Mitigating the BFL1-mediated antiapoptotic pathway in diffuse large B cell lymphoma by inhibiting HDACs

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Author's contribution

EP acquired, analyzed, and interpreted the experimental data and drafted the manuscript. CL performed bioinformatics and statistical analyses. JH, DYS, JMB, YK and SSY devised the study concept and design and JP, JL provided technical guidance for all aspects of the project. All authors discussed the results and contributed to the final manuscript. SSY and YK confirm the authenticity of all the raw data. All authors critically reviewed the manuscript before submission.

Running heads: BFL1 inhibition by HDAC inhibitor in DLBCL

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Data sharing statement

Whole transcriptome data of SUDHL-2 can be obtained from sequence read archive (SRA) with accession number PRJNA806671.

Word count

Abstract word count: 191 / 250

Main text count: 2996 / 4000

Figure/table count: 6 / 0

Supplemental material: 12 supplemental figures, 5 supplemental tables

Acknowledgements

This work was supported by the Korea Health Industry Development Institute (KHIDI; grant no. HI14C1277) and National Research Foundation of Korea (NRF; grant no. 2021R1A2C3005360).

Abstract

Endogenous BFL1 expression renders diffuse large B-cell lymphoma (DLBCL) cells insensitive to BCL2 and/or MCL1 inhibitors. Considering the difficulties of developing a direct BFL1 inhibitor, we intended to inhibit histone deacetylase (HDAC) to mitigate the adverse biologic role of BFL1 by modulating WT1 and NOXA. In vitro assays including western blotting, qRT-PCR, proliferation assay, and flow cytometry were performed using DLBCL cell lines. shRNA-mediated BFL1 knockdown DLBCL cells were generated to verify the role of BFL1 in HDAC inhibitors sensitivity. DLBCL cells expressing high BFL1 exhibited enhanced sensitivity to pan-HDAC inhibitor panobinostat compared to low BFL1 expressing cells, mainly attributable to the difference in the amount of apoptosis. HDAC inhibitors decreased BFL1 and WT1 expressions while increased NOXA level in DLBCL, as we hypothesized. The BFL1 knockdown experiment demonstrated that HDAC inhibitor's sensitivity depends on the expression level of BFL1 in DLBCL cells. Furthermore, we found the specific HDAC class was expected to play a critical role in BFL1 inhibition by comparing the results of several HDAC inhibitors. In summary, our study provides a rationale for using HDAC inhibitors to induce apoptosis in DLBCL patients using BFL1 as a predictive biomarker.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a B-cell lymphoma constituting ~40% of non-Hodgkin B-cell lymphomas.(1) DLBCL is genetically heterogeneous with various subtypes according to genetic irregularities, clinical outcomes, response to treatment, and prognosis.(2) Addition of rituximab to cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) chemotherapy improved the prognosis of DLBCL patients, but more than 30% of DLBCL patients still have a poor prognosis.(3-5) Accordingly, researchers have attempted to discover new treatment approaches for DLBCL to improve outcome.(6)

B-cell lymphoma 2 (BCL2) family proteins are key regulators of apoptosis(7) having distinct subgroups that function in complementary ways to retain their original apoptosis regulatory function; antiapoptotic proteins (BCL2, MCL1, BCL-W, BCL-XL, and BFL1), proapoptotic effector proteins (BAK and BAX), and pro-apoptotic activator proteins (BIM, PUMA, BID, BIK, BAD, BMF, HRK, and NOXA).(8) Several BH3 mimetics that bind to antiapoptotic proteins and trigger cell death are under active clinical development(9). Among them, BCL2 targeting venetoclax has been approved for hematologic malignancies.(10, 11) In DLBCL, overexpression of BCL2 is one of the factors associated with CHOP resistance.(12, 13) Although a recent clinical trial of venetoclax with R-CHOP in BCL2+ (by immunohistochemistry) DLBCL patients yielded a promising result,(14) unmet needs still exist regarding the therapeutic use of apoptosis for the following reasons: 1) No effective strategies are present for patients lacking BCL2 expression, 2) even in BCL2+ patients, resistance to venetoclax occurs easily because of the presence of alternative proteins that retain the antiapoptotic function of BCL2 family.(15)

Meanwhile, BFL1 is the main molecule that can replace the antiapoptotic function of BCL2 in DLBCL, which in turn might be related to both intrinsic and acquired resistance to venetoclax.(16, 17) Hence, targeting BFL1 is a rational and attractive strategy in DLBCL to improve clinical outcome.

Unfortunately, so far, there are only stapled peptides and early-stage small molecules targeting BFL1 and clinically available BFL1 inhibitors are not available.(18, 19) Considering the difficulty in developing direct BFL1 inhibitors, we decided to investigate indirect strategies for inhibiting BFL1 at transcriptional and post-translational levels. During BFL1 transcription, several transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), retinoid X receptor (RXR), and Wilms' tumor-1 (WT1) are involved.(20) WT1 directly upregulates BFL1 in hematologic malignancies,(21, 22) while NOXA is a pro-apoptotic activator protein that closely interacts with BFL1.(23) At the post-translational level, NOXA is a counterpart of BFL1 and decreases BFL1 activity by sequestering activator or effector proteins.(23) Therefore, increased NOXA expression can increase the apoptosis rate. Based on the above evidence and hypothesis, inhibition of histone deacetylase (HDAC), which decreases WT1 and increases NOXA levels, was investigated to indirectly regulate BFL1. Various HDAC inhibitors were tested and compared to identify the most important HDAC subclass that effectively inhibits BFL1. Our study aimed to suggest mechanism-based drug treatment in BFL1-high DLBCL patients.

Methods

Cell culture and drugs

We cultured 6 DLBCL cell lines, 1 kidney cell lines for experiments. The drugs used are 4 HDAC inhibitors (Panobinostat, trichostatin A, belinostat, and vorinostat) and venetoclax. The detailed culture method can be found in the supplemental methods.

