**INTRODUCTION**

Activation of the genetic mutation of phosphatidylinositol-3 kinase (PI3K) is often observed in several types of cancer. The activation of PI3K is associated with promotion of cell growth, cell migration, and deregulated apoptosis. Therefore, inhibition of PI3K is considered to be a potential therapeutic target of cancer treatment. Several inhibitors targeting PI3K have been developed and clinical trials with these inhibitors are ongoing. However, the efficacy of PI3K inhibitors is limited. Furthermore, it has been reported that the simultaneous inhibition of PI3K and other genes enhances the cytotoxicity of PI3K inhibitors on cancer cell lines. For example, Divakar et al reported that the CDK4/6 and PI3K dual inhibitor exhibited strong in vitro and in vivo cytotoxicity against mantle cell lymphoma. Zhao et al reported that the HSP90 and PI3K dual inhibitor exhibited strong in vitro and in vivo cytotoxicity against mantle cell lymphoma. Meng et al (2019) reported that simultaneous inhibition of HDAC, PI3K, and mammalian target of rapamycin (mTOR) strongly induced cell death in the glioblastoma cell line. Propranolol, a beta-adrenoreceptor which is also a LPIN1 inhibitor, enhanced the in vitro and in vivo cytotoxicity and antitumor effect of FK-A11. These findings should help in the development of FK-A11 as a novel HDAC/PI3K dual inhibitor.
these results, it seemed like the simultaneous inhibition of another gene with HDAC and PI3K would be a potential strategy for enhancing the cytotoxicity of HDAC/PI3K dual inhibitor. Therefore, we hypothesized that there are several potential candidate genes that enhance the cytotoxicity of HDAC/PI3K dual inhibitor FK-A11.

Previously, we revealed ID1 as a lethal synthetic gene with the most common TP53 mutation R175H in glioblastoma cell line SF126 using high-throughput RNA interfering screening. We attempted to identify the gene that enhances the cytotoxicity of the novel HDAC/PI3K dual inhibitor FK-A11 by using the same RNA interfering screening technique in this study.

2 | MATERIALS AND METHODS

2.1 | Reagents and cell line

FK-A11 is produced by Hamari Chemicals. Reagents were dissolved in DMSO for the in vitro assay and in saline solution with 1.3% DMSO and 20% 4-hydroxypropyl β-cyclodextrin (ChemScene) for the in vivo experiments.

2.2 | Cell line

The human fibrosarcoma cell line HT1080 and prostate cancer cell line PC3 were used in the present study. Both HT1080 and PC3 were obtained from ATCC. Both HT-1080 and PC3 were cultured in RPMI-1640 medium containing 10% FBS.

2.3 | RNA interfering screening

Primary screening to identify the gene that enhances the cytotoxicity of FK-A11 was conducted using Decode Pooled Lentiviral shRNA Screening Libraries (Dharmacon), which contains 95 700 shRNA. Whole human genes (18 205 genes) were targeted. Each DNA was targeted by 5-6 shRNA that have different sequences. shRNA were integrated into HT1080 with lentiviral vector according to the manufacturer’s instructions. Multiplicities of infection (MOI) of lentivirus in the present study were .3. DNA extraction from HT1080 and PCR of shRNA, which was integrated into DNA, was also conducted according to the manufacturer’s instructions. Next-generation sequencing for shRNA quantification was commissioned to DNA Chip Research.

2.4 | Knockdown analyses of candidate genes using siRNA

Knockdown of ATG4A, EIF4B, HBXIP, LPIN1, and ML-IAP, which were identified from primary screening, were carried out using siRNA for ATG4A (sc-91197), EIF4B (sc-77253), HBXIP (sc-77371), LPIN1 (sc-60941), and ML-IAP (sc-37510) obtained from Santa Cruz Biotechnology, respectively. The other two types of siRNA for LPIN1 (Silencer Select siRNA, ID: s23206 and s23207) were purchased from Thermo Fisher Scientific. LPIN1 silencing was performed by transfecting siRNA into HT1080 cells using Lipofectamine RNAi Max (Thermo Fisher Scientific) according to the procedure manual.

