a clonogenic assay (described below). A survival graph was constructed based on the average values of triplicate experiments, and the IC_{50} was calculated.

4. Clonogenic assay

Cells were plated at the same density into wells of a 6-well culture plate for each of the different treatment groups and each radiation dose. After treatment, cells were irradiated with a 6-MV X-ray from a linear accelerator (Varian Medical Systems, Palo Alto, CA) at a dose rate of 2.46 Gy/min and then incubated for 14–23 days to allow for colony formation. The resulting colonies were fixed with methanol and stained with 0.5% crystal violet. Colonies containing 50 or more cells were counted, and the surviving fraction (SF) was calculated. The radiation-survival data were fitted to a linear-quadratic model using Kaleidagraph software, version 3.51 (Synergy Software, Reading, PA). The sensitizer enhancement ratio at 0.05 ($SER_{0.05}$) was defined as the ratio of the dose at $SF_{0.05}$ without an inhibitor to that with an inhibitor.

5. Western blot analysis

Cells were washed, scraped from the plate surface, and suspended in lysis buffer (Cell Signaling Technology, Danvers, MA). An equal amount of total protein from each sample was separated by SDS-PAGE and electroblotted onto polyvinylidenedifluoride membranes (Millipore Corp., Bedford, MA).

The membranes were blocked in Tris-buffered saline and Tween-20 solution with 5% skim milk or bovine serum albumin (BSA) and probed with primary antibodies directed against the PI4K isotypes (PI4K IIα ab68732, PI4K IIβ ab71823, PI4K IIIα ab111565, and PI4K IIIβ ab109418; Abcam, Cambridge, UK), p-AKT (Ser473), p-ERK (Tyr202/204), p-PKCβII (Ser660), p-ATM (Ser1981), Rad51 (all from Cell signaling), p-DNA-PKcs (Thr2609, Abcam) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed and incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:10,000 for 1 hour.

6. *In vitro* kinase assay

PI4K activity was confirmed using an ADP-Glo kinase assay (Cat# V1781, Promega, Madison, WI), according to the manufacturer's protocol. This luminescent assay measures ADP produced from the kinase reaction. Briefly, 5 ng/μl of PI4K IIIα (Cat# PV5689, Invitrogen) was incubated with the selected drugs at room temperature in reaction buffer, lipid substrate working solution including PI:3PS, and 5 μl of ATP in a final volume of 25 μl. After 1 hour, 25 μl of ADP-GloTM reagent was added and incubated for 40 min. Next, 50 μl of the kinase detection reagent was added to convert the ADP to ATP, and luciferase/luciferin was introduced to facilitate detection of ATP. The mixture was incubated for 1 hour, and the plate was read using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

7. Immunocytochemistry

Cells were plated into chamber slides and then cultured. At specified times after each treatment, cells were fixed in 4% paraformaldehyde and permeabilized in methanol for 20 min at room temperature. Cells were

subsequently rinsed and blocked in PBS containing 2% BSA for 1 hour and then incubated for 24 hours at 4°C with a primary antibody against γH2AX (Cell Signaling Technology) diluted 1:500 in PBS. AlexaFluor 488–conjugated donkey anti-goat antibody (Molecular Probes, Eugene, OR) diluted 1:1,000 in PBS was applied and incubated for 1 hour at room temperature as the secondary antibody. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/ml for 5 min for nuclear counterstaining and then washed. Slides were mounted using Prolong anti-fade mounting medium (Molecular Probes, Life Technology) and examined under an Axio Scope.A1 Imager fluorescent microscope. Images were acquired using an AxioCam MRc5 digital camera and processed with AxioVision v.4.4 software (Carl Zeiss, Gottingen, Germany).

8. Annexin V fluorescein isothiocyanate (FITC) assay

Apoptosis was assessed using an Annexin V FITC assay (BD Bioscience, Franklin Lakes, NJ). Cells were seeded in 8-well chamber slides and treated with pharmacologic inhibitor or DMSO prior to irradiation. At 24 hours, cells were double stained with annexin V FITC and propidium iodide according to the manufacturer's instructions. The cells were then analyzed under a fluorescent microscope.

9. Lysotracker staining

LysoTracker (Molecular Probes), a membrane-permeating dye, was used as a probe for autophagy. Cells were seeded in 8-well chamber slides and treated with drug or DMSO prior to irradiation. After 2 hours, LysoTracker staining solution (500 nM) was added. Two hours later, cells were fixed with 4% paraformaldehyde and stained with DAPI. The cells were then analyzed under a fluorescent microscope.

10. Cellular senescence–associated β-galactosidase assay

Cellular senescence was evaluated by detecting the activity of β-galactosidase. Cells were seeded in 8-well chamber slides and treated with drug or DMSO prior to irradiation. After 7 days, the cells were fixed and stained using a Senescence β-galactosidase staining kit (Cell Signaling Technology), according to the manufacturer's protocol. The cells were then examined under a light microscope.

11. Wound healing assay

Cells were grown to confluence in 6-well plates (Sonic-Seal Slide, Nalgene Nunc, Rochester, NY) and then starved by culturing in DMEM without FBS for 24 hours. Each well was then divided into a 2 × 3 grid. Using a 1-ml pipette tip, a line was scratched in each hemisphere of the well to wound the cells, and the medium was changed to the starvation medium. Images were taken of the intersections of the linear cell wounds and grid lines. Images were captured immediately after wounding and after 24 hours. The distance between the wound edges was measured using Image J software. The cell migration rate was calculated using the following equation: (initial distance – final distance)/initial distance × 100.

12. Vasculogenic mimicry (VM) assay

A commercial Matrigel assay kit (BD Bioscience) was used to assess VM. Extracellular matrix matrigel (200 μl) was placed in wells of 48-well plates and incubated at 37℃ for 2 hours. Cells were treated with prespecified drug and then seeded onto the coated plate. After incubation for 24 hours, VM was assessed using an inverted microscope.

13. Statistical analyses

The experiments were repeated thrice. These results are expressed as the mean ± SD and analyzed with the two-tailed Student's t-test.

RESULTS

Specific inhibition of PI4K IIIα using RNAi increases the radiosensitivity of malignant glioma cells and breast carcinoma cells.

To characterize the effect of inhibition of specific PI4K isotypes on the radiosensitivity of cancer cells, U251 and BT474 cells were transfected with PI4K isotype–specific siRNA or a non-specific control siRNA. Specific inhibition of each PI4K isotype was verified by Western blotting. A clonogenic assay performed 48 hours after transfection showed that inhibition of PI4K IIIα resulted in an increase in radiation-induced death of both U251 cells ($SER_{0.05}$ 1.17, Fig. 1A) and BT474 cells ($SER_{0.05}$ 1.17, Fig. 1B). At a dose of 6 Gy, radiation-induced cell death was significantly increased following PI4K IIIα silencing in both cell lines (U251, $p = 0.0146$; BT474, $p = 0.0378$). In contrast, inhibition of the other PI4K isotypes did not affect radiosensitivity (Fig. 2). These results suggest that PI4K IIIα plays an important role in modulation of the response of malignant glioma cells and breast cancer cells to radiation.

