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2,3,6-Trisubstituted quinoxaline derivative, a small molecule inhibitor of the Wnt/beta-catenin signaling pathway, suppresses cell proliferation and enhances radiosensitivity in A549/Wnt2 cells

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ABSTRACT

GDK-100017, a 2,3,6-trisubstituted quinoxaline derivative, reduced β-catenin-T-cell factor/lymphoid enhancer factor (TCF/LEF)-dependent transcriptional activity and inhibited cell proliferation in a dose-dependent manner with an IC_{50} value of about 10 μM in A549/Wnt2 cells. GDK-100017 down-regulated the expression of Wnt/β-catenin pathway target genes such as cyclin D1 and Dkk1 but not c-myc or survivin. GDK-100017 inhibited cell proliferation by arresting the cell cycle in the G1 phase not only in A549/wnt2 cells but also in SW480 colon cancer cells. In addition to its wnt signaling inhibitory properties, GDK-100017 also enhanced the radiosensitivity of the A549 human NSCLC line. These results suggest that GDK-100017 possesses potential anti-cancer activity by inhibiting the Wnt/β-catenin signal pathway, blocking the β-catenin-TCF/LEF interaction, and enhancing radiosensitivity.

1. Introduction

Wnt signaling is one of the key mechanisms that regulates cell proliferation, differentiation, and morphogenesis [1]. Aberrant activation of the canonical Wnt signaling pathway is related to many human cancers, including colon carcinoma, melanoma, and lung cancer [2,3]. As the relevance of Wnt signaling in human cancers has been emphasized, Wnt/β-catenin-mediated signaling has become a promising target for therapeutic intervention.

β-Catenin plays an important a role as a transcriptional activator in the canonical Wnt/β-catenin signal pathway with T-cell factor (TCF)/lymphoid enhancer factor (LEF). In the absence of the Wnt protein, cytosolic β-catenin is maintained at low levels through β-catenin degradation by destruction complex machinery. Activation of Wnt signaling inhibits glycogen synthase kinase 3 beta (GSK3β) activity and increases cytosolic β-catenin level. The elevated β-catenin level leads to translocation of β-catenin to the nucleus and to complex formation with T-cell factor (TCF)/lymphoid enhancer factor (LEF), which induces expression of target genes involved in proliferation, invasiveness, and angiogenesis [4]. The three major areas of targeting in the Wnt pathway are receptor/ligand interactions, cytosolic signaling components, and nuclear signaling components [5]. Inhibiting the destruction complex composed of APC, axin, GSK3β, and other proteins leads to stabilization and nuclear translocation of cytoplasmic β-catenin, which is key for activating canonical Wnt signaling [6].

Lung cancer is the most common cause of cancer-related deaths worldwide. Lung cancers are classified into histological categories based on the initiating cell type. The two main groups are small cell lung cancer and non-small cell lung cancer (NSCLC), accounting for approximately 18% and 80% of lung cancer incidence, respectively [7]. Although advances in chemotherapy have provided some improvement in overall survival for patients with advanced NSCLC, the overall survival rate remains poor [8]. Wnt1 and Wnt2 expression are upregulated within NSCLC lung tumors. Wnt1 and Wnt2 are considered proto-oncogenes but are also critical for cell differentiation and development during embryogenesis [8,9].

Radiation therapy has long been used to treat patients with cancer, particularly for treating NSCLC. However, radioresistant tumors and radiation toxicity in normal tissues are still a major concern. The current strategy of combining radiation with standard cytotoxic chemotherapeutic agents can potentially lead to unwanted side effects. Thus, much interest has developed for radiosensitizers that could improve the efficacy of radiation killing of cancer cells and prevent damage to normal cells and tissues caused by radiation without systemic toxicity [10,11]. A number of molecules involved in the DNA damage response, apoptosis,

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transcription factors, growth factor receptors, and cytoplasmic signal transduction play a role to determine radiosensitivity. Because many of these molecules are mutated, abnormally expressed, or have alternative functions in cancer cells, they have received considerable attention as tumor-specific targets with radiosensitization activity [11].

We previously screened 1,434 compounds to identify small molecule inhibitors of the Wnt/β-catenin pathway using a cell-based Topflash reporter gene assay in A549/Wnt2 cells [12]. Some of these inhibitors reduced cell growth and inhibited transcription activity of β-catenin-TCF/LEF in dose-dependent manner. In this study, we demonstrated that GDK-100017 (Fig. 1A), a 2,3,6-trisubstituted quinoxaline derivative, is a novel Wnt/β-catenin pathway small inhibitor with radiosensitization activity.

