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Original Article

BRD4 Inhibitor OPT-0010 Exerts Anticancer Effects Through Cell Cycle Arrest and Apoptotic Cell Death in Hepatic Carcinoma Cells

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ABSTRACT

Objective: Bromodomain-containing protein 4 (BRD4) is a critical transcriptional regulator of cell growth and differentiation. BRD4 inhibitors have the potential to curb cancer cell proliferation by downregulating the expression of associated genes. This study investigated the anticancer effects of a novel BRD4 inhibitor, OPT-0010, on human hepatic carcinoma cell lines.

Materials and Methods: Two hepatic carcinoma cell lines, SK-Hep1 and Huh-7, were subjected to 48-h OPT-0010 treatment. Cell viability and proliferation were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and adenosine triphosphate assays, respectively. Flow cytometry was used to detect cell cycle arrest. Apoptotic cell death was evaluated using Annexin V and caspase 3 assays. BRD4 and apoptosis-related genes (e.g., *BCL2* and *BAX*) and proteins (e.g., cleaved caspase-3, cleaved PARP, and BCL2) were evaluated using real-time polymerase chain reaction and western blotting, respectively. Additionally, a mouse xenograft model was used to analyze tumor growth, weight, and messenger RNA (mRNA) levels.

Results: OPT-0010 significantly decreased cell viability and proliferation, inducing cell cycle arrest and apoptotic cell death in both SK-Hep1 and Huh-7 cell lines. OPT-0010 showed substantial anti-tumor efficacy in a mouse xenograft model, affecting tumor growth and the expression of BRD4 and apoptosis-related proteins. Furthermore, in synergy with sorafenib, OPT-0010 exhibited an enhanced effect, increasing apoptotic cell death, and effectively suppressing tumor growth in vitro and in vivo.

Conclusion: This study provided comprehensive insights into the mechanisms and therapeutic effects of OPT-0010 on human hepatic carcinoma cell lines and in vivo mouse xenograft models. These results suggest that OPT-0010 is a promising therapeutic agent for treating human hepatocellular carcinoma.

Keywords BRD4 inhibitor, OPT-0010, hepatic carcinoma, cell viability, apoptosis, tumor growth, synergistic effect

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths globally.[1] Chronic hepatitis B or C, viral infection is a primary risk factors for HCC.[2] Although surgery offers a potential cure for HCC,[3] it is often diagnosed at an advanced stage, limiting treatment options to palliative care.[4] Unfortunately, late-stage diagnosis hinders the efficacy of chemotherapy.[5] Sorafenib (Nexavar), an FDA-approved drug for advanced HCC, targets specific enzymes and receptors, including VEGF receptor tyrosine kinases, RAF family serine/threonine kinases, and platelet-derived growth factor receptor (PDGFR).[6-9] However, the survival benefit of sorafenib is modest, necessitating the exploration of novel treatment strategies.[10] Therefore, there is an urgent need to develop effective therapies for HCC.

The bromodomain and extra-terminal domain (BET) family proteins consist of four subtypes: BRD2, BRD3, BRD4, and BRD.[11,12] Each BET protein exhibited a conserved structure characterized by two consecutive bromodomains (BD1 and BD2) composed of approximately 110 amino acids.

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These bromodomains bind specifically to acetylated lysine residues. Moreover, BET proteins possess an extraterminal (ET) protein-protein interaction domain.[13]

Among the BET proteins, BRD4 has been extensively studied in gastrointestinal (GI) cancers because of its high expression in cancer tissues and cell lines, making it a promising target for investigation and treatment.[14] Increased BRD4 expression significantly promotes GI cancer cell growth, differentiation, and metastasis and correlates with poor patient outcomes.[15,16] BRD4 directly binds to the promoter regions of oncogenes, leading to their overexpression and cancer growth.[17-20] Moreover, BRD4 recognizes acetylated lysines on transcription factors such as Twist and Snail, activating the epithelial-to-mesenchymal transition (EMT) process, promoting the survival and differentiation of EMT cells, and facilitating metastatic growth in GI cancers.[21,22]

Bromodomain and extraterminal (BET) protein inhibitors represent a promising approach for the treatment of various cancers, including those affecting the GI system.[14] These inhibitors competitively bind to BET proteins, disrupting their interaction with acetylated lysines and exhibiting anticancer effects. Abnormal transcriptional regulation resulting from BET protein upregulation promotes tumor initiation and progression.[23] BET inhibitors exert anti-tumor effect by downregulating BET protein expression and inactivating its function. Currently, several BET inhibitors targeting BET bromodomains (BD) are undergoing clinical investigations, with preclinical data supporting their use in treating GI cancers.[14]

This preclinical study explored whether inhibition of the epigenetic reader BRD4 (OPT-0010) could be an effective therapy for liver cancer, either as a monotherapy or in combination with Sorafenib. By assessing the effect of BRD4 inhibition on liver cancer cells, we aimed to uncover the underlying molecular mechanisms and evaluate the therapeutic potential of targeting BRD4 in HCC.

MATERIALS AND METHODS

1. Cell lines and drugs

Human liver cancer cell lines (Huh7, SK-Hep1, and HepG2) were purchased from the American Type Culture Collection (Manassas, VA, USA). An immortalized normal human ovarian surface epithelial cell line (HOSEpic) was obtained from ScienCell Research Laboratories Inc. (Carlsbad, CA, USA). OPT-0010 was obtained from JBK Lab (Seongnam, Korea). OPT-0010 is a novel small-molecule inhibitor of BRD4, provided by JBK Laboratory. The compound was specifically designed to target the bromodomain of BRD4 and disrupt its transcriptional regulatory functions. Sorafenib was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorafenib was dissolved in dimethyl sulfoxide (DMSO) for in vitro studies and 0.9% s aline for animal studies. Statistical analyses were conducted using GraphPad Prism version 9.4.1 for Windows. Significant values were considered at $p < 0.05$, and more significant values were considered at $p < 0.01$, compared to the control.

2. Cell viability and proliferation assay

Cell viability was determined using PrestoBlue Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA). Cells were seeded in 96-well plates in completed media 100 μL (contained 10% FBS and 1% P/S-supplemented media) per well and maintained overnight at 95% humidity and 5% CO_2. After incubating overnight, OPT-0010 was treated with 0–100 μM in completed media 100 μL for 48 h. The final DMSO concentration in each well never exceeded 0.1%. The cells were incubated for 1 h with 10% PrestoBlue in the dark. The absorbance was measured at 540 nm using a plate reader and normalized to the cell number (absorbance/cell number).

Cell proliferation was determined using Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories Co. Ltd., Tokyo, Japan). The cells were seeded into 96-well plates at 1×10^4 cells/well μL density and treated with OPT-0010 0, 1, 5, 10, 50, and 100 μM for 48 h. CCK-8 assay was conducted 48 h after OPT-0010 treatment. The serum-free medium was replaced, and 10 μL of CCK-8 was added to each well. After incubation at 37°C and 5% CO2 for 1 h, optical density (OD) was measured at 450 nm. Each measurement was performed in triplicate.