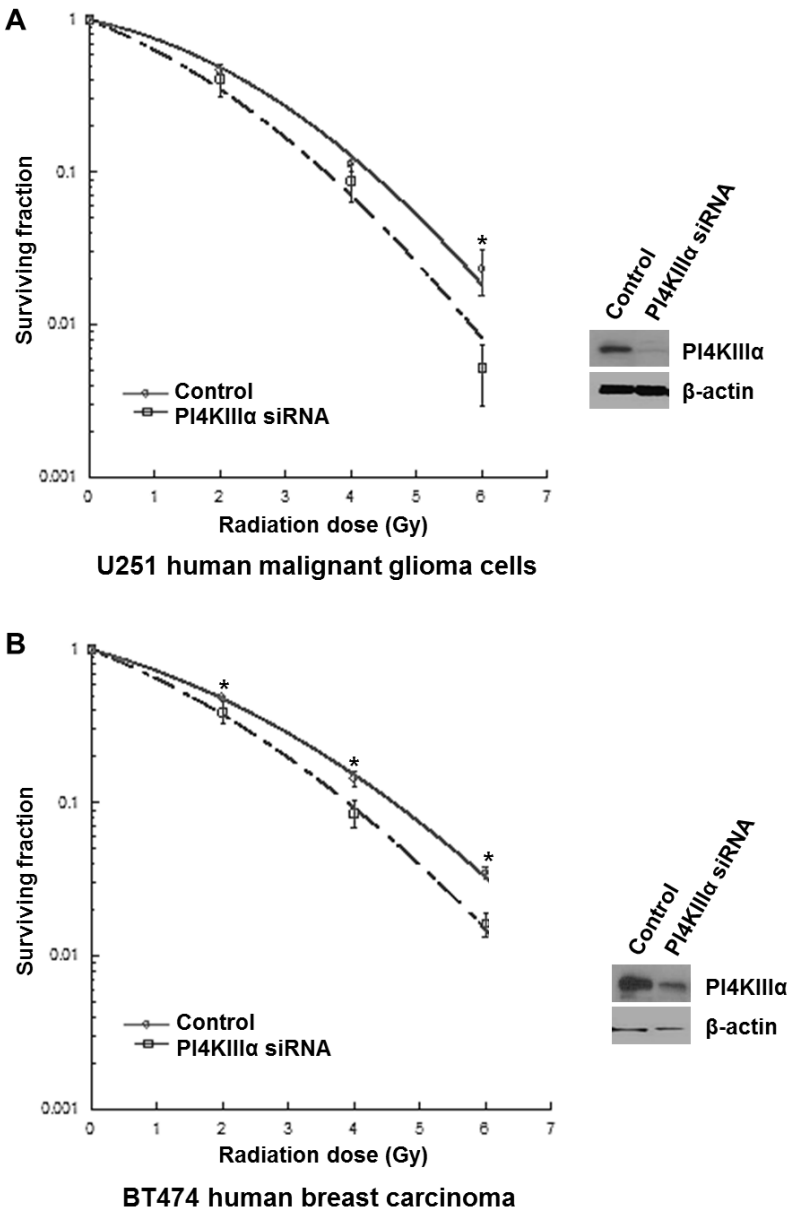


Figure 1. Specific inhibition of PI4K IIIα using RNAi increases the radiosensitivity of the U251 malignant glioma cells (A) and BT474 breast carcinoma cells (B). Survival curve points represent mean surviving fractions from three independent experiments. siRNA-mediated downregulation of PI4K IIIα expression was verified by Western blotting. (*: $p < 0.05$)

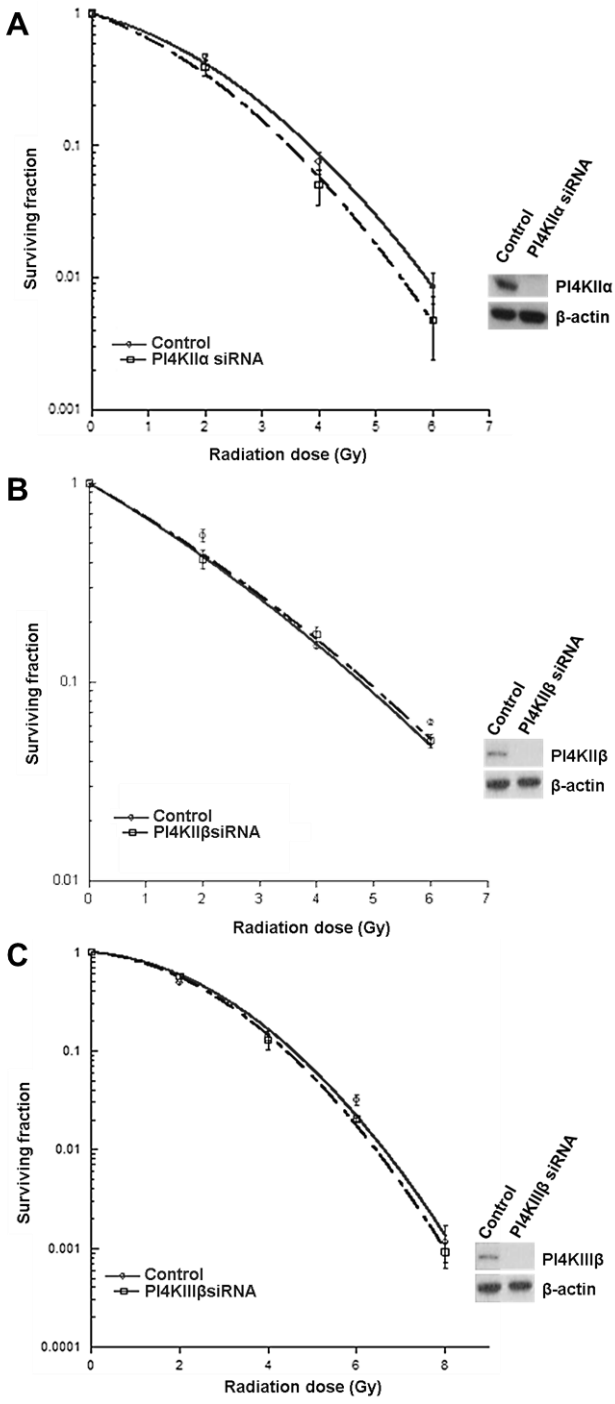


Figure 2. Specific inhibition of PI4K IIα (A), IIβ (B), or IIIβ (C) using RNAi was not associated with increased radiosensitivity in BT474 cells.

Pharmacologic inhibition of PI4K IIIα using anti-HCV agents.

To investigate the potential of anti-HCV agents as PI4K IIIα inhibitors, IC_{50} values for nine commercial anti-HCV agents were determined using U251 cells. Cells were treated with varying doses of each drug and incubated for 2 weeks. Colonies were then counted, and the IC_{50} of each drug was calculated (Fig. 3). Of the nine drugs screened, simeprevir effectively inhibited cell growth at all doses evaluated, with IC_{50} data indicating 200 nM as the appropriate dose for PI4K inhibition (Fig. 4A).