Western blotting

Equal amounts of protein were loaded on polyacrylamide gels containing SDS, transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA), and blocked for 1 h using 5% skim-milk (Tech&Innovation, Gang-won, South Korea). Membranes were incubated with primary antibodies overnight, followed by the appropriate secondary antibodies for 1 h. Antibodies used can be found in the supplemental methods. Membranes were stripped using Eastern-Blot Western Blot Stripping Buffer (BioMax, Seoul, South Korea).

Cell proliferation and apoptosis assay

Cell proliferation was assessed using the Quanti-Max WST-8 Cell Viability Assay Kit (Biomax, Derwood, MD) according to the manufacturer's instructions using a microplate reader (Molecular Devices, San Jose, CA). Cells were seeded in sterile 96-well plates and treated with drugs at increasing concentrations for indicated times. Growth inhibitory concentrations (IC50) of 50% were calculated using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA).

The ability of HDAC inhibitors and venetoclax to induce apoptosis in cells was measured using the Dead Cell Apoptosis Kit with Annexin V FITC and PI (Thermo

Fisher Scientific) and APC annexin V (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Apoptotic cells were analyzed using BD FACSymphony A3 (BD Biosciences). The results were analyzed using FlowJo 10.7.1 software (Tree Star, Ashland, OR, USA).

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Approximately 1–5 ug of RNA was used to synthesize cDNA using TOPscript RT DryMIX (Enzynomics, Daejeon, South Korea). Real-time PCR was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara) on a QuantStudio 1 real-time PCR system (Thermo Fisher Scientific). *GAPDH* was used to normalize the target mRNA expression. The primer sequences used are listed in Supplemental Figure 1.

Lentiviral shRNA-mediated BFL1 knockdown

The enhanced green fluorescent protein(EGFP) cloning vector was used for lentiviral mediated knockdown. The detailed construction method of the vector can be found in the supplemental methods. The BFL1 shRNA or scrambled shRNA plasmids were co-transfected with the lentiviral packaging vectors (detailed in the supplemental methods). After that, viral supernatant was concentrated using a Lenti-X concentrator (Takara) and added to the cells with 8 ug/mL polybrene (Sigma Aldrich) by the spinoculation method. Infected cells with GFP signals were selected using a FACS-sorting procedure (BD FACSAria II, BD Biosciences). The sequences used for BFL1 inhibition are listed in Supplemental Figure 2. (24, 25)

Statistical analysis

Statistical analyses for the efficacy of drugs were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA). Data are presented as means ± standard deviation (SD). Multiple comparisons among groups were performed using an analysis of variance (ANOVA). The combination index was calculated using Calcusyn v2.11 (Biosoft, Cambridge, UK).

Results

BFL1 expression highly correlates with venetoclax and HDAC inhibitor sensitivity

Six DLBCL cell lines were divided into two groups according to BFL1 expression level (BFL1+ group: SUDHL-2, OCI-LY-3, TMD-8, and RC-K8, BFL1- group: OCI-LY-1 and OCI-LY-19) (Figure 1A-B). Compared to the DLBCL cell lines in the BFL1- group, the BFL1+ group exhibited reduced sensitivity to venetoclax (Figure 1C). The cell lines in the BFL1+ group showed higher IC50 values than those in the BFL1- group with venetoclax treatment (Figure 1D). In contrast, BFL1+ cell lines were more sensitive to panobinostat, a representative HDAC inhibitor (Figure 1E, Supplemental Figure 3), than BFL1- cell lines (Figure 1F). In summary, DLBCL cell lines overexpressing BFL1 are sensitive to HDAC inhibitor while insensitive to venetoclax compared to DLBCL cell lines without BFL1 expression.

HDAC inhibitors inhibit BFL1 by repressing WT1 and upregulating NOXA

We hypothesized that the difference in the sensitivity to panobinostat between BFL1+/- groups originates from the pharmacological effect of HDACi in downregulating BFL1 and WT1, and upregulating NOXA. To determine the effect of panobinostat on WT1, NOXA, and BFL1, we examined the change in the expressions of molecules after panobinostat treatment between the BFL1+ group (SUDHL-2, OCI-LY-3) and BFL1- group (OCI-LY-19). In the BFL1+ group, we could detect decreases in BFL1 and WT1 levels and increases in NOXA levels in a dose-dependent manner (Figure 2A). This finding was also validated using whole transcriptome sequencing data of pre- and post-treatment samples (Supplemental

Figure 4). In contrast, NOXA was induced at low levels in the BFL1- group, and there was no change in BFL1 levels despite a reduction in WT1 level (Figure 2A). These results were reproduced with other HDAC inhibitors treatment (Supplemental Figure 5A-B). The decrease of WT1 and BFL1 levels and the increase of NOXA levels were not observed in venetoclax treatment (Figure 2B). Therefore, we could confirm the inhibition of BFL1 in WT1 and NOXA-dependent manner only with HDAC inhibitors as we have hypothesized.

Then, we confirmed the temporal consequences for reducing WT1 and BFL1 by assessing the response to the HDAC inhibitor in SUDHL-2. After panobinostat treatment, the decrease in WT1 level started from 2 h, which triggered a time-dependent reduction in BFL1 level from 6 h (Figure 2C).

To reveal the underlying mechanism of NOXA, we analyzed pre- and post-treatment WTS data of SUDHL-2. Via DEG analysis, we found upregulation of the *TXNIP* gene after panobinostat treatment. GSEA revealed that the P53 pathway and cell cycle arrest gene-sets were significantly enriched after panobinostat treatment (Figure 2D-E, Supplemental Figure 6). TXNIP increases P53 stability and activity,(26, 27) and P53 is a well-known transcription factor of NOXA and induces cell cycle arrest. Therefore, our WTS analysis suggests that the mechanism for NOXA upregulation is closely connected to TXNIP and P53.

Altogether, HDAC inhibitors inhibit BFL1 at both transcriptional and post-translational levels by suppressing its transcription factor WT1 and inducing its antagonist NOXA in DLBCL.