3. Caspase-3 activity

To evaluate apoptosis, caspase-3 activity and Annexin V-FITC/PI staining assays were performed. For the caspase-3 activity assay, cells were seeded at a density of 1×10^4 cells/well in white-walled 96-well plates and incubated at 37°C for 24 h. After incubation, the cells were treated with OPT-0010 at concentrations of 0, 1, 5, 10, 50, and 100 μM for 48 h. Following treatment, 100 μL of Caspase-Glo 3/7 Reagent (Cat# G8090, Promega, Madison, WI, USA) was added to each well, and the plates were incubated at room temperature for 30 min. Luminescence was measured using a luminometer (Molecular Devices), and all data were expressed as percentages relative to the control group.

4. Annexin V-FITC/PI Assay via Flow Cytometry

For the Annexin V-FITC/PI staining assay via flow cytometry, cells were seeded at a density of 5×10^5 cells/well in 6-well plates and incubated at 37°C with 5% CO_2 for 24 h to After incubation, the cells were treated with OPT-0010 at concentrations of 0, 1, and 10 μM for 48 h. Following treatment, cells were harvested by trypsinization, washed once with 1 mL of ice-cold PBS, and centrifuged at 300 g for 5 minutes. The cell pellets were resuspended in 100 μL of 1× binding buffer provided in the Annexin V FITC/PI Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA). Annexin V-FITC and Propidium Iodide (PI) were added to the suspension

according to the manufacturer's protocol, and the cells were gently mixed and incubated at room temperature for 15 minutes in the dark. After incubation, 400 μL of 1× binding buffer was added, and the samples were immediately analyzed using a flow cytometer (FACSCalibur, BD Biosciences, CA, USA) with 10,000 events recorded per sample.

5. Cell cycle arrest

The treated cells were washed with ice-cold PBS and fixed with 70% ethanol at −20°C overnight. The samples were then washed with PBS, resuspended in 0.5 mL of FxCycle™PI/RNase Staining Solution (Invitrogen) containing 50 μg/mL PI with 100 μg/mL RNase A, and incubated for 30 min at room temperature in the dark. Samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences).

6. Cell-derived mouse tumor xenografts model

Forty BALB/c nude female mice (7 weeks old) were provided by ORIENT BIO Inc. (Seongnam, Korea). The mice were housed in a pathogen-free room at controlled temperature (25 ±2°C) and humidity (65 ±5%) and alternating 12 h-light/-dark cycles. After the mice had been acclimated for 1 week, 100 μL of Matrigel containing 5 $\times 10^7$ Huh7 cells were subcutaneously injected into the right flanks of BALB/c nude mice. Mice with successful skin tumor formation were randomly divided into three groups (n = 10 per group) 4 weeks after implantation, as follows: group 1, tumor control (implanted Huh7 cells); group 2, tumor cell implantation and intravenous low-dose OPT-0010 (10 mg/kg); and group 3, tumor cell implantation and intravenous high-dose OPT-0010 (30 mg/kg). Tumor areas were measured once every 4 days and calculated as [width (mm) ×width (mm)] ×length [mm])/2 using a Vernier caliper. OPT-0010 was dissolved in PBS and intravenously administered once every 3 days. During the continuous 4-week treatment, body weight was measured twice a week. The mice were euthanized, and tumors were isolated. All animal experiments were performed in accordance with the guidelines of the Seoul National University Bundang Hospital Institutional Animal Care and Use Committee (IACUC) guidelines. Institutional Review Board Statement The IACUC of Seoul National University Bundang Hospital approved this study (No. BA-2111-332-010-02) (approval date: 26 November 2021)

7. Quantitative real-time PCR analysis

Total RNA was extracted from the isolated tumor samples using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Equal quantities of DNA-free RNA were reverse transcribed to generate cDNA using GoScript™Reverse Transcriptase (Promega). The real-time PCR was conducted in a 25 mL-reaction volume using 3 μL of a 1:10 cDNA dilution containing SYBR Green master mix (BioRad) and primers for PCR, BRD4, hypoxia-inducible factor 1 subunit alpha (HIF-1a), vascular endothelial growth factor (VEGF-a), octamer-binding transcription factor 4 (OCT-4), Nanog homeobox (NANOG), b-cell lymphoma 2 (BCL-2), and Bcl-2 associated X (BAX). All PCRs were performed using a Qiagen Rotor-Gene Q Real-Time PCR system, and fluorescence threshold values (Ct) were calculated. Relative mRNA levels were evaluated through standardization with 18s rRNA. The results are expressed as the fold difference in gene expression.

8. Protein preparation and western blot

The cells were treated with the indicated drugs for 48 h, and the treated cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl, 1% NP40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF) for 30 min at 4°C. Total cell extracts were separated using 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and incubated with primary antibodies diluted in the blocking solution. The signals were visualized using the chemiluminescent substrate method and Super Signal West Pico kit (Pierce, Thermo Fisher Scientific). β-actin was used as an internal control to normalize loading.

9. Statistical Analysis

Data were presented as mean ± standard deviation (SD). GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphing. For comparisons between two groups, Student's t-test was used. For multiple group comparisons, one-way or two-way ANOVA was performed, followed by Tukey's post-hoc test. A P-value < 0.05 was considered statistically significant. All experiments were performed in triplicate unless otherwise stated.

RESULT

1. BRD4 inhibitors (OPT-0010) as a Potential Therapeutic Target in Liver Cancer

To explore the potential of BRD4 as a therapeutic target in liver cancer, we conducted quantitative real-time PCR (RT-PCR) to evaluate BRD4 expression in three different liver cancer cell lines (HepG2, Huh7, and SK-Hep1) and compared it with that in a non-tumorigenic human hepatocyte cell line (HOSEpic). This study aimed to determine whether liver cancer cell lines exhibit elevated BRD4 expression, thereby indicating its potential as a promising therapeutic target.

The results demonstrated that the liver cancer cell lines HepG2, Huh7, and SK-Hep1 exhibited significantly higher levels of BRD4 expression than the non-tumorigenic hepatocyte cell line HOSEpic (Figure 1). Among the liver cancer cell lines tested, SK-Hep1 and Huh7 cells showed markedly higher BRD4 expression than HepG2 cells, suggesting that BRD4 expression levels may vary across different liver cancer subtypes. However, further functional

studies are needed to elucidate the potential role of BRD4 in liver cancer progression.