Next, an in vitro kinase assay was carried out to confirm that the growth inhibitory effect of simeprevir is due to the inhibition of PI4K IIIα activity. Simeprevir inhibited the activity of PI4K IIIα in a dose-dependent manner (Fig. 4B).

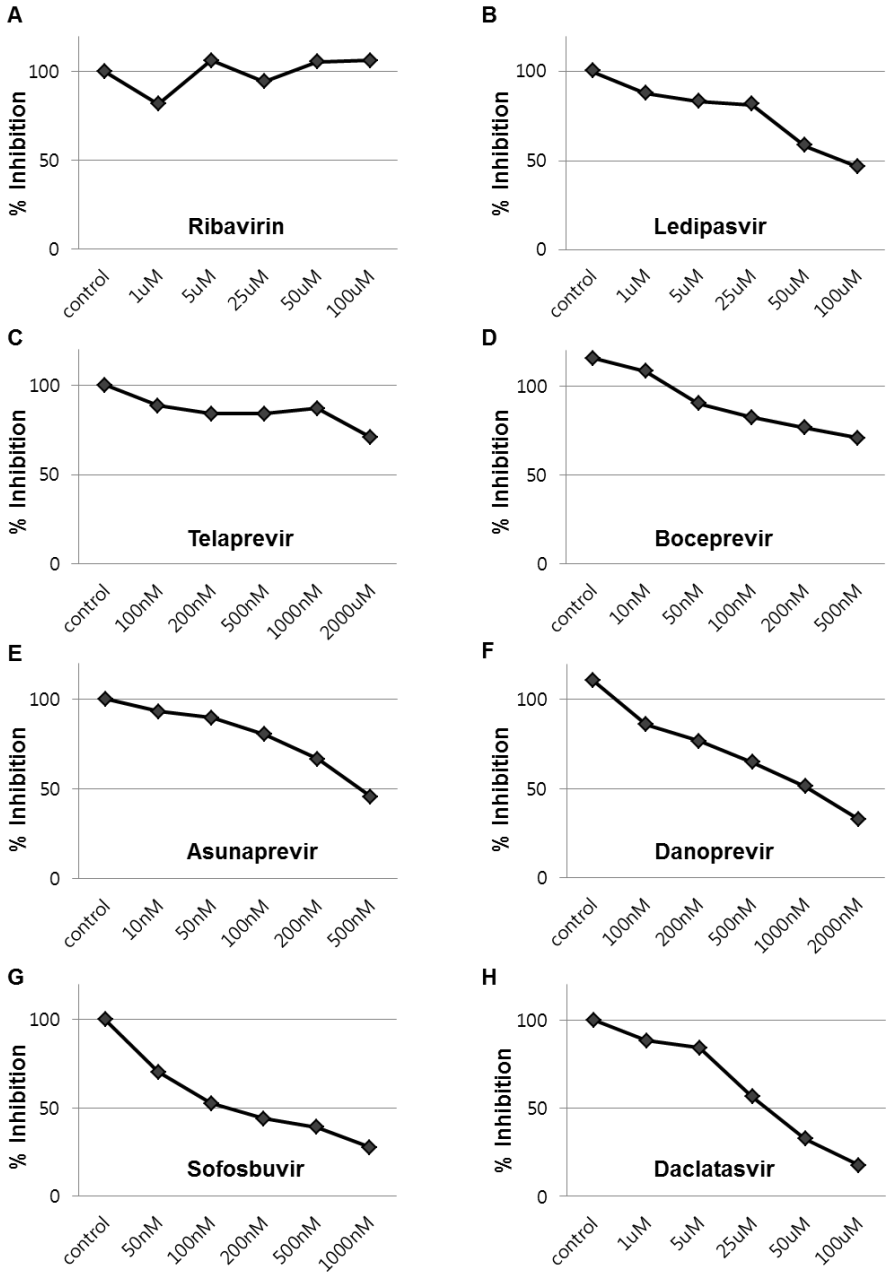


Figure 3. IC_{50} screening of various HCV direct acting antiviral agents.

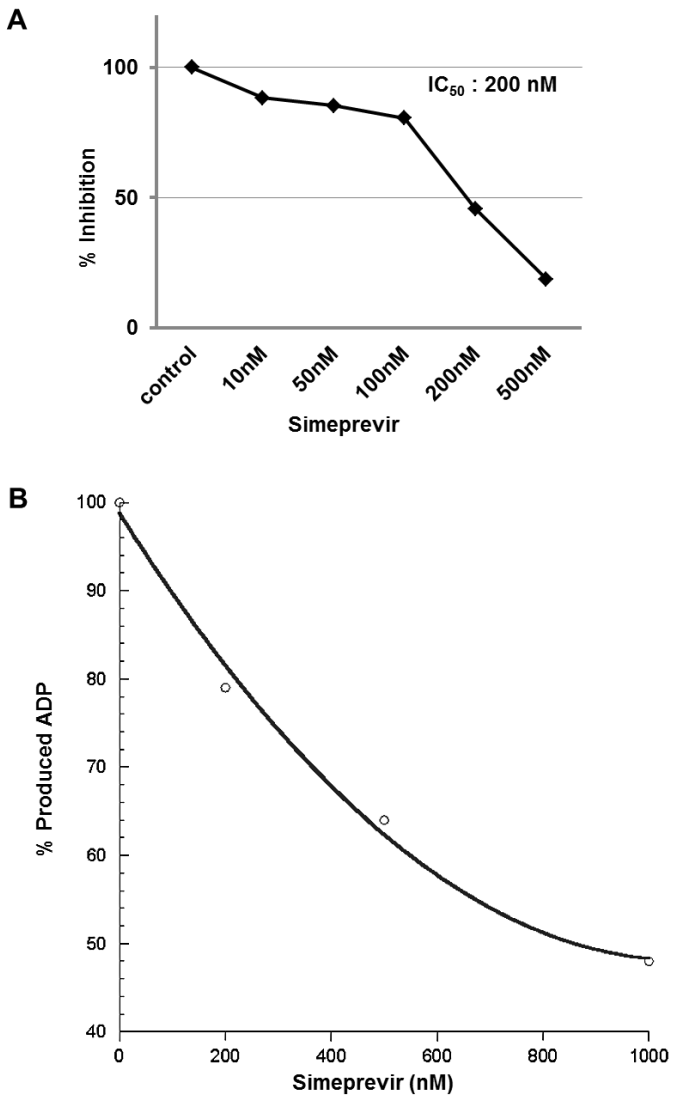


Figure 4. IC_{50} (A) and inhibitory effect on PI4K IIIα activity of simeprevir (B). The IC_{50} of simeprevir was determined as 200 nM as shown in the survival graph. Increasing simeprevir dose induced a decline in PI4K IIIα activity. This result directly demonstrates that simeprevir inhibits the activity of PI4K IIIα.

Pharmacologic inhibition of PI4K IIIα using simeprevir increases the radiosensitivity of a diverse array of human cancer cells through downregulation of both p-PKC and p-AKT.