2.5 | Knockout and overexpression analyses of gene LPIN1

Knockout of LPIN1 in HT1080 was performed by using CRISPR/Cas9 plasmid (Lipin-1 CRISPR/Cas9 KO plasmid sc-418483, Santa Cruz Biotechnology). Lipin-1 CRISPR/Cas9 plasmid and HDR plasmid were co–transfected into HT-1080 cells using the UltraCruz Transfection Reagent (sc-395739, Santa Cruz Biotechnology) according to the procedure manual. After treatment with puromycin, LPIN1-knocked-out cells were picked monoclonally. We developed an LPIN1-knocked-out HT-1080 cell line (HT-1080-LPIN1-KO cell) with these procedures.

Plasmid pcDNA/TO/LPIN1, which was a gift of sorts from Professor Christophe F. Deroanne, was transfected into HT-1080-LPIN1-KO cells using the Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to the procedure manual. After treatment with zeocin, the transfected cells were selected monoclonally. We developed LPIN1 overexpression cells in a doxycycline-dependent manner by applying these procedures. A concentration of 1 ng/mL of doxycycline was treated for in vitro overexpression of LPIN1.

2.6 | Cell proliferation assay

Cell proliferation assays were carried out with the Cell Counting Kit-8 (Dojindo Laboratories) as previously described.

2.7 | Western blot analysis

Western blot analysis was carried out as previously described using monoclonal antibodies of phospho-AKT (Ser473 and Thr308), AKT, phospho-S6, S6, acetyl-histone H3 (H3K9), and β-actin. All of the above antibodies, except for β-actin (Sigma-Aldrich), were purchased from Cell Signaling Technology.

2.8 | Xenograft model and in vivo antitumor analysis

Six to eight-week-old female BALB/cAJcl-nu/nu mice were used in this investigation. A total of 1 x 10⁶ HT1080 cells or PC3 cells in 100 μL PBS were subcutaneously injected into the flank of each mouse. After approximately 10 days, when tumor formation was confirmed, the mice were divided into each treatment group. Each test
compound was then administered. The length (L), width (W), and height (H) of each subcutaneous tumor mass were measured using digital calipers. Tumor volumes were calculated as \((L \times W \times H)/2\). The body weights of mice were also measured. Tumor weight, extracted from each mouse, was also measured.

2.9 | Statistical analysis

All data were presented as the mean ± standard deviation. Statistical analysis was performed using JMP Pro 12 software (SAS). Unless otherwise noted, the significance of differences was examined using Student’s t tests. \(P < .05\) indicated a statistically significant difference.

3 | RESULTS

3.1 | High-throughput shRNA screening

First, we conducted high-throughput shRNA screening to identify the candidate genes that enhance FK-A11 cytotoxicity in fibrosarcoma cell line HT1080, as described in Figure 1. The shRNA to whole human gene in each group was quantified using a next generation sequencing technique and then we compared the amount of shRNA to whole human gene from each group (Table S1). We identified the candidate shRNA, whose amount in the FK-A11-treated group was reduced to less than 0.8 when compared to that of the DMSO-treated group. The genes whose expression was targeted by these shRNA were candidate genes that enhance FK-A11 cytotoxicity. A total of 4492 candidate shRNA were identified from this primary screening. Of these 4492 shRNA, we further extracted the gene for which three or more different shRNA sequences were contained. For example, seven different sequences of shRNA targeting the ATG4A gene were contained in this screening kit. Of these seven different shRNA, three types of shRNA targeting ATG4A were contained in 4492 shRNA. Finally, five candidate genes (ATG4A, EIF4B, HBXIP, LPIN1, and ML-IAP [also known as BIRC7]) were selected and were used for further validation testing.