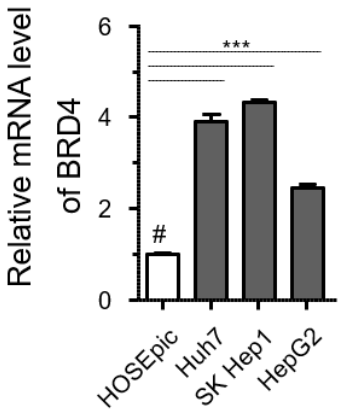


Figure 1. Relative expression levels of BRD4 in liver cancer cell lines (HepG2, Huh7, and SK-Hep1) and an immortalized non-tumorigenic human hepatocyte cell line (HOSEpic) RT-PCR analysis was conducted to evaluate the endogenous mRNA expression levels of BRD4 in these cell lines. The results indicate that BRD4 is significantly overexpressed in liver cancer cell lines (HepG2, Huh7, and SK-Hep1) compared to the non-tumorigenic hepatocyte cell line HOSEpic. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control.

2. Inhibition of Cell Survival by BRD4 Inhibitors (OPT-0010) in Liver Cancer Cells

Cell viability and proliferation assays were performed to evaluate the effect of the BRD4 inhibitor (OPT-0010) on the survival of human liver cancer cell lines. The half-maximal inhibitory concentration (IC50) of OPT-0010 was determined using the PrestoBlue assay in Huh7 and SK-Hep1 cells (Figure 2A and 2B). This assay measures the cellular metabolic activity and serves as an indirect indicator of cell viability and proliferation.

SK-Hep1 and Huh7 cells were subsequently treated with different concentrations of OPT-0010 (ranging from 0 to 100 μM) for 48 hours. Cell viability and proliferation were assessed using PrestoBlue and CellTiter-Glo assays, respectively. The results exhibited significant dose-dependent inhibition of cell viability in both SK-Hep1 and Huh7 cells (Figure 2C and 2D). Furthermore, OPT-0010 significantly suppressed the proliferation of both cell lines (Figure 2E and 2F).

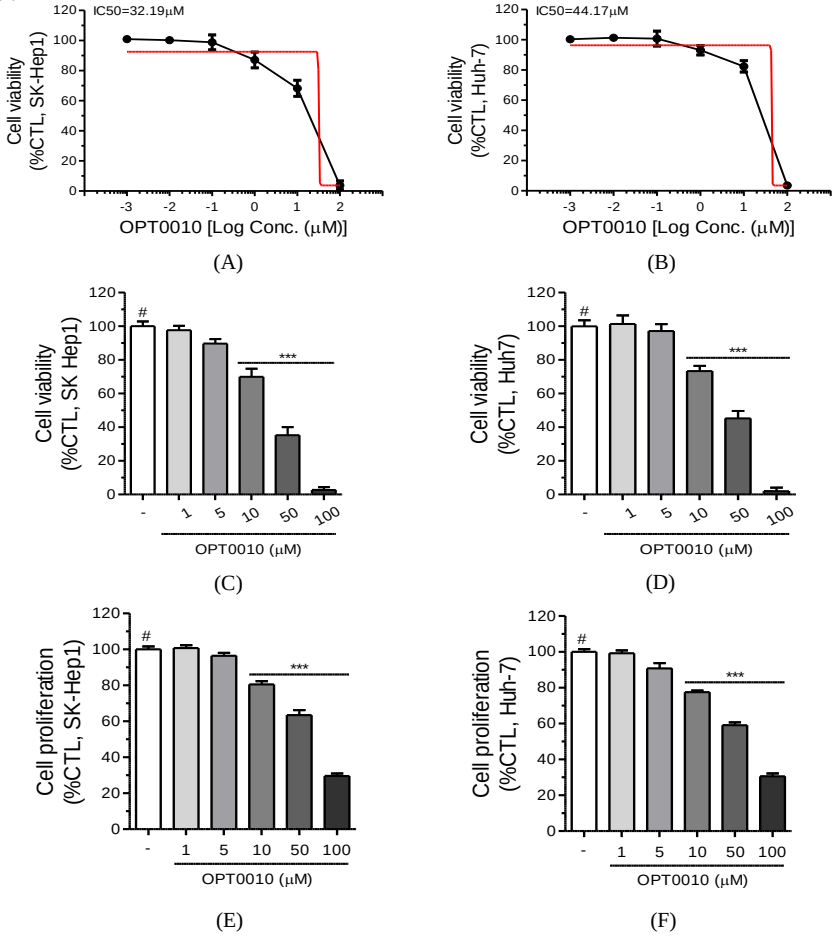


Figure 2. Inhibition of cell survival and proliferation by OPT-0010 in SK-Hep1 and Huh7 cells. (A, B) Inhibitory concentration (IC50) values of OPT-0010 in SK-Hep1 and Huh7 cells were 32.19 μM and 44.17 μM, respectively, as determined by the PrestoBlue assay. (C, D) Cell viability of SK-Hep1 and Huh7 cells was considerably reduced following treatment with varying concentrations of OPT-0010 (0–100 μM) for 48 h, as evaluated using the PrestoBlue assay. (E, F) Analysis of ATP content in OPT-0010-treated SK-Hep1 and Huh7 cells using the CellTiter-Glo assay demonstrated a marked suppression of cell proliferation in both cell lines. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control.

3. Induction of Apoptotic Cell Death by BRD4 Inhibitors (OPT-0010) in Liver Cancer Cells

Annexin V and Caspase-3 assays were used to analyze apoptosis and related signaling pathways to confirm that OPT-0010 induced apoptosis in human liver cancer cells. The results showed a substantial dose-dependent increase in apoptosis and caspase-3 activity with increasing concentrations of OPT-0010 (Figure 3A, 3B, 3C, and 3D).

Moreover, OPT-0010 treatment downregulated the expression of anti-apoptotic proteins, such as BRD4 and BCL-2, while upregulating the expression of cleaved caspase-3 (C-C3) and cleaved PARP (C-P) in both SK-Hep1 and Huh7 cells (Figure 3E and 3F). These findings provide strong evidence that OPT-0010 effectively inhibits cell survival and induces apoptosis in liver cancer cells, suggesting its potential as a therapeutic agent.