BFL1 level is associated with HDAC inhibitor-mediated apoptosis

To clarify that the different sensitivities to HDAC inhibitors and venetoclax are mainly attributable to the difference in apoptosis, we examined the expression levels of apoptogenic factors, including caspase-9, caspase-3, and PARP, in the BFL1+/- cells after drug treatment.

When panobinostat was added, SUDHL-2 cells began apoptotic process from 10 nM (Figure 3A), the concentration at which BFL1 expression was decreased (Figure 2A). In OCI-LY-3 cells, apoptosis was induced from 30 nM (Figure 3A), the concentration at which BFL1 was reduced (Figure 2A). In OCI-LY-19 cells, in contrast, we identified no apoptogenic induction in total form level at broad concentrations (Figure 3A). Contrary to the result of panobinostat, we identified no apparent apoptogenic induction in BFL1+ cells with venetoclax treatment. On the contrary, OCI-LY-19 cells underwent apoptogenic induction after venetoclax treatment (Figure 3B).

To confirm the level of apoptosis, we analyzed the apoptotic cells after panobinostat treatment using flow cytometry with annexin V and PI staining. BFL1+ cells showed enhanced apoptotic responses (Figure 3C-D). In BFL1+ cells, apoptosis rate was significantly increased at the concentration in which BFL1 expression was decreased (Figure 2A). Conversely, there was only slight increase in apoptosis rate of OCI-LY-19 cells compared to the previous two cell lines. By analyzing the apoptosis induction at different drug concentrations, we confirmed that panobinostat induces apoptosis by inhibiting BFL1, explaining the difference in drug sensitivity between BFL1 +/- groups.

BFL1 is a critical molecule for deciding cell vulnerability to HDAC inhibitors by inhibiting apoptosis induction

To evaluate the impact of BFL1 on cell vulnerability to HDAC inhibitors, BFL1 was knocked down in SUDHL-2 cells that have the most robust BFL1 expression using a lentiviral infection system. We confirmed the proper infection and BFL1 knockdown using several in vitro experiments (Supplemental Figure 7). BFL1 knockdown cells showed the repressed cell development compared to scrambled vector transduced cells (Figure 4A).

As we expected, knockdown of BFL1 recovered the venetoclax sensitivity (Figure 4B, Supplemental Figure 8). More importantly, we confirmed that panobinostat sensitivity depends on the presence of BFL1 (Figure 4C-D). BFL1 knockdown cells were insensitive to HDAC inhibitors, which is in contrast to the scrambled vector-transduced cells (Figure 4C-D, Supplemental Table 1-2). However, a decrease in WT1 level in BFL1 knockdown cells treated with panobinostat was observed (Supplemental Figure 9), which means that the expression of WT1 targets other than BFL1 was decreased. Hence, the sensitivity to HDAC inhibitors depends primarily on the presence of BFL1.

To examine whether the apoptosis induction by HDAC inhibitor correlates with the baseline BFL1 expression levels, we analyzed the apoptogenic factors after panobinostat treatment as previously described. The scrambled vector-transduced cells showed a higher apoptogenic response than BFL1 knockdown cells to panobinostat. Interestingly, a significant reduction in BFL1 levels in scrambled

vector-transduced cells started from 30 nM, different from that in NI cells (10nM) (Figure 5A).

In flow cytometry analysis, only few apoptotic cells were observed even at 30 nM of panobinostat in BFL1 knockdown cells (Figure 5B). On the other hand, there was a significant increase in the apoptosis rate at 30 nM (78.2 %) compared to that at 10 nM (20.3%) in scrambled vector-transduced cells, indicating the importance of BFL1 in apoptosis induction (Figure 5C). BFL1 knockdown cells showed lower apoptosis than scrambled vector-transduced cells following other HDAC inhibitor treatments as well (Supplemental Figure 10A-B). Taken together, we confirm the importance of BFL1 in inducing apoptosis following HDAC inhibitor treatment.

Differential sensitivity to HDAC inhibitors in DLBCL suggests a class-specific mechanism of action for BFL1 inhibition

Several HDAC inhibitors were used in our study, and the results differed depending on the type of HDAC inhibitors. The most exciting finding is observed with vorinostat, where differential drug sensitivity according to the BFL1 expression level was not identified (Figure 6A). Furthermore, no BFL1 inhibition following vorinostat treatment was observed (Figure 6B-C), unlike other pan-HDAC inhibitors. Mechanism-wise, vorinostat does not inhibit HDACs in class IIa (4, 5, 7, and 9) (28) unlike panobinostat, trichostatin A, and belinostat. Therefore, we suggest that inhibition of HDAC class IIa can be important to inhibit BFL1 in DLBCL.

Discussion

Venetoclax, a highly selective BCL2 inhibitor, faces resistance problems when BCL2 family antiapoptotic proteins are overexpressed.(29) Among them, MCL1 and BFL1 have been reported as key determinants for venetoclax efficacy.(30, 31) Significant progress has been made in recent years, including the development of MCL1 inhibitors.(32) Unlike MCL1, several modalities to target BFL1 indirectly have been identified, including CDK9, BET inhibitor(15, 17) due to the absence of BFL1 inhibitor. In this work, we identified another novel approach to inhibit BFL1, which is, HDAC inhibitors.

BFL1 has been suggested as a promising therapeutic agent to overcome resistance to various drugs such as R-CHOP and venetoclax in blood cancer.(25, 30, 33) In fact, BFL1 is a main antiapoptotic molecule replacing BCL2 and MCL1 by sequestering pro-apoptotic proteins. After BH3 mimetic exposures, BFL1 preserves cell survival while avoiding mitochondrial depolarization.(30) However, the development of the BFL1 direct inhibitor has not been successful yet. Some previous studies have reported the indirect inhibition of BFL1 through various pharmacological approaches.(15, 17) Our study aimed to elucidate a novel method for the indirect inhibition of BFL1.