2. Methods

2.1. Cell lines and culture conditions

A549, SW480, and L132 cell lines were obtained from the American Tissue Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin G, and 100 U/ml streptomycin (Gibco-BRL). A549/wnt2 cells were induced to over-express human Wnt2 by stable transfection of pUSEampWnt2, as described in Lee et al. [12]. Cell cultures were incubated at 37 °C in 5% CO_2/95% air, and the medium was replaced every third day.

2.2. Cell proliferation assay

Cells were seeded in 96-well plates (5×10^3 cells/well), incubated for 24 h and then treated with various concentrations of the GDK-100017 for the indicated times. Cell proliferation was determined by the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit, according to the manufacturer's protocol (Promega, Madison, WI, USA). After adding MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution, the plate was incubated at 37 °C in a CO_2 incubator for 30 min, and absorbance was measured on a Molecular Dynamic plate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

2.3. Preparation of RNA and reverse transcription-polymerase chain reaction (RT-RCR)

mRNA levels of the Wnt/β-catenin signaling pathway related genes were analyzed by RT-PCR to observe the effect of GDK-00017. Total RNA was extracted from cells using Easyblue solution following the manufacturer's procedure (Intron Co., Seoul, Korea). First-strand synthesis using 2 μg of total RNA as a template was performed at 42 °C for 1 h using the oligo-dT primer and M-MLV Reverse Transcriptase (Promega). Gene expression was investigated by PCR using the following primers: cyclin D1 5′- TGCTCCT GGTGAACAAGCTC-3′ (sense) and 5′-GTCAGAGATGGAAGGGGGAA-3′ (antisense) generating a 635 bp product, c-Myc 5′-TACCCTCTCAA CGACAGCAG-3′ (sense) and 5′-TCTTGACATTCTCCTCGGTG-3′ (antisense) generating a 550 bp product, Dickkopf-1 (DKK1) 5′-ATT-CCAACGCTATCAAGAACC-3′ (sense) and 5′- CCAAGGTGCTATGAT CATTACC-3′ (antisense) generating a 383 bp product and survivin 5′-GAGCTGCAGGTTCCTTATC-3′ (sense) and 5′-ACAGCATCGAGCCA AGTCAT-3′ (antisense) generating a 431 bp product.

2.4. Transfection and luciferase reporter gene assay

Cells were transfected with the luciferase reporter construct pSuperTopflash containing eight Tcf consensus binding sites upstream of firefly luciferase cDNA, or pSuperFopflash, a plasmid with mutated Tcf binding sites (kindly provided by Professor Ja-Hyun Baek at Korea University, Seoul, Korea). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), with the reporter construct and the pRL-TK plasmid, and incubated with various concentrations of the selected compounds at 37 °C. After 24 h, cells were lysed in 50 μl passive lysis buffer (Promega). Firefly and Renilla luciferase activity were determined using the Dual-Glo Luciferase Assay System (Promega). Results are expressed as mean ± standard error of normalized firefly and Renilla luciferase activity ratios for each triplicate set.

2.5. Preparation of cytoplasmic and nuclear extracts

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 400 ml buffer A [10 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF] supplemented with a complete protease inhibitor cocktail (Roche, Rockland IL, USA). The cells were allowed to swell on ice for 15 min, lysed gently with 12.5 ml of 10% Nonidet P-40, and centrifuged at 2000*g* for 10 min in ice. The supernatant was collected and used as the cytoplasmic extract. The nuclear pellet was resuspended in 40 ml of buffer C [20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF] supplemented with a complete protease inhibitor cocktail (Roche), incubated for 30 min in ice, and the nuclear debris was centrifuged at 20,000*g* for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen, and stored at −80 °C until used for analysis. Protein content was determined using the Bradford assay (Bio-Rad).

2.6. Western blot

Whole cell lysates were prepared by scraping cells into ice-cold RIPA buffer [150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 50 mM Tris–HCl (pH 7.4), 1 mM NaF, 1 mM Na_3OV_4] supplemented with complete protease inhibitor cocktail. Protein samples (20–30 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Whatman, Maidenstone, UK) for Western blotting. The primary antibodies used were β-catenin, GAPDH, poly-ADP ribose polymerase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and cyclin D1 (Cell Signaling Technology, Danvers, MA, USA). Immunoreactive proteins were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Amersham Biosciences, Parsippany, NJ, USA).