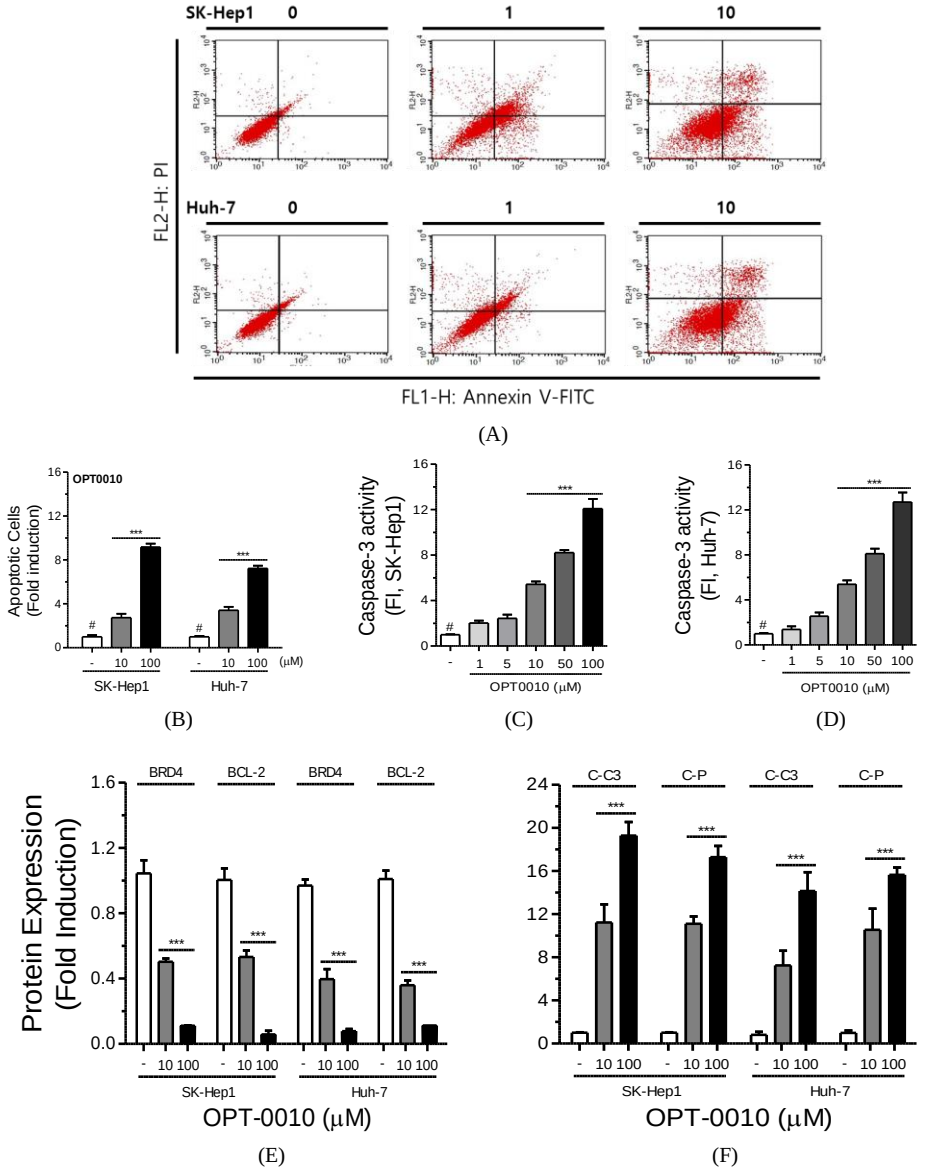


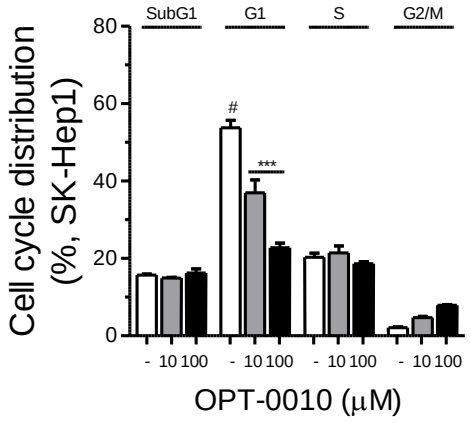
Figure 3. Inhibition of cell survival and induction of apoptotic cell death by OPT-0010 in SK-Hep1 and Huh7 cells. (A) Representative flow cytometry analysis results showing the induction of apoptosis in SK-Hep1 and Huh7 cells treated with varying concentrations of OPT-0010. Cells were double-stained with Annexin V and propidium iodide (PI) to differentiate between apoptotic and necrotic cells. (B) Flow cytometric analysis of Annexin V-FITC/PI-stained cells demonstrating apoptotic rates of SK-Hep1 and Huh7 cells treated with increasing concentrations of OPT-0010 (0–100 μM) for 48 h. (C, D) Caspase-3 activity in SK-Hep1 and Huh7 cells treated with OPT-0010 was measured using the Caspase-Glo 3/7 Assay system. A significant, dose-dependent increase in caspase-3 activity was observed, reflecting enhanced apoptosis in both cell lines. (E) Western blot analysis of anti-apoptotic proteins, including BRD4 and BCL-2, in SK-Hep1 and Huh7 cells treated with OPT-0010. Quantification analysis revealed a marked downregulation of these proteins, indicating activation of pro-apoptotic pathways. (F) Western blot analysis of cleaved caspase-3 (C-C3) and cleaved PARP (C-P) expression levels in SK-Hep1 and Huh7 cells treated with OPT-0010. Quantification analysis showed a dose-dependent upregulation of C-C3 and C-P, indicating that OPT-0010 induces apoptosis by modulating the expression of these key apoptotic markers. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control.

4. BRD4 Inhibitors (OPT-0010) Induce Cell Cycle Arrest and Apoptosis in HCC Cells

To elucidate the mechanisms by which OPT-0010 inhibited the proliferation of liver cancer cells, flow cytometry was conducted to analyze its effects on cell cycle distribution. Treatment with OPT-0010 decreased the percentage of cells in the G1 phase and increased the percentage of cells in the G2/M phase, whereas no significant changes were noted in the S phase (Figures 4A and 4B). These results indicated that OPT-0010 impedes cell cycle progression in SK-Hep1 and Huh7 cells, leading to cell cycle arrest at the G2/M phase. This suggests that OPT-0010 suppressed the growth and survival of liver cancer cells by inducing cell cycle arrest and promoting apoptosis.

5. Combination of OPT-0010 and Sorafenib Therapy Reduces Cancer Cell Survival and Promotes Apoptotic Cell Death in Liver Cancer Cells

Co-treatment experiments were performed to investigate the combined effects of OPT-0010 and sorafenib on SK-Hep1 and Huh7 cells. Cells were treated simultaneously with 5 μM OPT-0010 and 0.1 μM sorafenib for 48 h. Combination therapy significantly reduced cell viability compared to individual treatments with OPT-0010 or sorafenib alone, as shown in Figures 5A and 5B. Furthermore, the combination treatment led to enhanced induction of apoptosis, as evidenced by increased Annexin V staining (Figure 5C) and elevated caspase-3 activity (Figures 5D and 5E) in both SK-Hep1 and Huh7 cells. These results indicated that co-treatment with OPT-0010 and sorafenib exerted an additive effect, resulting in a more pronounced reduction in cancer cell survival and promotion of apoptotic cell death in liver cancer cells.