In this study, we confirmed that HDAC inhibitors hamper BFL1 at both the transcriptional and post-translational levels in DLBCL and that the sensitivity to HDAC inhibitors depends on the presence of BFL1. Furthermore, BFL1 knockdown cells showed higher responsiveness to venetoclax than scrambled vector-transduced cells (Supplemental Figure 8A–B), confirming a resistance mechanism to venetoclax

via BFL1. At the molecular level, our novel BFL1 inhibition approach is based on the biological mechanism, transcription and molecular interaction. Transcriptional changes, including WT1 or TXNIP, P53 decreases BFL1 transcription or increases the transcription of NOXA. Increased NOXA level inhibits antiapoptotic function of BFL1 by molecular interaction. To overcome the limitation of using a few cell lines, we additionally validated the association between BFL1 expression and panobinostat response using 12 DLBCL cell lines from Cancer Cell Line Encyclopedia (CCLE) and Genomics of Drug Sensitivity in Cancer (GDSC) (Supplemental Figure 11). As a result, we identified that BFL1 affects the sensitivity to various drugs, such as HDAC inhibitors and venetoclax.

For clinical application of our study result based on recent molecular classification of DLBCL, we conducted genetic subtyping using sequencing data which is available from CCLE and TCGA, using LymphGen algorithm (Supplemental Table 3-4).(34) Interestingly, 80% of the DLBCL cell lines with high BFL1 expression were the MCD subtype and more sensitive to HDAC inhibitors. In the analysis using TCGA data, MCD subtype is the only subtype showing the significant difference in patients' numbers between BFL1 high/low group. In the pivotal study, the MCD subtype has higher expression levels of NF-κB target genes than other subtypes,(34) and the association of BFL1 and NF-κB in DLBCL has been well documented in the previous studies.(35, 36) Although further large-scale studies are needed in the future, we suggest the possibility of using HDAC inhibitors in DLBCL patients with high BFL1 expression levels in the MCD group.

Moreover, we identified a synergistic effect of panobinostat combined with lenalidomide in cells with high BFL1 expression levels (Supplemental Table 5).

Lenalidomide is a salvage drug in some of relapsed and refractory DLBCL patients. Therefore, we empirically tested whether the combination of lenalidomide with panobinostat is synergistic in the cell line having high BFL1 expression. Identifying synergistic partners with HDAC inhibitors may improve clinical outcomes in high BFL1 expressing DLBCL.

In conclusion, we have demonstrated that HDAC inhibitors reduce the expression and activity of BFL1 in a WT1- and NOXA-dependent manner at both the transcriptional and post-translational levels. Additionally, we identified that the sensitivity to HDAC inhibitors is dependent on the presence of BFL1 in DLBCL (Supplemental Figure 12). Because of the absence of BH3 mimetics directly targeting BFL1 to date, HDAC inhibitors could provide a new therapeutic option for DLBCL patients using BFL1 as a predictive biomarker.

Disclosure of Conflicts of Interest

None

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Figure Legends

Figure 1. BCL2 family expression in DLBCL and drug sensitivity differences among BFL1 +/- cell lines (A) Relative mRNA expression of *BCL2A1*, *MCL1*, and *BCL2* in DLBCL cell lines. (B) Western blotting for identifying expression levels of BFL1, MCL1, and BCL2 in DLBCL cell lines. (C) Proliferation assay after 72 h from venetoclax treatment using WST-8 in DLBCL cell lines. The proliferation graph representing each group was plotted using the mean value of the proliferation graph of each cell line. (D) Calculated log IC50 values at 72 h after venetoclax treatment. The log IC50 values of BFL1+/- groups represent the average log IC50 of each cell line. (E) Proliferation assay after 72 h from panobinostat treatment using WST in DLBCL cell lines. (F) Calculated log IC50 values at 72 h after panobinostat treatment, N/A, could not be determined. **≤ 0.001, ***≤ 0.0001.

Figure 2. Inhibition of BFL1 by WT1 repression and NOXA overexpression following HDAC inhibitor treatment (A) WT1 reduction and NOXA induction following panobinostat treatment. Each cell line is treated with panobinostat for 24 h. (B) No decrease in WT1 level and no increase in NOXA level following venetoclax treatment. Each cell line was treated with venetoclax for 24 h. (C) Measurement of WT1, BFL1, NOXA, MCL1, and BCL2 protein levels in SUDHL-2 cells at the indicated time points following 30 nM panobinostat treatment. There was no correlation between the reduction in WT1 and MCL1 level in a time-dependent manner, suggesting that the decrease in MCL1 level in Figure 2A is attributable to a mechanism independent of WT1 (D) Volcano plot expressing down or upregulated genes after panobinostat treatment for 6 h. (E) Gene set enrichment

analysis results about P53 signaling pathway. Cells were exposed to panobinostat or DMSO for 6 h (n=3).

Figure 3. Identification of apoptosis following panobinostat treatment or venetoclax in BFL1 +/- cell lines (A) Identification of the apoptosis pathway following treatment of panobinostat in DLBCL cell lines. Each cell line was treated with panobinostat for 24 h. (B) Identification of the apoptosis pathway following treatment of venetoclax in DLBCL cell lines. Each cell line was treated with venetoclax for 24 h. (C) Induction of apoptosis following treatment of panobinostat in DLBCL cell lines. Each cell line was treated with panobinostat for 24 h. (D) Graph showing the percentage of apoptotic cells in vitro treated with different doses of panobinostat. The number of early apoptotic cells increased at 10 nM but decreased at 30 nM, indicating that the early apoptotic stage cells moved into the late apoptotic stage in SUDHL-2. Error bars indicate S.D for n = 3. *≤ 0.01, ***≤ 0.0001, ns>0.10.