Having confirmed that simeprevir inhibits PI4K IIIα, the response of simeprevir-treated U251, BT474, and HepG2 cells to radiation was assessed. Cells were pretreated with simeprevir or DMSO for 2hours, irradiated, and incubated for 24 hours after treatment to allow for colony formation. As shown by survival curves (Fig. 5 A-B, left panel), at a concentration of 200 nM, simeprevir (a potent inhibitor of PI4K IIIα) increased the radiation susceptibility of both U251 cells ($SER_{0.05}$ 1.16) and BT474 cells ($SER_{0.05}$ 1.29). Radiation-induced cell death following a dose of 6 Gy was significantly increased in the simeprevir group for both U251 ($p = 0.015$) and BT474 ($p = 0.003$) cells (Student's t-test). Inhibition of PI4K IIIα using simeprevir led to marked downregulation of p-PKC and p-AKT in both cell lines (Fig. 5 A-B, right panel). Although no radiosensitizing effect was observed in HepG2 cells at a simeprevir dose of 200 nM, treatment with a high dose (1000 nM) resulted in increased radiation sensitivity ($SER_{0.05}$ 1.12). Significantly enhanced cell death at a radiation dose of 6 Gy ($p = 0.007$) and downregulation of both p-PKC and p-AKT was also observed (Fig. 5C). These findings suggest that the radiosensitizing effect of simeprevir in U251, BT474, and HepG2 cells results from suppression of prosurvival signaling.

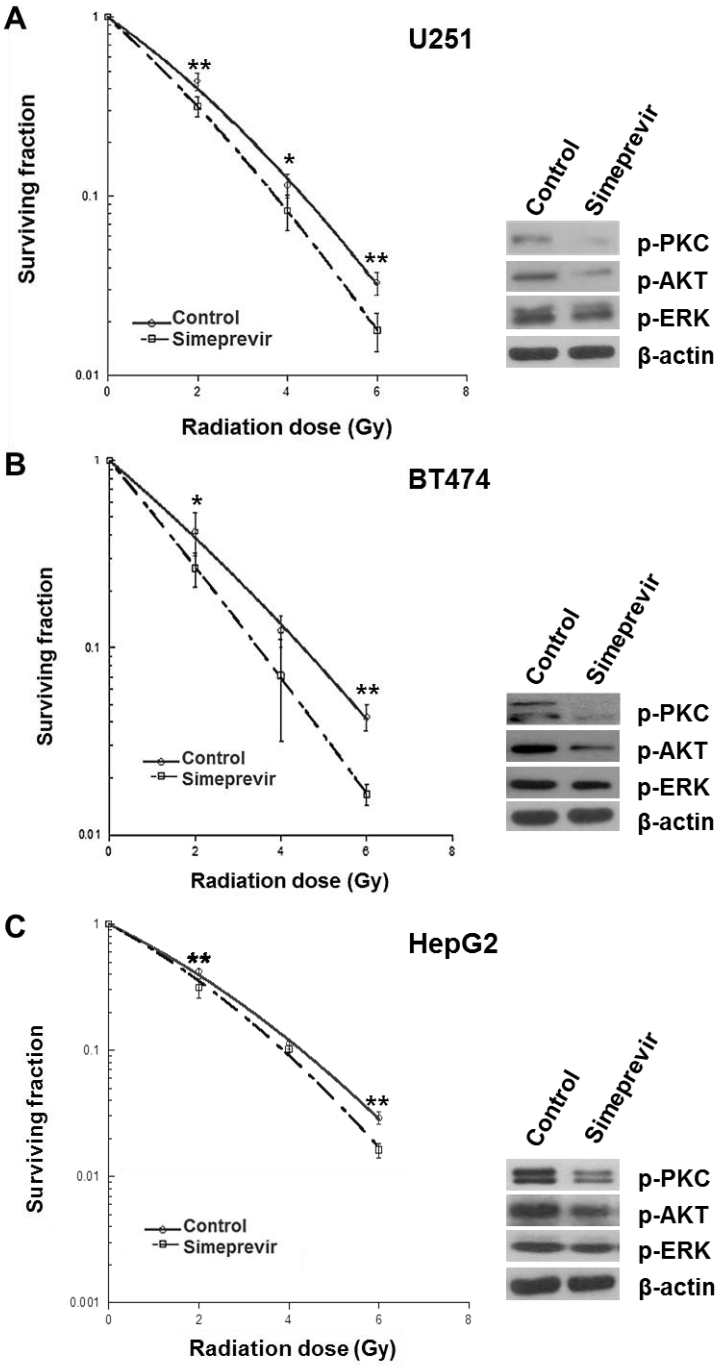


Figure 5. Pharmacologic inhibition of PI4KIIIα using simeprevir increases the radiosensitivity in U251 cells (A), BT474 cells (B), and HepG2 cells (C). Western blotting showed that pretreatment with simeprevir downregulated p-

PKC and p-AKT (right panel). Survival curve points represent the mean surviving fraction from three independent experiments. (*: $p < 0.1$, **: $p < 0.05$) (*: $p < 0.1$, **: $p < 0.05$)

Simeprevir-mediated PI4K IIIα inhibition impairs DNA damage repair mechanisms following irradiation.

It is well known that radiation induces DNA damage and that inhibition of the PI3K/Akt pathway inhibits DNA damage repair. Thus, a γH2AX assay was performed to evaluate the capacity of simeprevir-treated cells to repair radiation-induced DNA damage. Pretreatment with simeprevir induced prolongation of γH2AX foci in U251, BT474, and HepG2 cells compared with mock-treated controls at 6 hours following a 6 Gy radiation dose (Fig. 6A-C). Pretreatment with simeprevir led to downregulation of p-DNA-PKcs, which is indicative of impaired nonhomologous end-joining repair, whereas no discernable change was detected in Rad-51 expression (Fig. 6D).