2.7. Cell cycle analysis

Cell-cycle arrest was analyzed by measuring DNA staining with propidium iodide (Sigma, St. Louis, MO, USA), as described by the manufacturer. The cells were digested with 1 × trypsin (Gibco) and harvested at 48 h after GDK-100017 treatment and/or radiation. Cell pellets were washed twice with PBS and fixed in 70% ethanol at 4 °C for 1 h. The cells were washed twice with PBS and treated with 20 μg/ml ribonuclease A (Sigma) at 37 °C for 1 h. Then, the cells were stained with 50 μg/ml propidium iodide for 10 min at room temperature in the dark and analyzed by flow cytometry with a FACScan (Becton Dickinson, Fullerton, CA, USA) using the CELLQuest program (Becton Dickinson).

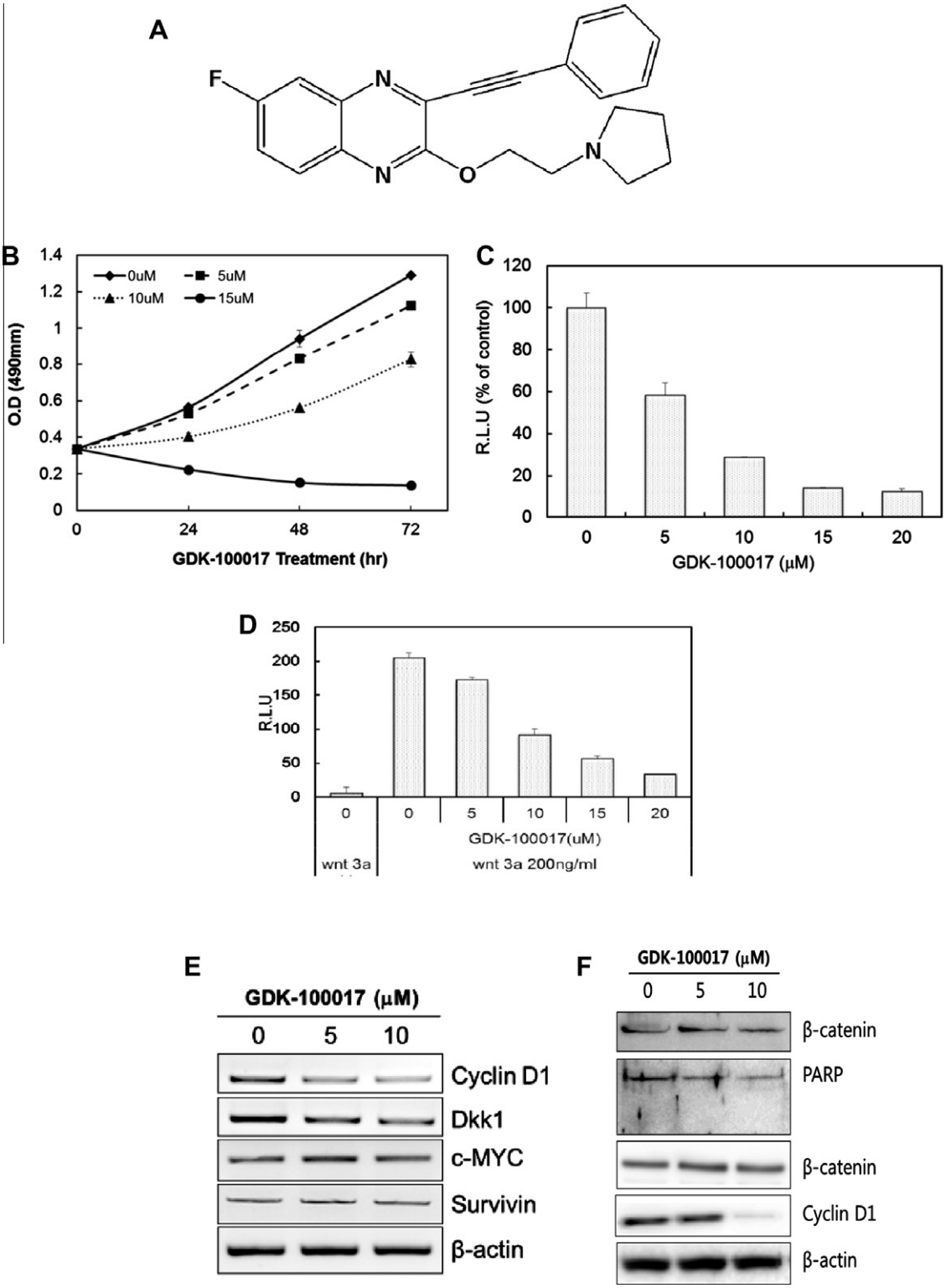

Fig. 1. GDK-100017 inhibits Wnt/β-catenin signaling in A549/wnt2 cells. (A) Chemical structure of GDK-100017 (B) Inhibited cell proliferation by GDK-100017. A549/wnt2 cells were treated with various doses of GDK-100017 for 24, 48, and 72 h and assessed by the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit. (C) and (D) GDK-100017 inhibited Wnt/β-catenin pathway activity in A549/wnt2 cells; A549/wnt2 cells were transiently co-transfected with (C) TOPFlash and pRL-TK plasmids or (D) TOPFlash and pRL-TK plasmids and incubated with various concentrations of GDK-100017 for 24 h. (E) The effect of GDK-100017 on Wnt/β-catenin target genes expression determined by reverse transcription-polymerase chain reaction (RT-PCR) (F) Western blot analysis of β-catenin protein levels in the nuclear and cytosolic fractions. A549 cells were treated with various dose of GDK-100017 for 24 h, and 50 μg protein extracts were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and detected with anti-β-catenin antibody.

2.8. Colony forming assay

A549 cells were seeded in 60 mm dishes (500 cells/dish) in triplicate. Cells were pretreated with 5 and 10 μM GDK-100017 for 4 h and then irradiated with 2 Gy of γ-rays. The cells were irradiated at room temperature with a ^{137}Cs source using an IBL 437C type H irradiator (CIS Bio International, Saclay (Essonne), France). After incubating the irradiated cells for 48 h, the chemical was washed off, and the cells were maintained for 10 days to allow for colony formation. The cells were fixed with methanol and stained with 0.5% crystal violet.

