

Investigating the impact of CFT7455 on activating pro-apoptotic genes, promoting apoptosis and treating Diffuse Large B-Cell Lymphoma

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Abstract. Diffuse Large B-Cell Lymphoma (DLBCL) usually occurs due to an overexpression of BCL6 protein. However, the indirect inhibition of BCL6 remains challenging. This work mainly aims to investigate the impact of BCL6-targeted PROTAC medicine--CFT7455 on degrading BCL6 and removing DLBCL tumour cells. By using SU-DHL-4 cell line and treating these cells with CFT7455 in different concentrations with different treatment durations, CFT7455' function could be tested. MTT assay is used to test cell viability. And Western Blot is used to detect BCL6 protein level. TUNNEL assay and Annexin V/PI assay are used to validate apoptosis. The results could show that CFT7455 might induce potent, dose-and time-dependent BCL6 degradation. This degradation would lead to a significant decrease in cell activities and an increase in apoptosis at the same time. These findings may suggest the potential of CFT7455 as a treatment of BCL6-driven DLBCL, promoting the further development of it.

Keywords: PROTACs, BCL6, Diffuse Large B-Cell Lymphoma, Targeted protein degradation, Apoptosis

1. Introduction

DLBCL is now one of the most common happening types of lymphoma, accounting for about 25% to 30% of all the non-Hodgkin lymphomas. This disease triggers healthy B cells to change into fast-growing cancer cells and they become incapable of fighting off pathogens. It clinically expresses as rapidly growing mass or enlarging lymph nodes in a nodal or an extranodal site. There are several normal causes of DLBCL, including rise in age, genetic mutations, infections with virus like HIV, weakened immune power, increased BMI and exposure to toxic substances. This disease is commonly happening around the worldwide, with a data in 2021 illustrates that there are 77240 new cases of DLBCL estimated in only US [1]. One of the most common treatments for DLBCL currently is R-CHOP (cyclophosphamide/doxorubicin/vincristine/prednisone) [2]. This therapy binds the monoclonal antibody drug rituximab with three chemotherapy drugs and a corticosteroid. Though the safety and efficacy of this treatment, it doesn't always work. Studies present that relapses or recurrent cancer could affect roughly 30%-40% of the people with DLBCL who received R-CHOP [3]. However, targeting its oncogenic drivers like BCL6 may be highly effective in treating

this particular cancer, promoting more a more effective way of treating DLBCL other than R-CHOP [4].

BCL6 is a transcriptional repressor which overexpresses in 40%-50% of the DLBCL cases [5]. This protein controls many vital aspects of immune cell development and functions. It inhibits pro-apoptotic genes (e. g. p53, PUMA) and other DNA damage sensors, preventing the genetic process of apoptosis to clear out the cancer cells [6]. The BCL6 protein consists of a trimodular structure comprise an N-terminal BTB/POZ domain, a central transcriptional repressor domain containing a PEST (proline, glutamic acid, serine and threonine) region (RD2-PEST domain); and C-terminal Zinc finger domain [7]. N-terminal BTB/POZ domain contains a charged pocket targeted by inhibitors, RD2-PEST domain is critical for transcriptional repression of target genes like tp53, and mutations in C-terminal Zinger Finger Domain disrupt BCL6's oncogenic function in lymphoma. Despite its therapeutic relevance, direct BCL6 inhibition remains challenging. Among other kinds of treatment provided currently which pertain to inhibition of overexpressed BCL6 gene, small molecule inhibitors (e. g. BI-3802) totally block co-expressor binding, preventing the interaction of the BTB/POZ domain of the BCL6 with several co-expressors. Meanwhile, they do not reduce BCL6 levels and resistance may occur. In addition, other current BCL6-targeted strategies like transcriptional modulators (e. g. HDAC inhibitors) indirectly suppress BCL6 which leads to off-target toxicity. Considering all the limitations of those current therapies, PROTAC (Proteolysis-targeting chimera) technology may offer a better solution by inducing targeted protein deration, inactivating BCL6 protein so the pro-apoptotic genes will be promoted again.

Proteolysis-targeting chimeras (PROTACs) are engineered techniques for targeted protein deration. A bifunctional PROTAC molecule with two covalently-linked ligands recruits target protein and E3 ubiquitin ligase together to trigger the degradation of target protein by the ubiquitin-proteasome system. This method has several advantages over aforementioned therapies. Firstly, there are many routes for administration, including intravenous injection, Peros and subcutaneous injection and these routes are all able to work. Also, catalytic action is taken out by this method effectively without requirement of active sites, eliminating scaffolding and pathogenic proteins significantly. Moreover, PTOTACs overcomes resistance to occupancy-driven inhibitors. Additionally, this therapy penetrates patients' tissue moderately and the delivery process is systemic.