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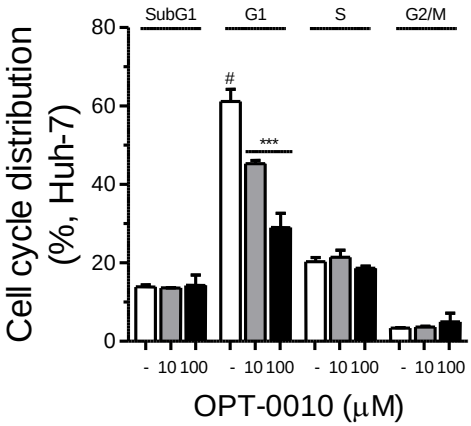


(B)

Figure 4. Cell cycle arrest in SK-Hep1 and Huh7 cell lines induced by OPT-0010 treatment. (A, B) Percentages of DNA content in each cell cycle phase (G1, S, and G2/M) for SK-Hep1 and Huh7 cells treated with OPT-0010. Cells were treated with OPT-0010 at varying concentrations, and the cell cycle distribution was analyzed via flow cytometry. The results reflect a significant decrease in the percentage of cells in the G1 phase and a corresponding increase in the G2/M phase, with no notable changes in the S phase. Data are presented as means ± S.E.M. compared to the vehicle control (***P < 0.01). These findings indicate that OPT-0010 induces cell cycle arrest at the G2/M phase and promotes apoptosis in both SK-Hep1 and Huh7 cell lines, highlighting its potential as an effective therapeutic agent.

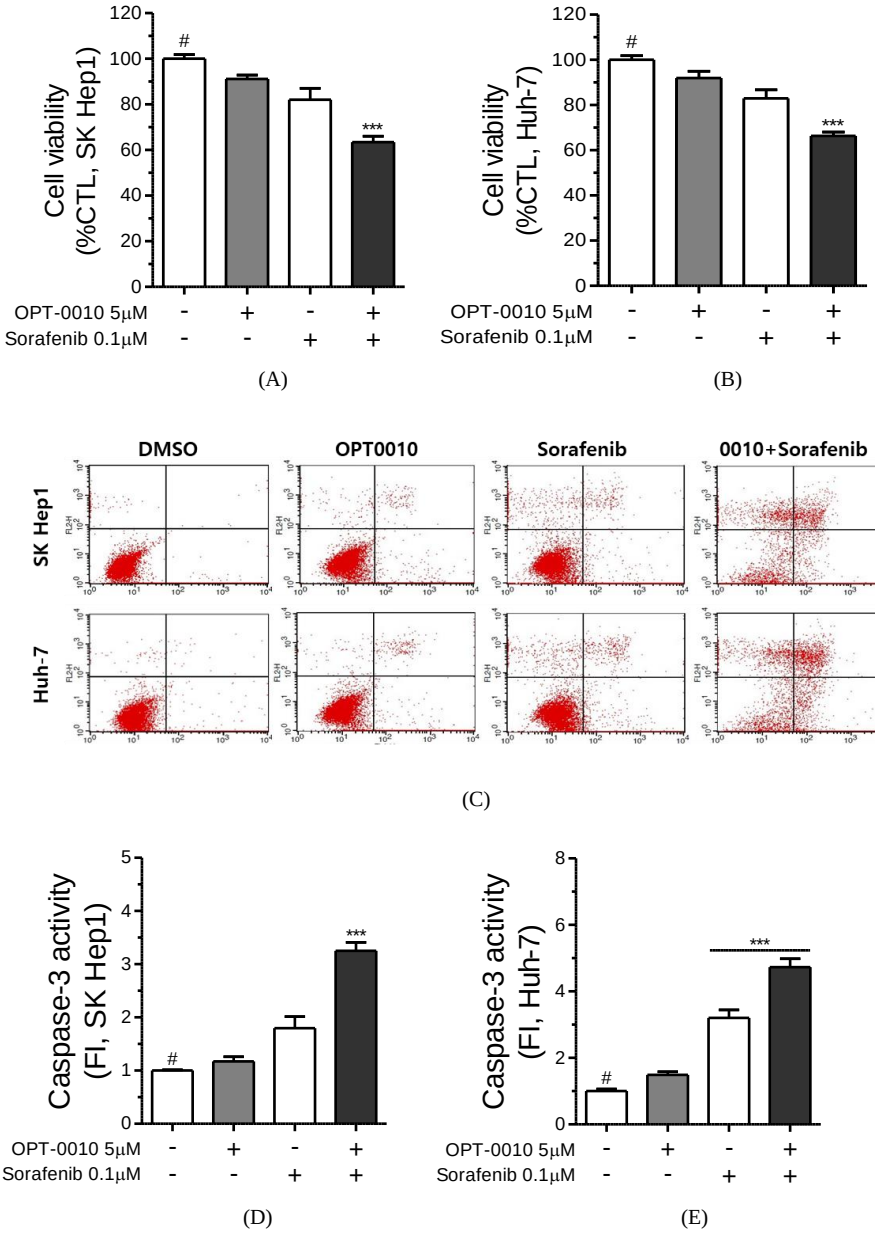
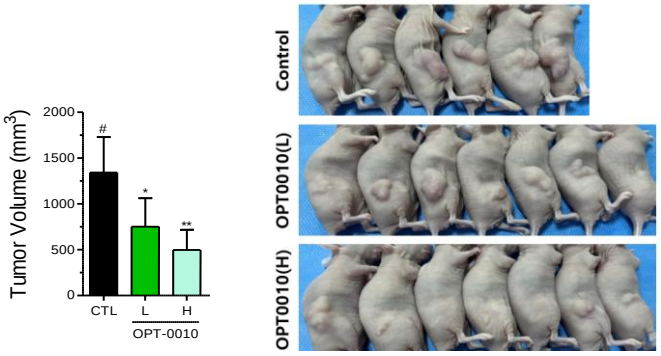


Figure 5. Effect of Combination Therapy Using OPT-0010 and Sorafenib on SK-Hep1 and Huh7 Cell Survival and Apoptotic Cell Death. (A, B) Cell viability of SK-Hep1 and Huh7 cells treated with 0.1 μM sorafenib and 5 μM OPT-0010 for 48 h, as determined by the PrestoBlue and CellTiter-Glo assays. The combination therapy significantly reduced cell viability compared to the control and individual treatments, indicating enhanced efficacy in inhibiting cell survival. (C) Representative flow cytometry analysis of apoptosis induced by the combination treatment of OPT-0010 and sorafenib in SK-Hep1 and Huh7 cells. Cells were double-stained with Annexin V and PI to differentiate between apoptotic and necrotic cells. Flow cytometry analysis showed a marked increase in apoptotic cell death with the combination treatment compared to the control and individual treatments. (D, E) Caspase-3 activity in SK-Hep1 and Huh7 cells treated with OPT-0010 and sorafenib, measured using the Caspase-Glo 3/7 Assay system. The combination treatment resulted in a significant increase in caspase-3 activity compared to the control and individual treatments, indicating that co-treatment with OPT-0010 and sorafenib effectively promotes apoptotic cell death. *p< 0.05, **p < 0.01, ***p < 0.001 compared with the control.