3. Results

3.1. Effect of GDK-100017 on the Wnt/β-catenin pathway in human NSCLC lines

A549/wnt2 cells were treated with various doses of GDK-100017 for 24, 48, and 72 h to evaluate whether GDK-100017 could inhibit NSCLC proliferation. GDK-100017 inhibited proliferation of A549/wnt2 cells in dose- and time-dependent manners with a 10 μM IC_{50} value at 72 h of culture (Fig. 1B). Next, we examined whether GDK-100017 could inhibit the intrinsic and wnt3a-activated Wnt/β-catenin signal pathway. A549/wnt2 and A549 cells were transiently co-transfected with TOPFlash and pRL-TK plasmids and incubated with various concentrations of GDK-100017 for 24 h (Fig. 1C and D). GDK-100017 significantly inhibited Wnt/β-catenin transcriptional activity in A549/wnt2 cells in a dose-dependent manner without affecting Fopflash activity (Fig. 1C). GDK-100017 also exhibited a dose dependent inhibitory effect on wnt3a-induced luciferase activity in A549 cells (Fig. 1D).

The change in Wnt/β-catenin target gene expression was determined in A549/wnt2 cells by RT-PCR after treating the cells for 24 h with 5 and 10 μM GDK-100017 (Fig. 1E). GDK-100017 down-regulated expression of the Wnt/β-catenin signal target genes cyclin D1 and DKK1 but not survivin or c-myc.

We determined β-catenin protein levels in nuclear and cytosolic cell lysates of A549/Wnt2 cells by immunoblot analysis to determine whether the changes were related to Wnt/β-catenin target gene expression (Fig. 1F). No changes in the amounts of cytosolic β-catenin or nuclear β-catenin were observed.

3.2. Effect of GDK-100017 on the Wnt/β-catenin pathway in the SW480 colon cancer cell line

SW480 cells were treated with GDK-100017 for 24, 48, and 72 h to observe whether GDK-100017 acts a constituent in the Wnt/β-catenin pathway activating system (Fig. 2A). GDK-100017 effectively inhibited SW480 cell proliferation by about 40% at 10 μM. GDK-100017 inhibited Topflash activity in dose-dependent manner in the Wnt/β-catenin transcriptional activation assay without affecting Fopflash activity (Fig. 2B). The cyclin D protein level in SW480 cells decreased significantly in a dose-dependent manner after a 24 h treatment with GDK-100017; however, the β-catenin protein level did not change in A549/Wnt2 cells (Fig. 2C).