Figure 4. DLBCL cell lines' vulnerability to HDAC inhibitors depends on BFL1 (A) Gene set enrichment analysis results about negative regulation of cell development-related gene-sets after BFL1 knockdown. (B) Proliferation assay after 72 h from venetoclax treatment using WST in transduced cells. (C) Proliferation assay after 72 h from panobinostat treatment using WST in transduced cells. (D) Calculated log IC50 values at 72 h after panobinostat treatment. * ≤ 0.01, ** ≤ 0.001, *** ≤ 0.0001. Error bars indicate S.D for n = 6 (B and C).

Figure 5. Apoptotic induction by HDAC inhibitor depending on BFL1 existence (A) Identification of the apoptogenic molecules following panobinostat treatment between BFL1 shRNA and scrambled transduced cells. (B) Analyzed apoptotic cells

by Annexin V and PI staining using flow cytometry after panobinostat treatment (0, 10, and 30 nM) for 24 h (C) Graph showing the percentage of apoptotic cells in vitro treated with different doses of panobinostat. Error bars indicate S.D for n = 3. * ≤ 0.05, *** ≤ 0.0001, ns>0.10.

Figure 6. The most important HDACs for BFL1 inhibition (A) Proliferation assay after 72 h from vorinostat treatment using WST in transduced cells. (B) No WT1, BFL1 decrease and NOXA increase after vorinostat treatment in transduced cells. (C) No WT1, BFL1 decrease and NOXA increase after vorinostat treatment in DLBCL cells.

Figure 1.

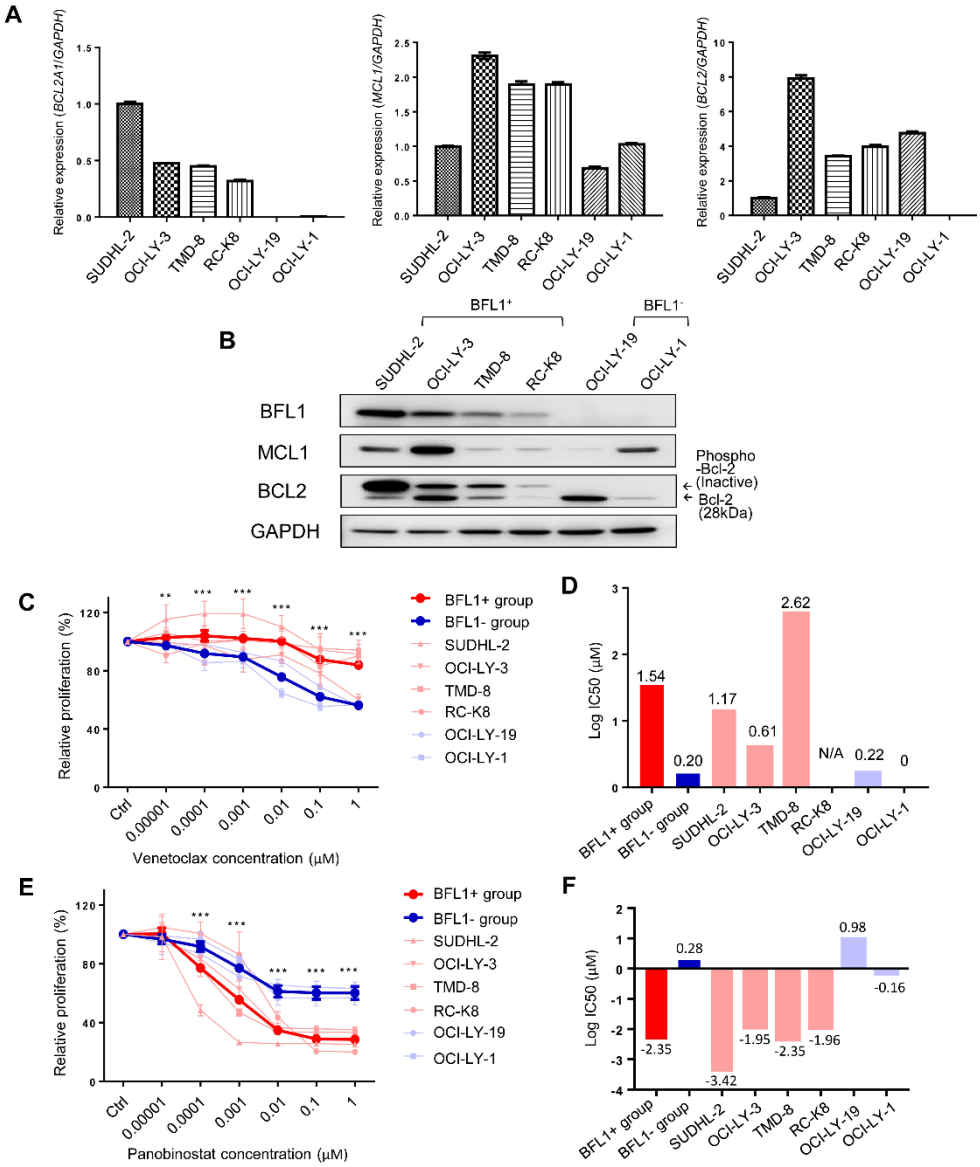

A
Relative expression (BCL2A1/GAPDH)
Relative expression (MCL1/GAPDH)
Relative expression (BCL2/GAPDH)
SUDHL-2
OCI-LY-3
TMD-8
RC-K8
OCI-LY-19
OCI-LY-1
B
BFL1+
BFL1-
BFL1
MCL1
BCL2
GAPDH
Phospho -Bcl-2 (Inactive)
Bcl-2 (28kDa)
C
Relative proliferation (%)
Venetoclax concentration (μM)
Ctrl
0.00001
0.0001
0.001
0.01
0.1
1
BFL1+ group
BFL1- group
D
Log IC50 (μM)
1.54
0.20
1.17
0.61
2.62
N/A
0.22
0
E
Panobinostat concentration (μM)
F
-2.35
0.28
-3.42
-1.95
-2.35
-1.96
0.98
-0.16

Figure 2.