3.2 | Suppression of candidate genes by siRNA in the HT1080 cell line

To investigate whether the suppression of candidate genes by siRNA resulted in the enhancement of cell death following the treatment with FK-A11, we transfected the siRNA of five candidate genes (ATG4A, EIF4B, HBXIP, LPIN1, and ML-IAP) into HT1080. We purchased siRNA products containing five different siRNA sequences for each candidate gene and transfected these siRNA products to the knockdown candidate gene in HT1080. Under the transfection of scramble siRNA or candidate gene siRNA, HT1080 was treated with 10 nmol/L of FK-A11. As shown in Figure 2, transfection of LPIN1 siRNA significantly enhanced cell reduction by FK-A11 treatment when compared to that by the scramble siRNA. Therefore, we purchased two different siRNA for the LPIN1 gene (siRNALPIN1-1 and siRNALPIN1-2) and confirmed the reproducibility of enhancing the effect of FK-A11 by LPIN1 knockdown. As shown in Figure 3A, both siRNA LPIN1-1 and siRNA LPIN1-2 downregulated LPIN1 expression. As shown in Figure 3B, FK-A11 cytotoxicity was significantly enhanced under LPIN1 downregulation. We have previously reported that even though the inhibition of either PI3K or HDAC did not enhance cell death, simultaneous inhibition of HDAC and PI3K strongly induced cell death through apoptosis.13 As shown in Figure 3C, LPIN1 knockdown enhanced HDAC inhibition and PI3K dual inhibition by FK-A11 which resulted in the enhancement of downregulation of phosphorylated AKT and induction of acetylated histone. Apoptosis was also strongly induced by FK-A11 under LPIN1 downregulation, which is shown from the enhancement of strong induction of cleaved poly (ADP-ribose) polymerase (PARP) under LPIN1 downregulation. As shown in Figure 3D, enhancement of induction of apoptotic cells by the treatment of FK-A11 under the LPIN1 downregulation was also confirmed by flow cytometry analysis using annexin V and propidium iodide (PI) double staining.

To investigate whether the downregulation of LPIN1 enhances the cytotoxicity of HDAC inhibitor alone or PI3K inhibitor alone, either SAHA (HDAC inhibitor) or GDC-0941 (PI3K inhibitor) was added to HT1080 under the condition of LPIN1 downregulation by LPIN1 siRNA transfection. As shown in Figure 5A, the cytotoxicity of SAHA or GDC-0941 was not enhanced by the downregulation of LPIN1.
As shown in Figure S1B, the induction of acetylated histone was not enhanced by the knockdown of LPIN1. The downregulation of phosphorylated AKT was not enhanced by the treatment of 100 nmol/L or higher concentration of FK-A11.

3.3 Overexpression of LPIN1 rescued cell death by treatment with FK-A11

We performed LPIN1 knockout using the CRISPR-Cas9 technique in HT1080 cells and named the developed cell line "HT1080-LPIN1-KO." As shown in Figure 4A, LPIN1 knockout in HT1080-LPIN1-KO cells was validated by western blot analysis. As shown in Figure 4B, enhanced cytotoxicity of FK-A11 was shown in...
HT1080-LPIN1-KO cells when compared to that of the control cells. Also shown in Figure 4C is the confirmation that apoptosis was strongly induced in HT1080-LPIN1-KO cells through the simultaneous enhancement of HDAC and PI3K inhibition by FK-A11. As shown in Figure 4D, the enhancement of induction of apoptotic cells through the treatment of FK-A11 by the LPIN1 knockout was also confirmed by flow cytometry analysis using annexin V and PI double staining.

Next, we established the monoclonal cell line that overexpresses LPIN1 in a doxycycline-dependent manner in HT1080-LPIN1-KO cells using the plasmid that was used in the previous report.14 We investigated whether or not LPIN1 overexpression in HT1080-LPIN1-KO cells rescued cell death with treatment of FK-A11. Figure 4E shows treatment of doxycycline-induced LPIN1 overexpression in HT1080-LPIN1-KO cells. As shown in Figure 4F, the enhanced cytotoxicity from the treatment of FK-A11 in HT1080-LPIN1-KO cells was partly rescued by LPIN1 overexpression. Also shown in Figure 4G is that the dual inhibition of PI3K and HDAC through treatment of FK-A11 was weakened by LPIN1 overexpression in HT1080-LPIN1-KO cells. The induction of cleaved PARP by treatment of FK-A11 in HT1080-LPIN1-KO cells was also suppressed by LPIN1 overexpression. As shown in Figure 4H, the induction of apoptotic cells through the treatment of FK-A11 was reduced by the overexpression of LPIN1 in flow cytometry analysis using annexin V and PI double staining. The apoptotic cells induced by the treatment of FK-A11 were reduced by the overexpression of LPIN1.