3.3. Effect of GDK-100017 on L132 human embryonic pulmonary epithelial cells

The purpose of cancer chemotherapy is to efficiently eradicate tumor cells with minimal side-effects to surrounding normal cells and the whole organism. Thus, we evaluated whether GDK-100017 caused cytotoxicity and cell-cycle arrest in the L132 normal lung cell line, which are human embryonic pulmonary epithelial cells. Incubating the cells with 0–15 μM GDK-100017 for 24, 48, and 72 h affected cell proliferation less compared to that in A549/Wnt2 and SW480 cells (Fig. 3A). The IC_{50} value of cell proliferation by GDK-100017 in L132 cells was about 32 μM after a 72 h incubation. GDK-100017 treatment did not result in any significant changes in the cell cycle analysis (Fig. 3B). These results suggest that GDK-100017 has low toxicity in normal cells and could be useful as a potential chemotherapeutic drug.

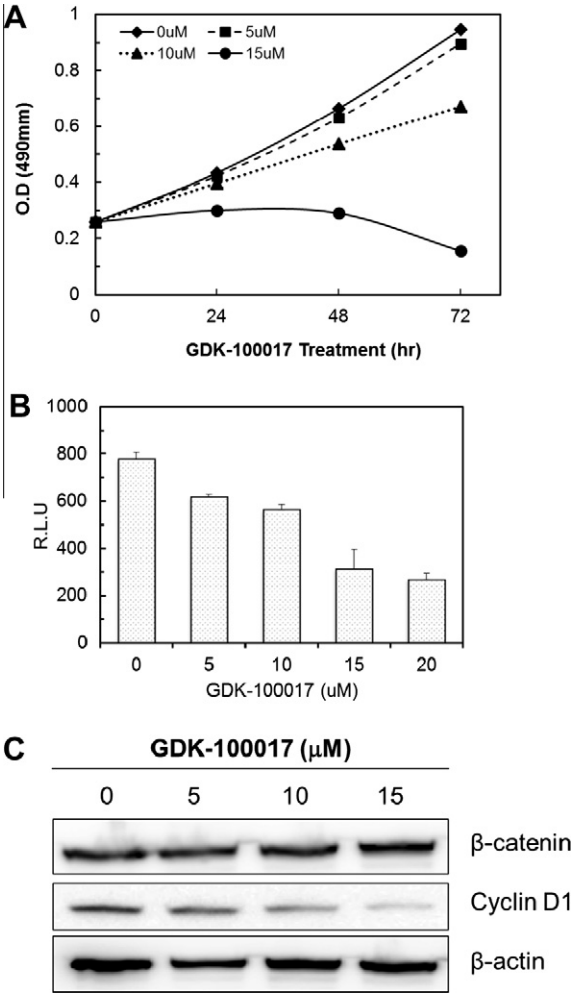

Fig. 2. GDK-100017 inhibits Wnt/β-catenin signaling in SW480 cells. (A) Inhibited cell proliferation following GDK-100017 treatment. SW480 cells were treated with various doses of GDK-100017 for 24, 48, and 72 h and assessed with the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit. (B) GDK-100017 inhibited Wnt/β-catenin pathway activity in SW480 cells. (C) Immunoblot analysis of β-catenin protein levels in the nuclear and cytosolic fractions. SW480 cells were treated with various doses of GDK-100017 for 24 h, and 50 μg of protein extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and detected with anti-β-catenin and cyclin D1 antibodies.

3.4. GDK-100017 acts as a potential radiosensitizer in A549 cells

Diverse chemotherapeutic agents against potential molecular targets have received attention as radiosensitizers. We tested whether GDK-100017 could potentially act as a radiosensitizer using cell cycle analysis and a colony forming assay in A549/Wnt2 cells (Fig. 4). GDK-100017 treatment alone caused dose-dependent cell cycle arrest at the G1 phase, whereas 2 Gy of γ-irradiation alone did not affect the cell cycle. However, the combined treatment of GDK-100017 and 2 Gy of γ-irradiation arrested the cells in the G2 phase. Our results showed that a combination of GDK-100017 and irradiation significantly induced cell cycle arrest