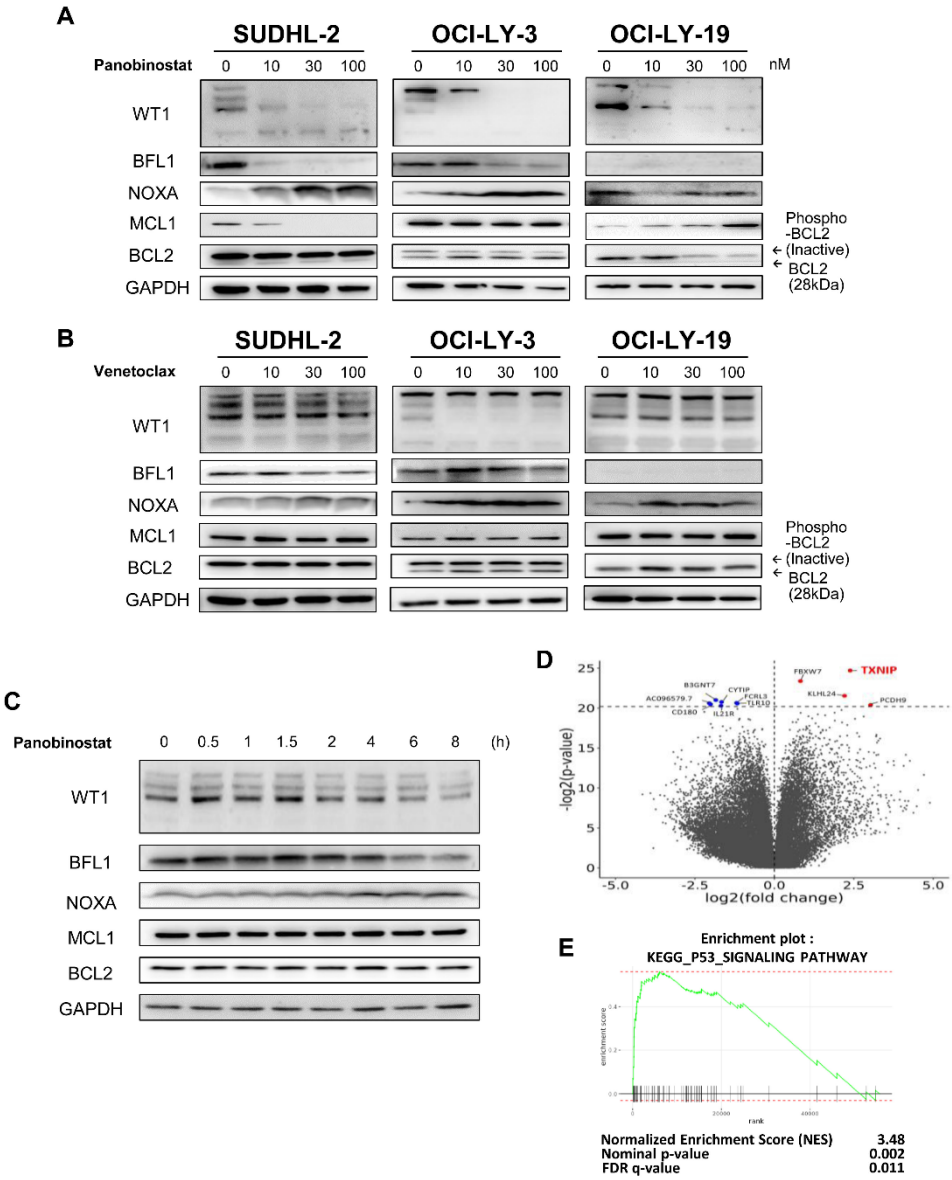

A
SUDHL-2
OCI-LY-3
OCI-LY-19
Panobinostat
0 10 30 100
nM
WT1
BFL1
NOXA
MCL1
BCL2
GAPDH
Phospho -BCL2 (Inactive)
BCL2 (28kDa)
B
Venetoclax
C
0 0.5 1 1.5 2 4 6 8 (h)
D
TXNIP
-log2(p-value)
log2(fold change)
E
Enrichment plot :
KEGG_P53_SIGNALING PATHWAY
Normalized Enrichment Score (NES) 3.48
Nominal p-value 0.002
FDR q-value 0.011

Figure 3.