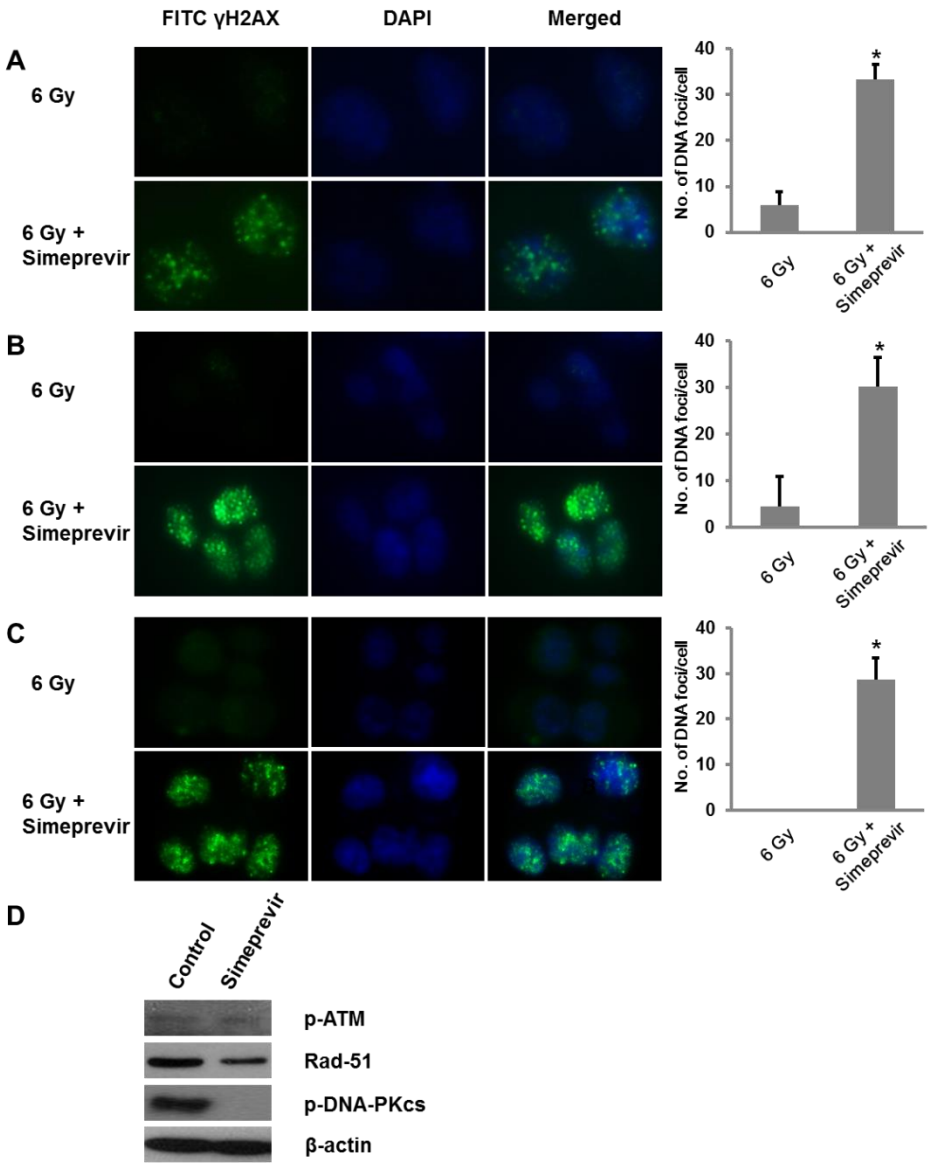
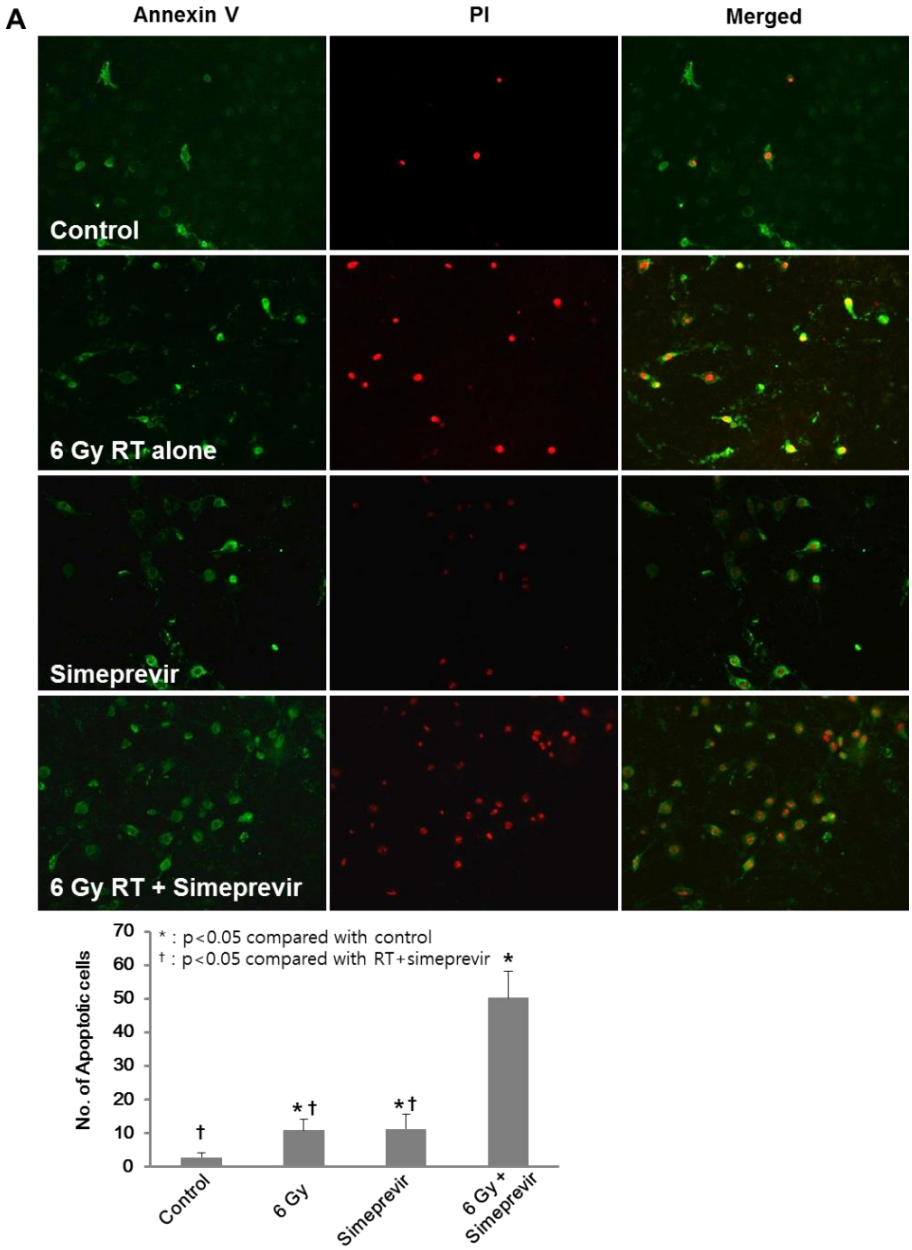


Figure 6. Simeprevir inhibits DNA damage repair following irradiation. U251 (A), BT474 (B), and HepG2 (C) cells were pretreated with simeprevir or DMSO and irradiated at a dose of 6 Gy. After 6 hours, prolongation of γH2AX foci was detected in simeprevir-treated cells along with decreased phosphorylation of DNA-PKcs, indicating impaired nonhomologous end-joining repair (D). (*: $p < 0.05$)

Modes of cell death

Next, the modes of cell death associated with enhanced radiosensitivity were investigated using an Annexin V FITC assay, LysoTracker assay, and β-galactosidase staining. Annexin V has high affinity to phosphatidylserine (which translocates from the inner to the outer surface of the plasma membrane in the early stages of apoptosis) and can be detected by green fluorescence. To identify dead and damaged cells, the assay is used in conjunction with PI, which exhibits red fluorescence. The combined treatment with simeprevir and irradiation increased drastically both green and red fluorescence in U251 cells. (Fig. 7A). A LysoTracker assay was performed to detect autophagy. Pretreatment of U251 cells with simeprevir prior to irradiation also resulted in increased punctate fluorescence indicative of lysosomal localization at 2 hours (Fig. 7B). Senescence was evaluated by β-galactosidase staining; no discernable change was noted in U251 cells up to 7 days after treatment (Fig. 7C).