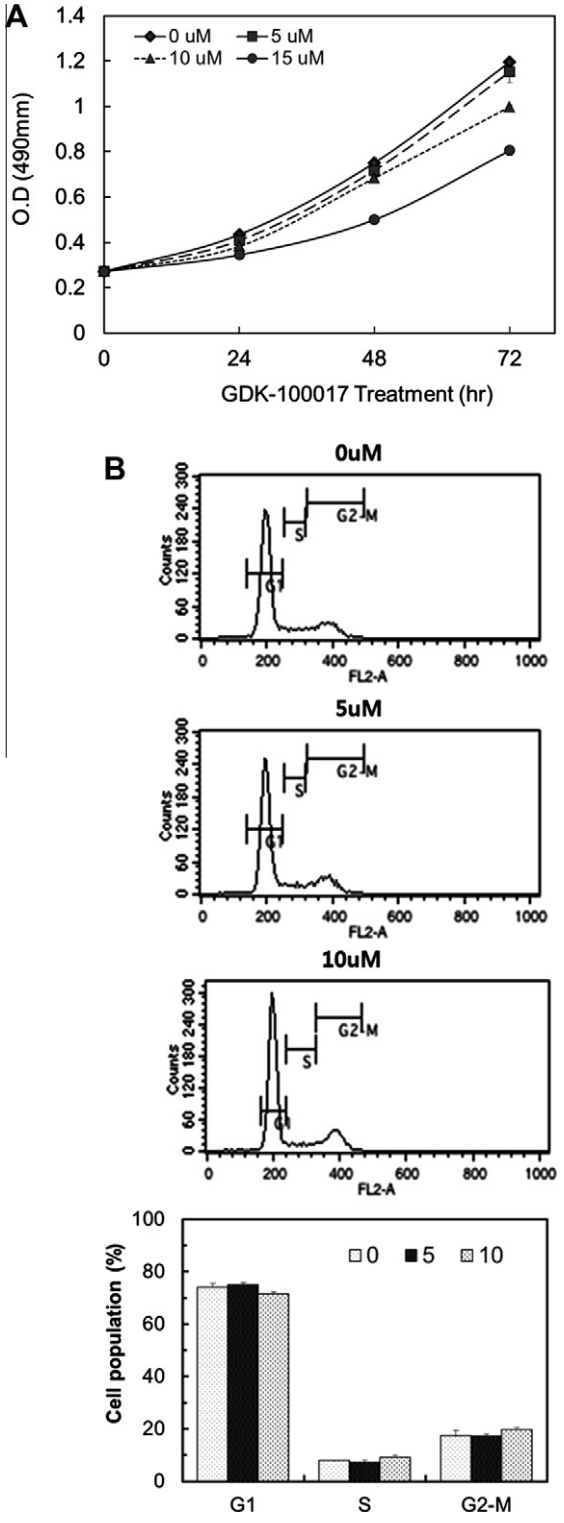

Fig. 3. GDK-100017 does not affect L132normal lung cells. (A) The effect of various concentrations of GDK-100017 on L132 cell proliferation. L132 cells were treated with various doses of GDK-100017 for 24, 48, and 72 h and assessed by the CellTiter 96 Non-radioactive Cell Proliferation Assay kit. (B) Flow cytometric analysis of the L132 cells treated with various doses of GDK-100017 for 48 h.

in A549 cells compared to that of GDK-100017 or irradiation alone (Fig. 4A). We performed the colony forming assay by combining GDK-100017 and γ-irradiation to confirm the radiosensitization effectiveness of GDK-100017 (Fig. 4B). A549/Wnt2 cells were pretreated with 10 μM GDK-100017 or DMSO for 4 h, subsequently radiated with 2 Gy of γ-irradiation, and incubated for 14 days. GDK-100017 pretreatment alone suppressed colony formation in a dose-dependent manner, whereas γ-radiation alone did not affect A549/Wnt2 cells. The combined treatment of GDK-100017 and γ-irradiation effectively decreased cell colony number compared with that of GDK-100017 alone. These results suggest that GDK-100017 may be a candidate chemical to enhance the radiotherapy effect against NSCLC cells.

4. Discussion

Altered function and expression of Wnt/β-catenin pathway components are associated with a wide range of cancers, including colorectal cancer, lung cancer, melanoma, breast cancer, and prostate cancer. In this study, we describe the discovery of a novel small molecular inhibitor, GDK-100017, that inhibited cell proliferation by interfering with the Wnt/β-catenin pathway and enhanced radiosensitivity in a human A549 NSCLC line.