3.4 | Treatment with LPIN1 inhibitor-enhanced cytotoxic and in vitro anti-tumor effect of FK-A11

A previous study reported that propranolol, a non–selective beta-adrenoreceptor blocker that is commonly used for hypertension or arrhythmias, inhibited the enzymatic activity of LPIN1.15,16 We investigated whether or not treatment with propranolol enhances the in vitro cytotoxic effect of FK-A11. As shown in Figure 5A, treatment with 100 μmol/L propranolol significantly enhanced the cytotoxic effect of FK-A11 in HT1080 cells in the MTT assay. As shown in Figure 5B, dual inhibition of HDAC and PI3K and induction of cleaved PARP by FK-A11 were both enhanced in HT1080 cells through treatment with 100 μmol/L of propranolol. Kim et al (2016) showed that the downregulation of LPIN1 attenuated the stability of insulin receptor substrate (IRS)-1.17 Therefore, we assessed the stability of IRS-1 with the treatment of LPIN1 inhibitor. As shown in Figure 5B, the stability of IRS-1 was strongly attenuated by the treatment of propranolol in HT1080. As shown in Figure 5C, the enhancement of induction of apoptotic cells through the treatment of FK-A11 with the addition of propranolol treatment was also confirmed by flow cytometry analysis using annexin V and PI double staining. The apoptotic cells induced by the treatment of FK-A11 were enhanced by the addition of propranolol.

Bromoenol lactone has also been previously reported as an LPIN1 inhibitor.15,18 As shown in Figure 5D, treatment with bromoenol lactone also enhanced the cytotoxic effect of FK-A11 in the MTT assay. As shown in Figure 5E, dual inhibition of HDAC and PI3K and induction of cleaved PARP by FK-A11 were both enhanced in HT1080 cells through treatment with 10 μmol/L bromoenol lactone. As shown in Figure 5E, the stability of IRS-1 was strongly attenuated by the treatment of bromoenol lactone in HT1080. As shown in Figure 5F, the enhancement of induction of apoptotic cells through the treatment of FK-A11 with the addition of bromoenol lactone treatment was also confirmed by flow cytometry analysis using annexin V and PI double staining. The apoptotic cells induced by the treatment of FK-A11 was enhanced by the addition of bromoenol lactone.

As in the case of the HT1080 cell line, and prostate cancer cell line PC3, combination treatment of propranolol or bromoenol lactone and FK-A11 enhanced both the inhibition of HDAC and PI3K by FK-A11 and strongly induced apoptosis (Figure S3).

3.5 | FK-A11 and propranolol combination therapy enhanced the in vivo anti–tumor effect of FK-A11

Anti–tumor efficacy induced by combination treatment with FK-A11 and propranolol was evaluated using HT1080 xenograft mouse models. In the HT1080 fibrosarcoma tumor-bearing BALB/cAJcl-nu/nu mice, 2 mg/kg of FK-A11 and 2 mg/kg of propranolol were administered intraperitoneally every 3 days and every day, respectively. As shown in Figure 6A, the tumor growth in the FK-A11 alone group was significantly inhibited when compared to that in the control group or the propranolol group, respectively. Moreover, the tumor growth in the FK-A11 and propranolol combination group was significantly inhibited when compared to that in the control group or the propranolol group, respectively. Tumor weight in the FK-A11 and propranolol combination group was significantly smaller than that in the control group or the propranolol group. Tumor weight in the FK-A11 alone group was significantly smaller than that in the FK-A11 alone group. Moreover, the tumor weight in the FK-A11 and propranolol combination group was significantly smaller than that in the FK-A11 alone group. The body weight of tumor-bearing mice was measured to assess toxicity. As shown in Figure 6C, the body weights of the FK-A11 group or combination group were slightly lower than those of control or propranolol groups. However, body weight reduction was not enhanced by the FK-A11 and propranolol combination treatment when compared to that of FK-A11 alone. As shown in Figure 6D, the tumor weight in the FK-A11 alone group was significantly smaller than that in the FK-A11 alone group. As shown in Figure S4, there is a negative correlation coefficient between the tumor volume and the mouse body weight (r = −.33938).