A
Annexin V
PI
Merged
Control
6 Gy RT alone
Simeprevir
6 Gy RT + Simeprevir
* : p<0.05 compared with control
† : p<0.05 compared with RT+simeprevir
No. of Apoptotic cells
70
60
50
40
30
20
10
0
Control
6 Gy
Simeprevir
6 Gy + Simeprevir

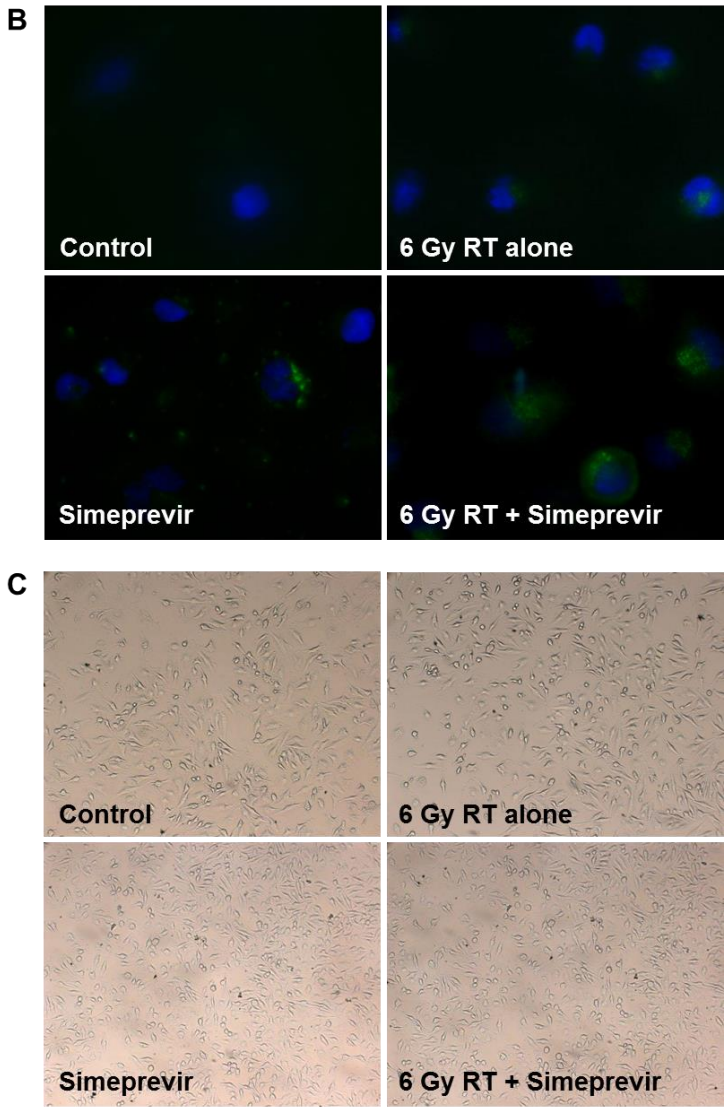


Figure 7. Modes of cell death. (A) Annexin V FITC/PI double staining images acquired at 24 hours (×200). The combined treatment with simeprevir and irradiation drastically increased both green (annexin V FITC) and red (PI) fluorescence in U251 cells (upper panels). (B) Treatment of U251 cells with simeprevir prior to irradiation induced punctate fluorescence indicative of lysosomal localization of LysoTracker. (C) β-galactosidase staining was employed to examine senescence; no discernable changes were observed in U251 cells up to 7 days after treatment.

Effect of PI4K IIIα inhibition on invasion, migration, and VM.

Cell invasion and migration are not only important in tumor progression; they also play key roles in tumor metastasis and angiogenesis. Thus, inhibition of cell invasion and/or migration could be helpful in anti-cancer therapy. A wound healing assay was performed to assess the effect of simeprevir treatment on the invasion and migration of cancer cells. As shown in Figure 8A, treatment with simeprevir at 200 nM markedly inhibited the migration of U251 cells.

Recent evidence suggested that tumors have multiple unexpected mechanisms of neovascularization (11). VM is a process that involves de novo generation of microvascular channels by genetically deregulated, aggressive tumor cells, without the participation of endothelial cells, and independent of angiogenesis (12). The phenomenon is associated with poor prognosis, and metastasis involving VM has been confirmed in glioblastoma, breast cancer, and liver cancer (13). In the present study, the inhibition of VM following treatment of U251 cells with simeprevir was evaluated using a VM assay. As shown in Figure 8B, simeprevir treatment significantly inhibited VM compared with controls.

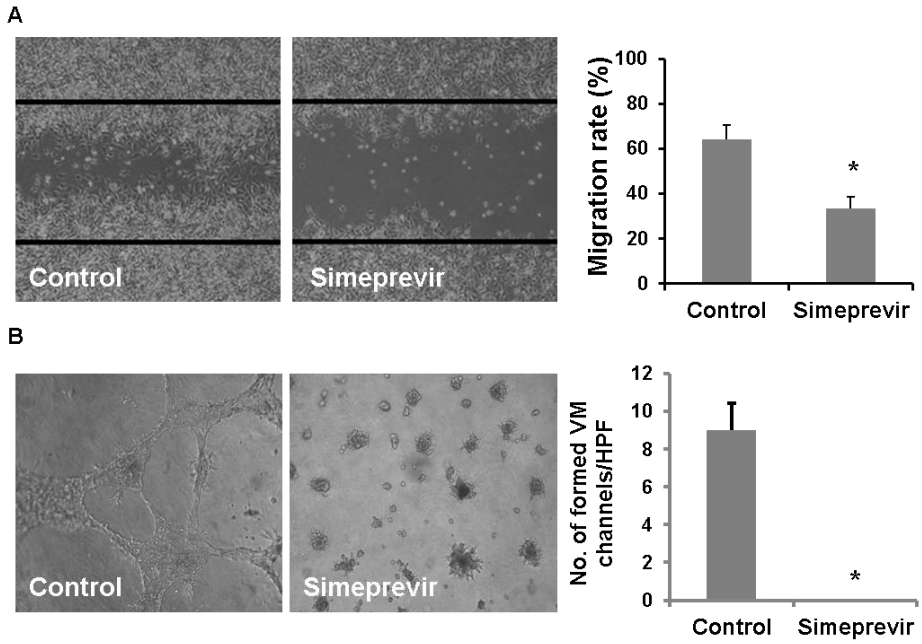


Figure 8. Effects of simeprevir on invasion, migration, and VM. The effects of simeprevir on invasion and migration of U251 cells were assessed using a wound healing assay (A). Stained cells were analyzed in representative fields (100×). Treatment with simeprevir (200 nM) markedly inhibited migration of U251 cells. The VM potential of U251 cells when plated on matrigel was determined with and without simeprevir (B). Simeprevir treatment inhibited VM, as shown in representative photographs (×200). (*: $p < 0.05$)

Toxicity of simeprevir to normal cells.

The cytotoxicity of simeprevir was evaluated using a clonogenic assay. At 200 nM, simeprevir exhibited no observable toxicity to normal human astrocytes (Fig. 9).