6. BRD4 Inhibitors (OPT-0010) Inhibit Tumor Growth in liver cancer (LC) Cell Mouse Models

The anti-tumor effects of OPT-0010 were assessed in vivo using a Huh7 cell-derived xenograft model. LC cells were injected subcutaneously into the flanks of nude mice. After four weeks, palpable xenograft tumors were established, and the mice were randomly divided into three groups: control (n = 6), low-dose OPT-0010 (10 mg/kg; n = 7), and high-dose OPT-0010 (30 mg/kg; n = 7). OPT-0010 was administered intravenously every three days for four weeks. Tumor volume was measured every four days, and body weight was recorded twice a week throughout the treatment period.

As demonstrated in Figure 6A, the average tumor volumes in the OPT-0010-treated groups were significantly lower than those in the control group from day 14 onwards. OPT-0010 treatment appeared to delay tumor growth in the Huh7 xenograft model, as shown in the time-course data (Figure 6B). No apparent signs of toxicity were noted in OPT-0010-treated mice, as evidenced by their stable body weight, normal food and water intake, and normal activity levels throughout the experiment (Figure 6C and 6D).



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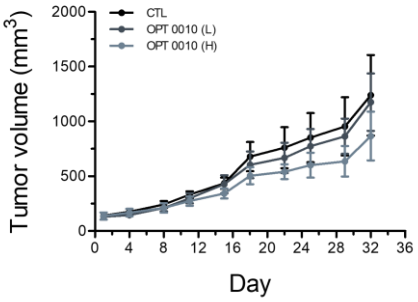

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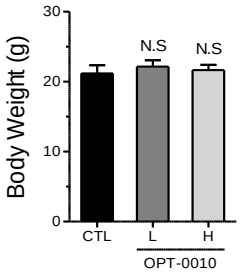

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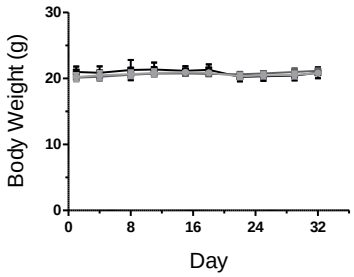

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Figure 6. Tumor Volume and Body Weight Changes in LC Cell Mouse Models Treated with Low and High Doses of OPT-0010. (A) Tumor volume changes over time in LC cell mouse models treated with low-dose (10 mg/kg) or high-dose (30 mg/kg) OPT-0010. Tumor volumes were measured every 4 days, and statistical analysis was conducted using two-way ANOVA (**$p < 0.05$), indicating significant differences between the treatment and control groups starting from day 14. (B) Tumor growth curves of Huh7 xenograft models treated with control, low-dose, or high-dose OPT-0010. (C, D) In each treatment group, body weight measurements of mice were recorded twice a week during the 4-week treatment period. No significant changes in body weight were observed, suggesting that treatment with OPT-0010 did not cause noticeable toxicity. These findings demonstrate that both low and high doses of OPT-0010 effectively inhibit tumor growth in LC cell mouse models, supporting its potential therapeutic value for treating liver cancer.

7. Downregulation of Target Protein Expression by BRD4 Inhibitors (Low and High Dose of OPT-0010) in LC-Derived Mouse Xenograft Models

The suppressive effects of low and high doses of OPT-0010 on the expression of BRD4, VEGF-A, Bcl-2, Oct-4, BAX, Nanog, and HIF-1α were assessed in LC-derived mouse xenograft models. Real-time PCR analysis revealed that both low- and high-dose OPT-0010 treatments significantly decreased the mRNA levels of NANOG and Bcl-2 (Figure 7D and 7F). Additionally, high-dose OPT-0010 treatment resulted in a marked reduction in the mRNA levels of BRD4, HIF-1α, VEGF-A, and Oct-4 in a dose-dependent manner after 24-h treatment (Figure 7A, 7B, 7C, and 7E). Conversely, Bax mRNA levels were increased following treatment with high-dose OPT-0010 (Figure 7G). BAX is a key protein involved in the regulation of programmed cell death and apoptosis. These results indicated that OPT-0010 modulates the expression of several apoptosis-related genes, supporting its potential role in inhibiting tumor growth in LC-derived mouse xenograft models.

8. Combination of BRD4 Inhibitors (OPT-0010) and Sorafenib Inhibits Tumor Growth in LC Cell Mouse Models

The anti-tumor effects of the combination of OPT-0010 and sorafenib were evaluated in vivo using an LC-derived xenograft model. After subcutaneous injection of Huh7 cells into the flanks of nude mice, palpable xenograft tumors were established after 4 weeks. The mice were randomly divided into three groups: group 1 (n = 6), which served as the tumor control; group 2 (n = 7), which received intravenous OPT-0010 (10 mg/kg); and group 3 (n = 7), which received intravenous OPT-0010 (10 mg/kg) combined with sorafenib.

As shown in Figures 8A and 8B, combination treatment with OPT-0010 and sorafenib resulted in significantly smaller tumor volumes and weights than the control and OPT-0010 alone groups starting from day 14. Combination treatment with OPT-0010 and sorafenib appeared to reduce tumor growth in the LC xenograft model (Figure 8C). No signs of toxicity were observed in any treatment groups, as assessed by stable body weight, food and water intake, activity levels, and general health conditions (Figures 8D and 8E).