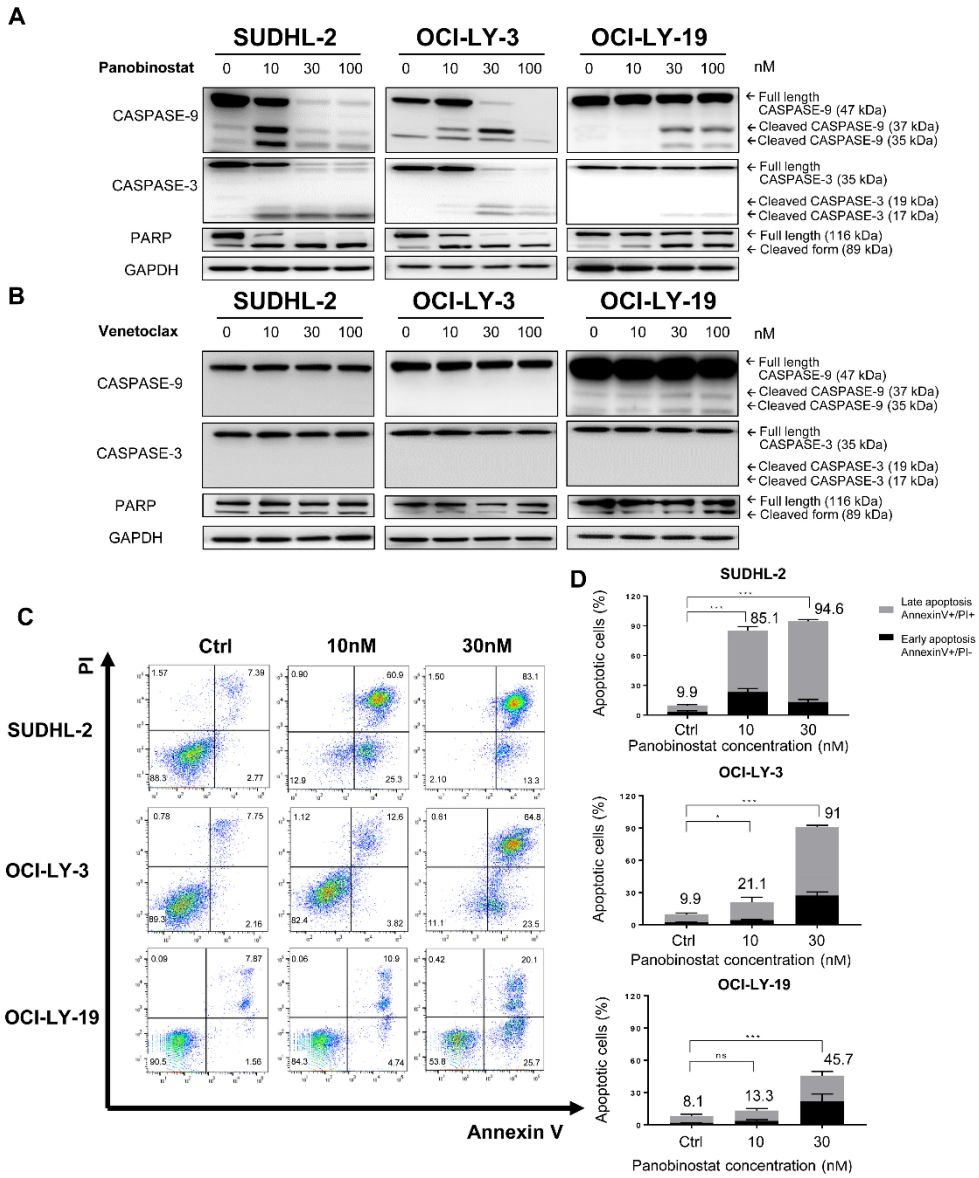

A
SUDHL-2
OCI-LY-3
OCI-LY-19
Panobinostat
0 10 30 100
nM
CASPASE-9
CASPASE-3
PARP
GAPDH
Full length CASPASE-9 (47 kDa)
Cleaved CASPASE-9 (37 kDa)
Cleaved CASPASE-9 (35 kDa)
Full length CASPASE-3 (35 kDa)
Cleaved CASPASE-3 (19 kDa)
Cleaved CASPASE-3 (17 kDa)
Full length (116 kDa)
Cleaved form (89 kDa)
B
Venetoclax
C
Ctrl
10nM
30nM
PI
Annexin V
D
Apoptotic cells (%)
Panobinostat concentration (nM)
Late apoptosis AnnexinV+/PI+
Early apoptosis AnnexinV+/PI-
9.9
85.1
94.6
21.1
91
8.1
13.3
45.7

Figure 4.

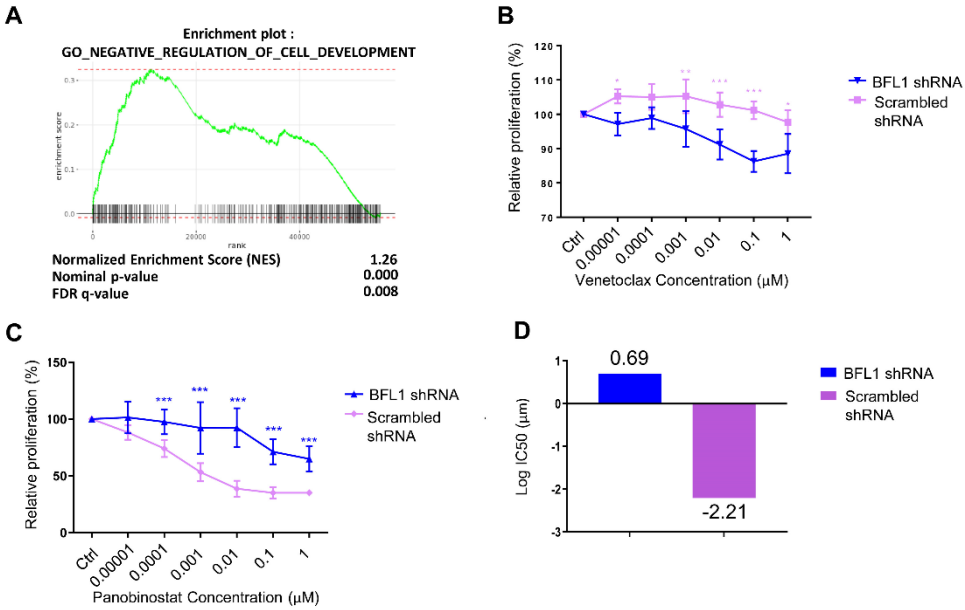

A
Enrichment plot :
GO_NEGATIVE_REGULATION_OF_CELL_DEVELOPMENT
Normalized Enrichment Score (NES) 1.26
Nominal p-value 0.000
FDR q-value 0.008
B
Relative proliferation (%)
Venetoclax Concentration (µM)
BFL1 shRNA
Scrambled shRNA
C
Relative proliferation (%)
Panobinostat Concentration (µM)
BFL1 shRNA
Scrambled shRNA
D
Log IC50 (µm)
0.69
-2.21
BFL1 shRNA
Scrambled shRNA

Figure 5.