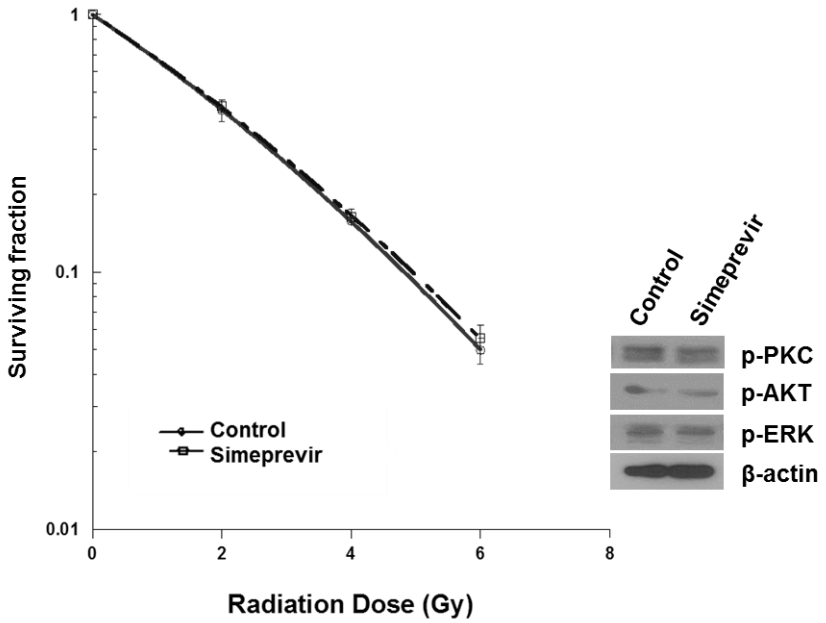


Figure 9. Cytotoxicity of simeprevir against normal cells. Compared with controls, the clonogenic survival of normal human astrocytes was not significantly affected by treatment with simeprevir.

DISCUSSION

PI4K catalyzes the phosphorylation of phosphoinositide to synthesize PI4P, which is converted to PI(4,5)P2 by subsequent phosphorylation. This process is critical for supplying an essential substrate for activation of not only Akt through PI3K, but PKC via PLC.

Considerable research has focused on PI3K as a promising target molecule. PI(3,4,5)P3 synthesis catalyzed by PI3K phosphorylation contributes to the activation of a wide range of downstream targets, including Akt, which activates mTOR (11). The activated PI3K/Akt pathway enhances cell survival in response to a variety of cell stressors, including radiation. Previously, we demonstrated that inhibition of the PI3K/Akt pathway downregulates phosphorylation of Akt and increases the radiation sensitivity of a diverse array of human cancer cell lines (6,12–14). Our previous reports also showed that the radiosensitizing effects of targeting PI3K/Akt signaling are associated with prolongation of γH2AX foci and downregulation of pDNA-PKcs, suggesting that inhibition of the PI3K/Akt pathway impairs the DNA double-strand-break repair response following irradiation (12,15,16). Based upon these preclinical data, clinical studies attempting to verify the efficacy of PI3K/Akt/mTOR pathway inhibition combined with radiation therapy are ongoing (17).

PKC activation is mediated by PLC, which generates diacylglycerol and PI(1,4,5)P3 from PI(4,5)P2. PKC has numerous downstream targets, including extracellular-related kinase 1/2 (ERK1/2), nuclear factor–kappa beta, and P-glycoprotein. Although short-term activation of PKC affects physiological processes such as secretion and ion-influx, sustained activation is thought to be involved in proliferation, differentiation, apoptosis, and tumorigenesis (18). In particular, PKC plays a role in activating survival- or

proliferation-associated signaling pathways, such as Ras/MAPK or PI3K/Akt in cancer. However, in terms of RT, few studies have examined radiosensitization. Choi et al. showed that PKC inhibitors increase radiation sensitivity by inducing apoptosis (19).

PI4K catalyzes the production of PI(4,5)P2, a common substrate for PI-dependent signaling pathways. Thus, inhibition of PI4K would be expected to inhibit both the PI3K/Akt and PLC/PKC pathways. However, no results of studies targeting PI4K for radiosensitization have been reported. In the present study, an enhanced response to radiation in various human cancer cells was observed following PI4K IIIα inhibition using either specific siRNA (Fig. 1) or simeprevir (Fig. 5). Downregulation of both p-Akt and p-PKC after treatment with simeprevir (Fig. 5, right panels) suggests that this approach simultaneously inhibits both the PI3K/Akt and PLC/PKC pathways. The prolongation of γH2AX foci and downregulation of pDNA-PKcs after inhibition of PI4K are indicative of impaired nonhomologous end-joining repair (Fig. 6). It has been reported that inhibition of the PI3K/Akt pathway results in impaired DNA damage repair; however, this is the first report demonstrating that inhibition of PI4K also impairs DNA repair via DNA-PK.

In this study, we examined breast cancer, glioblastoma, and hepatocellular cancer cells. Both breast cancer and glioblastoma tumors are considered radioresistant (20,21). In hepatocellular carcinoma, delivery of a tumoricidal dose of therapeutic agent is often impossible due to compromised liver reserve and damage to the adjacent luminal gastrointestinal tract (22,23). Thus, effective radiosensitizers could improve RT outcomes. Aberrantly enhanced PI3K/Akt signaling is a common feature of various cancers, occurring in more than half of breast cancer, glioblastoma, and hepatocellular carcinoma cases (24–26). Furthermore, expression of phosphatase and tensin homolog (PTEN), which negatively regulates the PI3K/Akt pathway by dephosphorylating

PI(3,4,5)P3 to PI(4,5)P2, is reduced by mutation or loss of heterozygosity in up to 80% of glioblastomas (24,25). Inositol polyphosphate-4-phosphatase type II (INPP4B) has recently emerged as new breast cancer biomarker. INPP4B also functions as a tumor suppressor through negative regulation of the PI3K/Akt pathway. The substrate of INPP4B, PI(3,4)P2, binds to the Pleckstrin Homology domain of Akt, which facilitates full activation of Akt. PI(3)P (converted from PI[3,4]P2 by INPP4B) inhibits PI3K-dependent Akt activation. Thus, reduced INPP4B expression seems to be associated with enhanced PI3K/Akt signaling. In breast cancer, mutations in *PI3KCA*, the gene encoding PI3K, are mostly observed in the ER+ luminal type, whereas loss of PTEN and INPP4B usually occurs in triple-negative breast cancer (24,27). These observations suggest that PI4K, which supplies the substrate common to the PI-dependent pathways involving PI3K, PTEN, and INPP4B, is a promising target in consideration of the cancer-specific expression patterns of these molecules.

Despite the above-mentioned data, PI4K has not been actively investigated in terms of cancer. Only a few studies have suggested that PI4K isotypes are involved in tumorigenesis and might thus be suitable targets for cancer treatment (2,28–31). Li et al. showed that blocking PI4K IIα expression results in tumor growth suppression *in vivo* and that PI4K IIα triggers hypoxia-inducible factor 1-alpha accumulation via PI3K and ERK1/2 pathways (7). Their subsequent study demonstrated that PI4K IIα knockdown downregulates HER2 activity and revealed a strong correlation between EGFR and PI4K IIα expression in breast cancer patients (30). Chu et al. demonstrated an anti-apoptosis role for PI4K IIα (32). PI4K IIβ is known to reduce the motility of hepatocellular carcinoma cells by facilitating the formation of CD81-enriched vesicles through functional interaction with tetraspanin CD81 (31). PI4K IIIβ appears to play a similar role in breast

cancer. In human breast cancer cells, overexpression of PI4K IIIβ has anti-apoptotic effects and disrupts *in vitro* acinar morphogenesis (32).