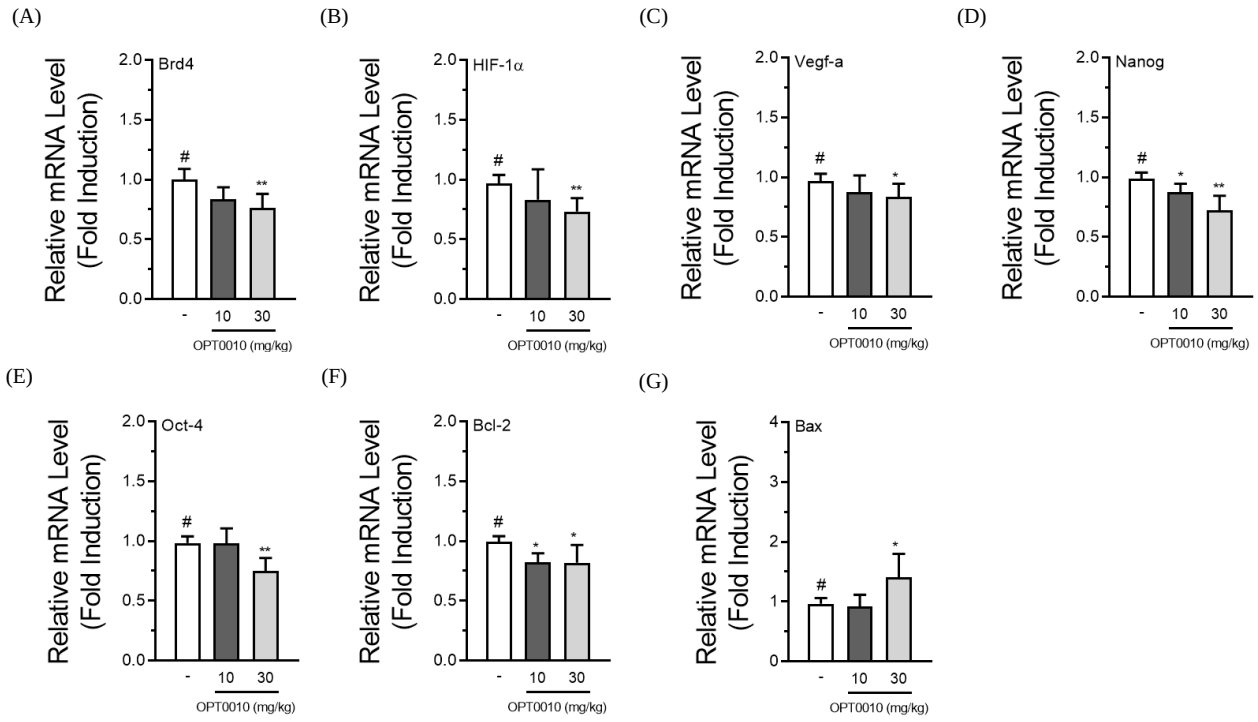

Figure 7. Downregulation of Target Gene Expression by BRD4 Inhibitors (OPT-0010) in LC-Derived Mouse Xenograft Models. (A) mRNA expression levels of BRD4, (B) HIF-1α, (C) VEGF-A, (D) Nanog, (E) Oct-4, and (F) Bcl-2 in LC-derived mouse xenograft models treated with low and high doses of OPT-0010. The mRNA expression levels were measured 24 h after treatment and quantified relative to GAPDH. High-dose OPT-0010 treatment significantly decreased the mRNA levels of BRD4, HIF-1α, VEGF-A, and Oct-4 in a dose-dependent manner (A, B, C, E). Both low- and high-dose treatments also reduced the mRNA expression of Nanog and Bcl-2 (D, F). The data are presented as mean ± SEM compared to the vehicle control. Statistical analysis demonstrated significant differences (*$p < 0.05$, ***$p < 0.01$) between the treated groups and the control, indicating the effective downregulation of target gene expression by OPT-0010. These results show that OPT-0010 modulates the expression of key genes involved in tumor growth and survival, supporting its potential therapeutic role in treating liver cancer.

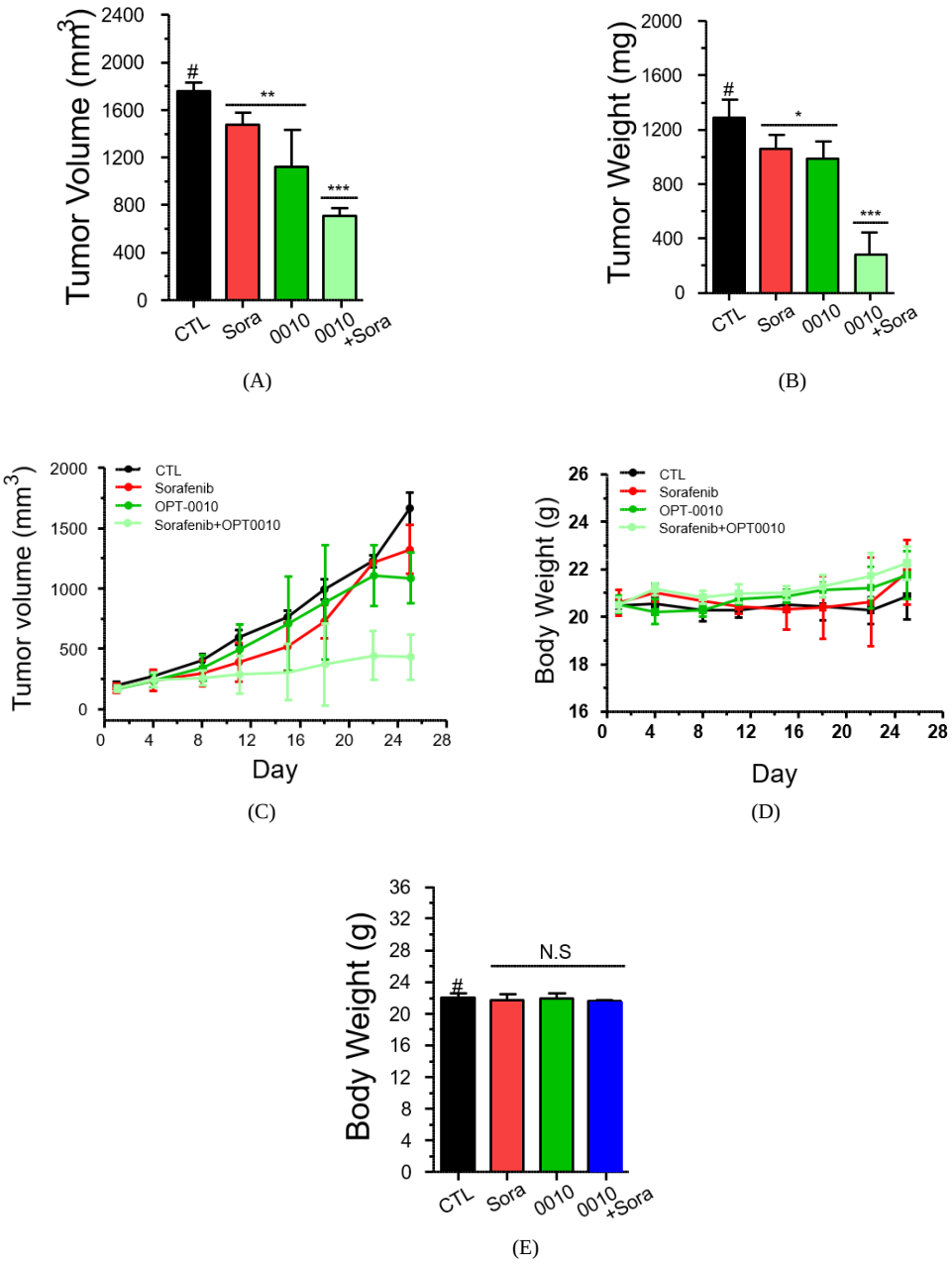

Figure 8. Tumor Growth Inhibition by OPT-0010 in Combination with Sorafenib in LC Cell Mouse Models. (A, B) Tumor volume and weight of LC cell-derived xenografts in mice treated with control or 10 mg/kg OPT-0010 combined with sorafenib. Tumor volumes were measured at regular intervals throughout the treatment period, and tumor weights were recorded at the end of the experiment. Data are presented as mean ± SE, and statistical analysis was conducted using two-way ANOVA (**, $p < 0.05$ vs. control). (C) Individual tumor volumes of LC cell-derived xenografts in control and treatment groups. (D, E) Body weight measurements of mice in each treatment group recorded twice a week during the treatment period. No significant changes in body weight were observed, indicating no apparent toxicity of the combination treatment.