Many small molecule inhibitors have been reported to act at different steps in the Wnt/β-catenin pathway. IWR compounds stabilize axin2 with a consequential increase in β-catenin destruction [13]. ICRT 3, −5, and −14 disrupt the interaction between β-catenin and Tcf by directly binding to β-catenin [14]. AV-65 enhances the interaction between β-catenin and β-TrCP, resulting in increased β-catenin ubiquitination and degradation [15]. ICG-001 is a selective low molecule-weight inhibitor that antagonizes β-catenin/TCF-mediated transcription [16]. ICG-001 specifically downregulates expression of a subset of β-catenin/TCF-responsive genes without changing β–catenin level and also inhibits growth in a CRC xenograft model. ICG-001 disrupts the interaction between β-catenin and CBP, but not p300. GDK-100017 decreased Topflash activity and decreased the expression of β-catenin/TCF-responsive genes in a dose dependent manner. However, it had no effect on the level of cytosolic and nuclear β-catenin protein, similar to ICG-001. β-catenin in the nucleus binds to TCF/LEF and several cofactors such as CBP, p300, BCL9, Pygopus, and Brg1 to regulate gene expression [5]. Thus, our results suggest that GDK-100017 could disrupt the interaction between β-catenin and TCF/LEF or a variety of co-activators. Because canonical Wnt signaling in many cancers including NSCLC is activated by APC or β-catenin mutations [17], GDK-10017 might be more effective in cancer cells activated by Wnt signaling and an APC or β-catenin mutation. GDK-100017 inhibited cell proliferation and arrested the cell cycle in the G1 phase not only in A549/Wnt2 cells but also in SW480 colon cancer cells, which have a constitutively activated Wnt signaling pathway due to a β-catenin mutation. However, interestingly, GDK-100017 did not inhibit L132 cell proliferation, which is a normal lung cell line, and did not change the cell population distribution in the cell cycle analysis. Thus, we expect that GDK-100017 could be developed as an anticancer drug with minor side effects on normal cells. Many target genes are up-regulated by the Wnt/β-catenin pathway such as c-myc, cyclin D, claudin-1, survivin, and DKK1. These genes may play an important role in tumorigenesis and also be cancer therapy targets. GDK-100017 down-regulated the expression of cyclin D1 and DKK1 in A549/Wnt2 cells but not c-myc or survivin. Therefore, down-regulation of cyclin D1 by GDK-100017 resulted in G1 phase arrest in A549/Wnt 2 cells (Figs. 2C and 4A).

Diverse chemotherapeutic agents and certain phytochemicals against potential radiosensitized molecular targets have received attention as radiosensitizers. Curcumin, genistein, and resveratrol are good radiosensitizers due to their ability to increase damage, inhibit pro-survival signaling, inhibit the repair process induced by DNA damage, potentiate proapoptotic factors, decrease antioxidant potential, and raise reactive oxygen species within the cell