PI4K IIIα, which was targeted to enhance the effect of radiation in the present study, was identified as a mediator of resistance to chemotherapy in medulloblastoma cells and pancreatic cancer cells in two separate studies. Based on these findings, Waugh et al. hypothesized a novel role for PI4K IIIα in the constitutive chemoresistance of cancers (2). Overexpression of PI4K IIIα has also been associated with a more invasive or metastatic cancer phenotype (28). In a study of human hepatocellular carcinoma, overexpression of PI4K IIIα was shown to be related to an undifferentiated status and to tumor proliferation rate (29), resulting in a poor clinical outcome in terms of disease-specific survival and recurrence-free survival in patients with high levels of PI4K IIIα mRNA expression. In addition, a series of studies on HCV revealed that PI4K IIIα is a crucial host factor for viral replication (8,9).

Newly developed anti-HCV agents, so-called "direct-acting anti-HCV agents," have enabled clinicians to overcome the resistance associated with traditional HCV treatments, such as pegylated interferon-α and ribavirin, and are now widely used in practice. A new NS5A inhibitor suppresses viral replication through inhibition of PI4K IIIα (9,33,34). However, simeprevir, which was used in our study, inhibits the NS3/4A protease, which functions as both an RNA helicase and a serine protease, and is required for viral precursor processing (34). There is no evidence of simeprevir's inhibitory effect on PI4K, in contrast to the NS5A inhibitor. However, our *in vitro* kinase assay confirmed inhibition of PI4K after treatment with simeprevir (Fig. 4A), resulting in enhanced radiosensitization of human cancer cells, similar to the effect of siRNA-mediated PI4K inhibition.

In the present study, we demonstrated that simeprevir increases the

radiosensitivity of cancer cells by impairing the DNA damage repair processes (Fig. 6), resulting in apoptosis and autophagy as the final modes of cell death (Fig. 7). Reduced migration, invasion, and VM potential could also play important roles in the radiosensitizing effect (Fig. 8).

Our study is the first to propose PI4K IIIα as a target for radiosensitization. Furthermore, our data suggest that it would be useful to reposition simeprevir for use as a PI4K IIIα inhibitor. After validation of simeprevir's anti-tumor effect, especially with regard to radiosensitization, in *in vivo* studies, simeprevir could be translated easily into the clinical setting as an alternative anti-cancer drug based on its previously established safety.

In conclusion, the results of the present study indicate that targeting PI4K IIIα has a significant radiosensitizing effect on human cancer cells via inhibition of prosurvival signaling, DNA damage repair, and migration and invasion potential. The anti-HCV agent simeprevir holds great potential for use in cancer treatment as a pharmacologic inhibitor of PI4K IIIα. Inhibition of PI4K IIIα via either siRNA or simeprevir represents an attractive approach for simultaneously inhibiting both the PI3K/Akt and PLC/PKC pathways and an alternative scheme to increase the therapeutic index in combination with irradiation.

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국문 초록

서론: 포스파티딜이노시톨-4-키나아제 (PI4K)는 PLC/PKC 그리고 PI3K/Akt 신호전달 체계의 공통된 필수적인 기질인 포스파티딜이노시톨-4-인산염의 인산화를 담당하고 있다. PI4K 의 억제는 이러한 PI4P 에 의존적인 두 신호전달 체계를 동시에 억제할 수 있을 것으로 생각된다. 이 연구에서는, 인체 유래 교모세포종, 유방암, 그리고 간암 세포주에서 RNA 간섭 기법을 이용하여 PI4K 의 어떤 아이소타이프가 방사선 민감도에 영향을 주는 지 밝히고, 일부에서 PI4K IIIα 의 활동을 억제하는 것으로 알려져 있는 C 형간염 항바이러스제의 항암제, 특히 방사선 민감제로 전환 사용 가능성에 대해 알아보고자 하였다.

방법: 인체 유래 종양 세포주인 교모세포종 U251 세포주, 유방암 BT474 세포주, 간암 HepG2 세포주를 사용하였다. PI4K 의 특정 아이소타이프를 각기 억제하기 위하여 RNA 간섭 기법을 사용하였고, 각각의 아이소타이프 억제가 방사선 증강 효과를 가져오는지 평가하기 위하여 클론원성 세포 생존 분석을 시행하였다. PI4K IIIα 의 약리적 억제를 위해 사용될 C 형 간염 항바이러스제를 선별하기 위하여 9 가지 시판 약물의 반수 최대 억제 농도(IC_{50})를 구하였다. 선별된 약물의 PI4K 아이소타이프에 대한 특정 억제 효과는 체외 키나아제 실험으로 확인하였다. 방사선 민감도 증감 효과의 기전을 밝히고자, 면역블로팅과 면역세포화학기법, 세포사 분석, 세포 침습/이주와 모방 혈관형성분석의 실험을 수행하였다.

결과: 세포 생존 분석은 siRNA 를 이용하여 PI4K IIIα 특정 억제 시 U251 세포주와 BT474 세포주에서 방사선 유도 세포사가 유의하게 증가됨을 보였다. 이와 대조적으로, 다른 PI4K 아이소타이프의 억제는 방사선 민감도에 영향을 주지 않았다. 이 결과들은 PI4K IIIα가 악성 교모세포와 유방암 세포의 방사선에 대한 민감도 조절에 중요한 역할을 함을 암시한다.

PI4K IIIα의 약리적 억제를 위하여, IC_{50} 분석을 통해 시메프레비르(simeprevir)를 선별하였고, 시메프레비르의 PI4K IIIα 활성에 대한 억제 효과를 체외 키나아제 실험을 통해 확인하였다. 시메프레비르와 방사선의 병용은 실험된 세포주에서 PKC 와 Akt 의 인산화를 유의하게 감소시켰고, 방사선-유도 세포사를 증가시켰다. 시메프레비르의 전처치는 γH2AX foci 를 연장시키고, DNA-PKcs 의 인산화를 감소시켰는데, 이는 시메프레비르가 비상동 말단 연결에 의한 수복과정에 장해를 초래함을 시사한다. 시메프레비르 처리된 세포들은 세포 자살과 자식 작용이 혼재된 세포사를 나타냈다. 시메프레비르에 의해 종양 세포의 침습/이주 그리고 모방 혈관형성이 현저히 억제되었다.

결론: C 형 간염 항바이러스제를 이용한 PI4K IIIα의 표적화는 교모세포종, 유방암, 그리고 간암과 같은 다양한 인간의 암종에서 방사선 치료 효능을 증가 시키기 위해 실행 가능한 접근이겠다.

주요어 : 포스파티딜이노시톨-4-키나아제, 방사선치료, C 형 간염 항바이러스제, 약물 재창출 전략

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