To validate whether or not the treatment of propranolol enhanced in vivo dual inhibition of HDAC and PI3K by FK-A11, we performed western blot analysis using a protein extracted from the tumors of each group. As shown in Figure 6D, propranolol and FK-A11 combination treatment enhanced HDAC and PI3K dual inhibition when compared to that of FK-A11 alone. Induction of cleaved PARP was also enhanced by propranolol and FK-A11 combination treatment when compared to that of FK-A11 alone.
FIGURE 4 Overexpression of LPIN1 in LPIN1 knockout cells. A, Using the CRISPR-Cas9 technique, LPIN1 id was knocked out in HT1080 cells (HT1080-LPIN1-KO). Deletion of LPIN1 in HT1080-LPIN1-KO was verified through western blot analysis. B, The cytotoxic effects of 10 nmol/L in the control cells and HT1080-LPIN1-KO cells in a cell proliferation assay. The vertical axis corresponds to the absorbance ratio. The ratio was calculated as (absorbance of well with HT1080-LPIN1-KO cell)/(absorbance of well with control cell). The values shown are means ± SD (n = 3). *P < .05. C, Enhancement of HDAC/PI3K dual inhibition and cleaved poly (ADP-ribose) polymerase (PARP) induction by 10 nmol/L of FK-A11 in HT1080-LPIN1-KO cells were verified through western blot analysis. D, Flow cytometry of apoptosis using annexin V and PI double staining in control cell and HT1080-LPIN1-KO cell which was treated with 10 nmol/L of FK-A11 for 24 h. E, LPIN1 overexpression after treatment with 1 ng/mL doxycycline was verified through western blot analysis. F, Cytotoxic effect of 10 or 100 nmol/L of FK-A11 under Dox-off or on condition in a cell proliferation assay. The vertical axis corresponds to the absorbance ratio. The ratio was calculated as (absorbance of well with FK-A11 treated cell)/(absorbance of well with DMSO treated cell). The values shown are means ± SD (n = 3). *P < .05. G, Diminishment of the inhibition of dual HDAC and PI3K and induction of cleaved PARP by FK-A11 were verified through western blot analysis. H, Flow cytometry of apoptosis using annexin V and propidium iodide (PI) double staining of HT1080 under Dox on/off condition which was treated with 10 or 100 nmol/L of FK-A11 for 24 h

4 | DISCUSSION

We previously reported that even though the simultaneous inhibition of both PI3K and HDAC strongly induced apoptosis in the colorectal cancer cell line, the inhibition of either PI3K or HDAC weakly induced apoptosis. This phenomenon was also recently confirmed in vitro in the B-cell lymphoma cell line. It could be considered from these results that simultaneous enhancement of HDAC and PI3K inhibition is warranted to enhance the cytotoxicity of HDAC/PI3K dual inhibitor FK-A11 efficiently. In this investigation, only downregulation of LPIN1 did not enhance either the inhibition of PI3K or of HDAC in the HT1080 cell line (Figures 3C and 4C). However, under LPIN1 downregulation conditions, inhibition of both HDAC and PI3K through treatment with FK-A11 were enhanced. The strong induction of apoptosis in HT1080 cells through treatment with FK-A11 in the present study was considered to be attributable to the simultaneous enhancement of inhibition of HDAC and PI3K under LPIN1 downregulation.

LPIN1 is a gene that encodes phosphatidic acid phosphatase, which has a critical role in the de novo synthesis of phospholipids or triglycerides. It has been reported that LPIN1 deficiency resulted in rhabdomyolysis or exercise-induced myalgia in muscle-specific LPIN1 knockout mice. It was also reported that LPIN1 overexpression correlated with poor prognosis in patients with lung adenocarcinoma and that LPIN1 downregulation resulted in a decrease of cell viability and proliferation of lung adenocarcinoma cell lines through regulation of endoplasmic reticulum homeostasis and autophagy. Report also mentioned that LPIN1 downregulation enhances the sensitivity of lung adenocarcinoma cell lines to cisplatin. From that report, LPIN1 appears to play a critical role in the progression or chemosensitivity of cancer. However, there have been no previous reports on the relationship between LPIN1 expression level and chemosensitivity. First, we demonstrated that LPIN1 suppression enhanced cytotoxicity of the novel HDAC/PI3K dual inhibitor FK-A11 through simultaneous enhancement of HDAC and PI3K inhibition.