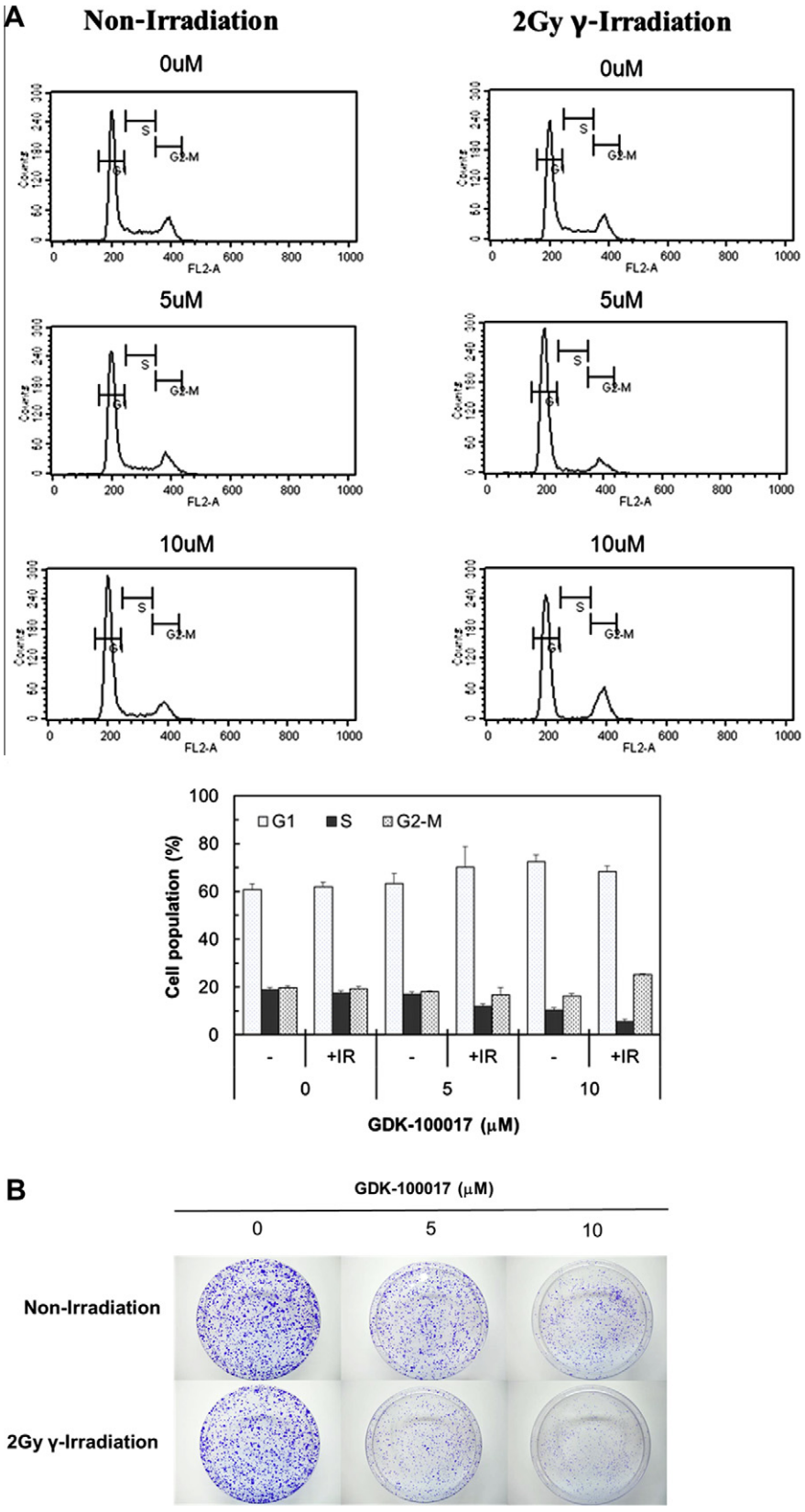

Fig. 4. GDK-100017 enhances radiosensitivity of A549/Wnt2 cells. (A) Flow cytometric analysis of A549/Wnt2 cells treated with GDK-100017 and 2 Gy γ-radiation. (B) The effectiveness of the combination of GDK-100017 and irradiation was assessed by the colony-forming assay. Images show the colonies formed by A549 cells.

[10]. Roscovitine, which is a small molecule cyclin-dependent kinase inhibitor, increases radiosensitivity in A549 cells by affecting cell cycle distribution, promoting caspase activity, and blocking the sublethal DNA damage repair process [18]. We analyzed the cell cycle distribution in cells pretreated with GDK-100017 to determine whether radiosensitization was associated with inhibitor-

mediated cell cycle redistribution. GDK-100017 induced cell cycle arrest at the G1 phase by reducing cyclin D1 expression. However, γ-radiation combined with a 10 μM GDK-100017 pretreatment significantly reduced clonogenic survival in A549 cells and increased the percentage of cells in G2 phase by approximately 1.6-fold (Fig. 4). These findings were similar to cell cycle arrest by NVP-BEZ235, a dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, and PI-103, a PI3 K inhibitor [19,20]. NVP-BEZ235 alone results in FaDu cell growth arrest at the G1 phase; however, BEZ235 induces a G2 block when combined with irradiation. A number of chemotherapeutic agents including PI-103, rapalogs, and MLN4924 also increase G2 phase population arrest as radiosensitizers [20–22]. Interestingly, although the growth and cell cycle distribution of A549 cells was not affected by 2 Gy of γ-radiation, GDK-100017 significantly reduced clonogenic survival of A549 cells (Fig. 4). This result suggests that GDK-100017 might render NSCLC more susceptible to radiotherapy with a minimum radiation dose.

In summury, we confirmed that 2,3,6-trisubstituted quinoxaline derivative, GDK-100017, not only effectively inhibited cell proliferation by blocking the Wnt/β-catenin pathway but also acted as a radiosensitizer in human NSCLC. We suggest that GDK-100017 possesses potential anti-cancer activity against human NSCLC by inhibiting the Wnt/β-catenin pathway and enhancing radiosensitivity.

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