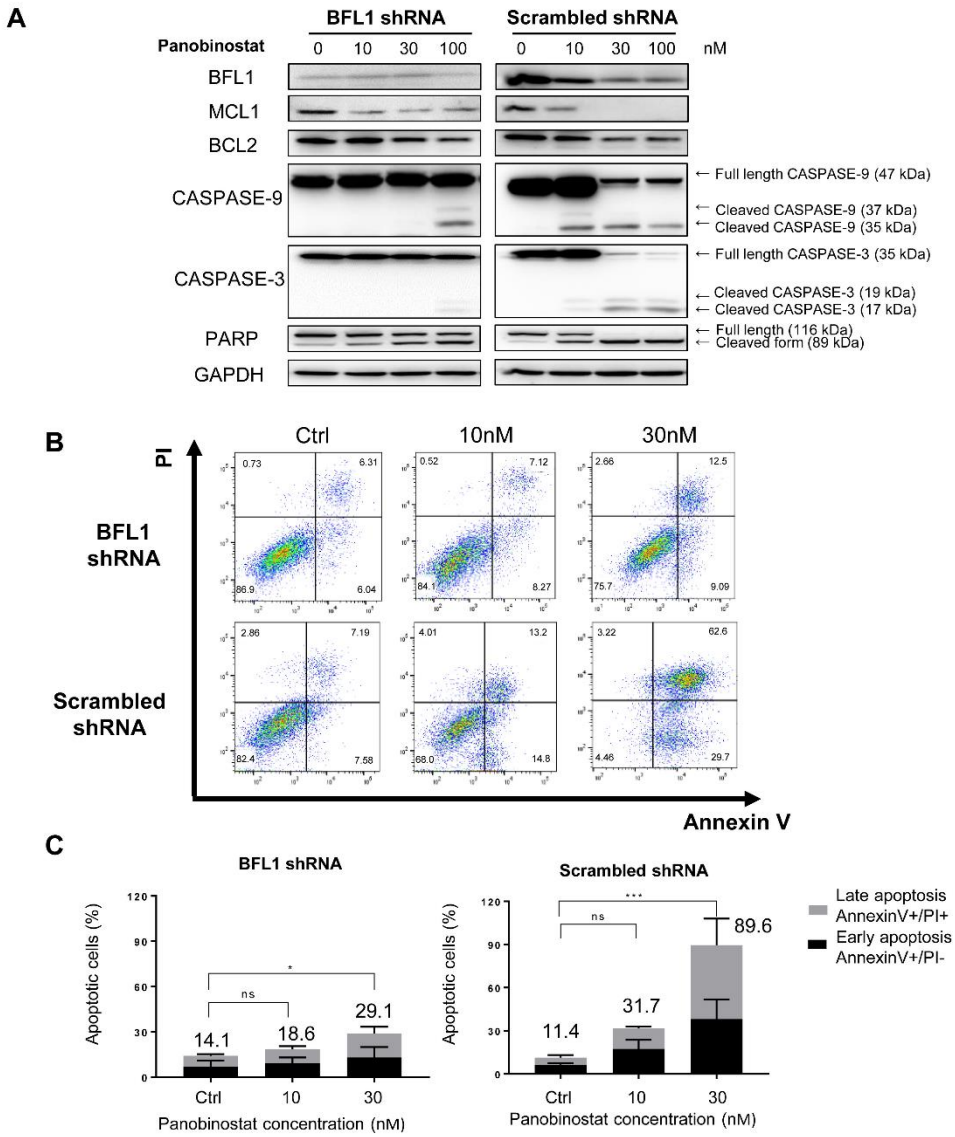
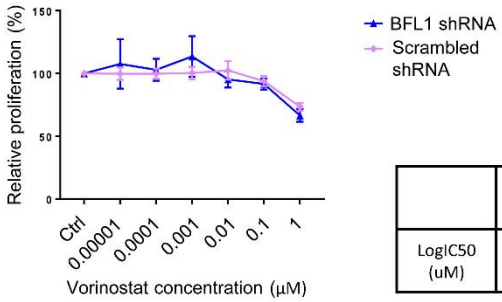

A
BFL1 shRNA
Scrambled shRNA
Panobinostat
0 10 30 100
0 10 30 100
nM
BFL1
MCL1
BCL2
CASPASE-9
CASPASE-3
PARP
GAPDH
← Full length CASPASE-9 (47 kDa)
← Cleaved CASPASE-9 (37 kDa)
← Cleaved CASPASE-9 (35 kDa)
← Full length CASPASE-3 (35 kDa)
← Cleaved CASPASE-3 (19 kDa)
← Cleaved CASPASE-3 (17 kDa)
← Full length (116 kDa)
← Cleaved form (89 kDa)
B
Ctrl
10nM
30nM
PI
BFL1 shRNA
Scrambled shRNA
Annexin V
C
BFL1 shRNA
Scrambled shRNA
Apoptotic cells (%)
ns
*
***
14.1
18.6
29.1
11.4
31.7
89.6
Late apoptosis AnnexinV+/PI+
Early apoptosis AnnexinV+/PI-
Ctrl
10
30
Panobinostat concentration (nM)

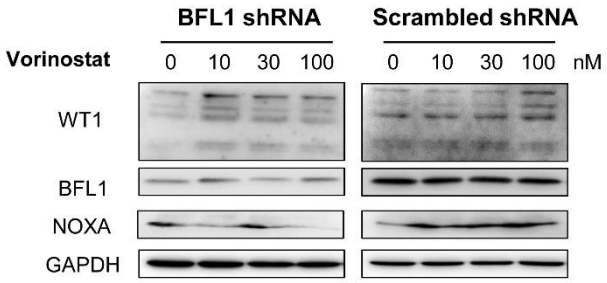
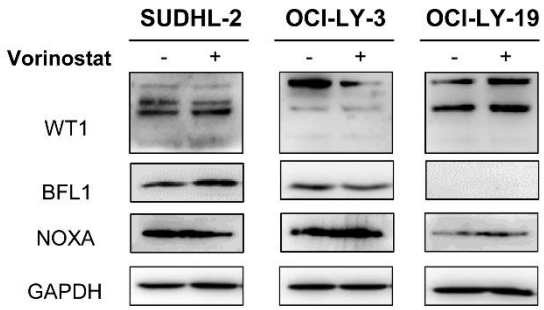
Figure 6.

A



	BFL1 shRNA	Scrambled shRNA
LogIC50 (uM)	0.389	0.523

B



C