The mechanisms for downregulation of LPIN1 mediated by the enhancement of inhibition of both HDAC and PI3K by FK-A11 has not yet been resolved. However, overexpression of LPIN1 by the Dox-on system in LPIN1 knockout cells significantly weakened the inhibitory effect of PI3K and HDAC by FK-A11 and rescued cell death through treatment with FK-A11 (Figure 4F, G). It was revealed that the LPIN1 expression level was definitely related to the inhibitory effect of HDAC and PI3K by FK-A11. Based on this result, if we could confirm the negative correlation between LPIN1 expression level and sensitivity to FK-A11 in several cancer cell lines, the expression level of LPIN1 might be established as a predictive biomarker for FK-A11. Further investigation by our team regarding this point is under way.

Propranolol is safely used as a non-selective beta-adrenoreceptor blocker in daily practice for treatment of patients with hypertension or arrhythmia. It has been reported in previous studies that beta-adrenoreceptor blockade using propranolol induced in vitro apoptosis in gastric cancer cells and in vitro and in vivo apoptosis in neuroblastoma cells. It was considered in these two previous investigations that these effects were attributable to cyclooxygenase-2 (COX2) downregulation caused by beta-adrenoreceptor blockade through treatment with propranolol. It was also determined that COX2 downregulation mediated in vitro PI3K inhibition in the lung adenocarcinoma cell line. Therefore, beta-adrenoreceptor inhibition through treatment with propranolol possibility mediates PI3K inhibition. In the present investigation, treatment with propranolol alone slightly downregulated the level of phosphorylated AKT, which implies PI3K inhibition in HT1080 cells (Figure S1B). However, treatment with propranolol alone did not inhibit HDAC activity and did not induce apoptosis in HT1080 cells. These results support the idea that the strong induction of apoptosis was attributable to simultaneous enhancement of HDAC and PI3K inhibition through treatment with FK-A11 under LPIN1 downregulation.

As shown in Figure S1A, LPIN1 downregulation did not enhance the cytotoxicity of SAHA, the HDAC inhibitor or GDC-0941, the PI3K inhibitor. As shown in Figure S1B, the induction of acetylated histone by the treatment of SAHA or the downregulation of phosphorylated AKT by the treatment of GDC-0941 were not enhanced. From these results, it was considered whether the enhancement of cytotoxicity through the enhancement of HDAC and PI3K inhibition under the condition of LPIN1 downregulation was FK-A11 specific.

As shown in Figure S2, the cytotoxicity of 10 nmol/L concentration of FK-A11 was strongly enhanced by the downregulation of LPIN1. However, the cytotoxicity of 100 nmol/L or higher concentration of FK-A11 was not enhanced by the downregulation of
LPIN1. This fact could be the basis for dose setting when FK-A11 is introduced into early phases of clinical study in the future.

As shown in Figure S4, there was a negative correlation between tumor volume and mouse body weight in the present in vivo investigation. From this result, it could be considered that the growth of the tumor resulted in the reduction of the food intake by the investigated mice. It might be one reason why the body weight of mice in each group on day 21 was smaller than that on day 0.

LPIN2 shares a strong homology and similar activity with LPIN1. 29 It was reported that LPIIN1 and LPIN2 double silencing strongly inhibited in vitro prostate cancer cell proliferation. 14 In this study, cell

![Figure 5](image)