DISCUSSION

Our study revealed that liver cancer cell lines generally exhibit higher BRD4 expression levels than non-neoplastic liver tissue cell lines, highlighting the potential of BRD4 as a promising therapeutic target for liver cancer treatment. These data indicate that BRD4 significantly affects the development and survival of patients with liver cancer. Moreover, these results are consistent with those of a previous study, which showed that increased BRD4 expression promotes cell growth. Simultaneously, its suppression inhibited HCC cell growth.[15,24] This agreement with prior research strengthens the validity of our observations and further emphasizes the therapeutic potential of BRD4 inhibition.

Beyond confirming the oncogenic role of BRD4, our study provides evidence for the anticancer activity of OPT-0010, a novel BRD4 inhibitor. OPT-0010 effectively suppressed cell viability and proliferation, induced apoptosis, and caused cell cycle arrest in liver cancer cells. These effects were dose-dependent and consistent with previous reports on the impact of BRD4 inhibition on HCC cells.[14,18,19,25]

Cell cycle analysis and apoptosis assays showed that OPT-0010 treatment induced G1 cell cycle arrest and apoptosis of HCC cells. The increase in cells in the sub-G1 phase, which is indicative of apoptosis, exhibited a dose- and time-dependent relationship, providing further evidence for the therapeutic effect of OPT-0010. These results suggested that the anti-proliferative effects of OPT-0010 on HCC cell lines were mediated by G1 arrest and apoptosis. The apoptotic cell death observed was attributed to the reduced expression of anti-apoptotic proteins BRD4 and BCL-2, along with the increased expression of C-C3 and C-P in SK-Hep1 and Huh-7 cells, indicating that OPT-0010 exerts its effects on the apoptosis pathway in liver cancer cells.

Consistent with previous studies on BRD4 inhibition, OPT-0010 treatment led to downregulation of BRD4 and its key transcriptional targets, such as BCL2, VEGF-A, and c-Myc, which are well-known regulators of cell survival and proliferation. These findings strongly suggest that the antitumor effects of OPT-0010 are mediated through BRD4 inhibition. However, further experiments, such as direct binding assays or genome-wide transcriptional profiling, would provide more definitive insights into its specificity for BRD4 compared to other BET family members such as BRD2 and BRD3.

Combining OPT-0010 with sorafenib demonstrated synergistic antitumor effects, resulting in significant tumor growth inhibition and increased apoptosis. Sorafenib (Nexavar), the first FDA-approved drug for advanced HCC, is a tyrosine kinase inhibitor that targets the VEGF receptor tyrosine kinases, RAF family kinases, c-Raf, B-Raf, and numerous growth factor receptors, specifically the PDGFR [6-9]. However, the survival benefits are limited, highlighting the need for alternative treatment strategies.[10] Based on the distinct action pathways of OPT-0010 and sorafenib as anti-tumor agents, the conceptual framework for investigating their combination in HCC treatment has emerged from different pathways.[8,9,19,25]

This study showed that combining OPT-0010 with sorafenib results in a significant reduction in cancer cell survival and an increase in apoptotic cell death. OPT-0010, a novel small-molecule BRD4 inhibitor, exhibits a dual bromodomain inhibition mechanism leading to the downregulation of oncogenic transcription programs. Furthermore, OPT-0010 showed favorable pharmacokinetic properties in preliminary studies, including high bioavailability and stability in serum. Thus, OPT-0010 is ideal for combination therapy with other chemotherapeutic agents, such as sorafenib. Future studies should further elucidate OPT-0010's binding kinetics and broader pharmacodynamic effects to establish a more comprehensive understanding of its therapeutic potential.

Moreover, the combination therapy downregulated the expression of target proteins, including BRD4, VEGF-A, Bcl-2, Oct-4, Nanog, and HIF-1α, while upregulating Bax expression. These molecular changes provide further evidence of the enhanced efficacy of OPT-0010 and sorafenib in inducing apoptosis and tumor growth inhibition. The modulation of these essential regulatory proteins suggests that the combination therapy exerts its anticancer effects through multiple pathways, effectively suppressing tumor growth and promoting cancer cell death.

In vivo studies using mouse models further validated the efficacy of OPT-0010 in inhibiting tumor growth. Combined with sorafenib, enhanced tumor growth inhibition without noticeable toxicity was noted, suggesting a promising clinical application potential. These findings align with previous research suggesting the potential of BRD4 inhibitors as viable therapeutic targets for various cancers, including liver cancer.[14,25-27] These results support the notion that targeting BRD4 may be a promising treatment strategy for liver cancer; however, further research is warranted to validate these findings. Moreover, the combination of BRD4 inhibitors and sorafenib is worth investigating as a potential therapy for liver cancer.

To support the potential therapeutic value of OPT-0010 in the treatment of liver cancer cells, we investigated the anti-tumor effects of a BRD4 inhibitor (OPT-0010) in vivo. Our study demonstrated a smaller tumor in the OPT-0010 treated group, with no apparent signs of toxicity, as well as in the combination group with sorafenib, as we observed downregulation of the expression of target proteins by BRD4 in LC-derived mouse xenografts.

The strength of this study lies in the comprehensive evaluation of the anticancer effects of the inhibitors using multiple assays, including the *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide* (MTT) assay, ATP assay, flow cytometry, Annexin V assay, caspase three activity assay, RT-PCR, and Western blot analysis. Furthermore, in vivo

experiments using a mouse xenograft model confirmed the anti-tumor effects of the inhibitors by reducing tumor growth, tumor weight, and mRNA levels.

Despite the promising results, this study has certain limitations. First, the study only evaluated the anticancer effects of the inhibitors in two human hepatic carcinoma cell lines. Further studies need to be conducted using other cell lines and primary tumor samples. Second, this study did not explore the potential side effects of these inhibitors, which require further investigation. Third, while OPT-0010 was designed as a selective BRD4 inhibitor, direct experimental data on its selectivity and binding affinity to other BET family members (BRD2, BRD3) are not provided in this study. Lastly, the proprietary nature of OPT-0010 limits the availability of structural information, which could aid in understanding its specificity. These limitations emphasize the need for additional experiments, such as biophysical binding assays or transcriptional analyses, to further validate its selectivity and mechanism of action. Future studies addressing these points will be essential to fully elucidate the therapeutic potential of OPT-0010.

Overall, the findings of this study suggest that BRD4 inhibitors, either as monotherapy or in combination with sorafenib, can potentially inhibit the growth of liver cancer cells and may be a promising treatment strategy for liver cancer. The study findings provide valuable insights into the efficacy of BRD4 inhibition in liver cancer and pave the way for potential clinical trials, leading to the development of more effective treatment options for patients with HCC.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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