**FIGURE 5** Treatment with two types of LPIN1 inhibitors with FK-A11. A, Cytotoxic effects of 10 or 100 nmol/L of FK-A11 after treatment with 100 μmol/L of propranolol in HT1080 cells in the cell proliferation assay. The vertical axis corresponds to the absorbance ratio. The ratio was calculated as (absorbance of well with FK-A11 treated cell)/(absorbance of well with DMSO treated cell). The values shown are means ± SD (n = 3). **P < .01. B, Enhancement of HDAC/PI3K dual inhibition and cleaved poly (ADP-ribose) polymerase (PARP) induction by 10 or 100 nmol/L of FK-A11 in HT1080 cells after treatment with 100 μmol/L of propranolol were verified through western blot analysis. C, Flow cytometry of apoptosis using annexin V and propidium iodide (PI) double staining in control cell and propranolol treated cell which was treated with 10 mg/L of FK-A11 for 24 h. D, Cytotoxic effects of 10 or 100 nmol/L FK-A11 after treatment with 10 μmol/L bromoenol lactone in HT1080 cells in the cell proliferation assay. The vertical axis corresponds to the absorbance ratio. The ratio was calculated as (absorbance of well with FK-A11 treated cell)/(absorbance of well with DMSO treated cell). The values shown are means ± SD (n = 3). **P < .01. E, Enhancement of HDAC/PI3K dual inhibition and cleaved PARP induction by 100 nmol/L of FK-A11 in HT1080 cells after treatment with 10 μmol/L of bromoenol lactone was verified through western blot analysis. F, Flow cytometry of apoptosis using annexin V and PI double staining in a control cell and a bromoenol lactone treated cell which was treated with 10 or 100 nmol/L of FK-A11 for 24 h

![Figure 6](image)

**FIGURE 6** In vivo anti–tumor efficacy of FK-A11 and propranolol combination treatment. A, 1.0 × 10⁶ HT1080 cells were subcutaneously injected into 6-week-old female BALB/c nu/nu nude mice. Tumor-bearing mice were divided into test groups, each with 5 mice. Solvent only, 2 mg/kg/day of propranolol, 2 mg/kg/3 days of FK-A11, or 2 mg/kg/day of propranolol and 2 mg/kg/3 days of FK-A11 combination were administered intraperitoneally. Length, height, and width of the subcutaneous tumor were measured and the tumor volumes were calculated as (length × height × width)/2. *P < .05. **P < .01. B, Box whisker plot of the weight of tumors that were extracted from mice in each group. **P < .01. C, Body weights in each group were also measured. Data are the means ± SE calculated for five mice. D, Western blot analysis of HDAC and PI3K inhibition or induction of cleaved poly (ADP-ribose) polymerase (PARP) in tumors of each group. Subcutaneous tumors were extracted from each mouse on day 21. Protein was extracted from each tumor and subjected to western blot analysis. NS, not significant
count was strongly lowered by the FK-A11 and propranolol or FK-A11 and bromoenol lactone combination treatment (Figure 5A,D) when compared to treatment with FK-A11 under LPIN1 downregulation (Figures 3B and 4B). It was determined that both propranolol and bromoenol lactone are pan LPIN inhibitors and inhibit all LPIN.15,16,30 Thus, treatment with propranolol or bromoenol lactone in this investigation inhibited both LPIN1 and LPIN2. The strong cell count reduction by combining FK-A11 with propranolol or bromoenol lactone was considered to be attributable to inhibition of both LPIN1 and LPIN2.

The acetylation status on histone protein in the present study is H3K9. The acetylation of H3K9 of the LPIN1 gene promoter region has not been investigated yet. However, the expression level of LPIN1 is enhanced by the treatment of 100 nmol/L or higher concentration of FK-A11 (Figure 4G; Figures S1B and S2). It had been reported that the acetylation of H3K9 results in gene activation and, consequently, induces gene expression.31 The enhancement of expression of LPIN1 might be attributable to the acetylation of LPIN1 H3K9 in the promoter region by the 100 nmol/L or higher concentration of FK-A11. Detailed examination of changes of gene expression with the treatment of FK-A11 might lead to the elucidation of the mechanisms of the enhancement of cytotoxicity of FK-A11 by the downregulation of LPIN1.

In conclusion, LPIN1 suppression is a potential strategy for enhancing the cytotoxicity of the novel HDAC/PI3K inhibitor FK-A11 through simultaneous enhancement of HDAC and PI3K inhibition by FK-A11. Further investigation of the mechanism for enhancing the cytotoxic effect of FK-A11 is warranted.

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DISCLOSURE

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ORCID

Ken Saijo https://orcid.org/0000-0003-0179-3789
Chikashi Ishioka https://orcid.org/0000-0002-3023-1227

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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