CFT7455, as a VHL-recruiting BCL6-directed PROTAC, may distribute to the development of this therapy [8]. CFT7455 is a degrader consists of a structure contains a BCL6 binder and a ligand for the E3 ubiquitin ligase VHL. It can simultaneously bind BCL6 and VHL, promoting the ubiquitination of BCL6 and degradation by the proteasome. In SU-DHL-4 (a validated BCL6-driven cell line, this degrader could achieve over 90% of BCL6 degradation at nanomolar doses. Nevertheless, quantitative relationships between CFT7455 dose, exposure time, BCL6 degradation, and apoptotic response remain uncharacterized, which this research will try to address.

This research predicts that increasing concentrations and treatment durations with CFT7455 decrease viability of SU-DHL-4 DLBCL (Diffuse Large B-Cell Lymphoma) cells and decrease BCL6, and increases apoptosis.

2. Material method

2.1. MTT assay

MTT assay is used to measure the viability (the proportion of living cells in a sample) of the cells, relying on the metabolic activity of the cells. MTT is added to cell culture and living cells will reduce MTT (a yellow tetrazolium salt) into formazan (a purple insoluble crystal) via mitochondrial

enzymes [9]. The cell culture will be put in a same volume of solution of the SU-DHL-4 cells, the negative control is pbs/dmso and the positive control is BI-3802 (10 μ M). CFT7455 (0. 01nM, 0. 1nM, 1nM, 10nM, 100nM, 1000nM, 24-72h) will be added to show its effect on treating tumour cells. After MTT reagent is added for 4 hours under 37 degree Celcius, DMSO will be added to dissolve formazan formed in order to make it possible to be tested. Then, we'll measure absorbance (log10 (incident light/transmitted light) of lights of formazan at 570nm wavelength. Only metabolically active cells (living cells) can change MTT into formazan and dead or dying cells will lose mitochondrial function, which will not result in formazan production. The results can be used to calculate viability. The formula of it is $\text{Viability (\%)} = (\text{Absorbancetreated} / \text{Absorbancepbs/dmso}) \times 100$.

2.2. Western blot

Western blot is a technique to detect specific proteins' expression in a complex sample. We can use this method to detect the BCL6 protein level to investigate CFT7455's function. Firstly, SU-DHL-4 cells with DLBCL will be treated by CFT7455 (0. 01nM, 0. 1nM, 1nM, 10nM, 100nM, 1000nM, 24-72h) and both negative (pbs/dmso) and positive (BI-3802 (10 μ M)) controls. Then the proteins will be extracted using RIPA buffer. After that, we'll run a SDS-PAGE gel electrophoresis (90min, 100-120V) to separate proteins by molecular sizes, the BCL6 protein migrates near 95 kDa. Proteins will then be transferred to PVDF/nitrocellulose membrane by wet transfer (100V, 60min). Then we'll use BSA to prevent non-specific antibody binding (2 hours). Anti-BCL6 as the primary antibody and HRP-conjugated antibody as the secondary antibody will be added to ensure specificity to BCL6 (4°C, overnight). Next, the bands on the membrane will become visualized with exposure to X-ray film/ECL chemiluminescence and the intensity of the bands could be measured and compared using computer software's.

2.3. TUNNEL assay

TUNNEL assay is a technique that detects DNA fragmentation, which is a hallmark of apoptosis. It can label broken DNA ends in dying cells, allowing quantification of cells underwent apoptosis [10]. Firstly, SU-DHL-4 cells will be treated with negative control (pbs/dmso), positive control (BI-3802 (10 μ M)) and CFT7455 (0. 01nM, 0. 1nM, 1nM, 10nM, 100nM, 1000nM, 24-72h). After that, cells will be fixed in 4% paraformaldehyde (15min, RT) and then be permeabilized with 0. 1% Triton X-100 (10min, on ice). Afterwards, add TdT enzyme and FITC-dUTP and incubate the mixture (60min, 37°C, dark). Next, add DAPI to stain the nuclear into blue. After all these processes are done, count FITC+ nuclei (green) in at least 5 different random fields (≥ 50 cells present) under microscopy with 20 $\times$ objective. The level of apoptosis can be calculated by $\% \text{Apoptosis cells} = (\text{TUNNEL}^+ \text{ cells} / \text{Total DAPI}^+ \text{ cells}) \times 100$.

2.4. Annexin V/PI assay

Annexin V is a protein that binds to phosphatidylserine (a phospholipid normally inside the plasma membrane) [11]. During early phase of apoptosis, phosphatidylserine flips to the outer surface. Propidium Iodide (PI) is a DNA dye that enters cells only when membranes of the cells are damaged (late phase of apoptosis). Firstly, SU-DHL-4 cells will be treated with negative control (pbs/dmso), positive control (BI-3802 (10 μ M)) and CFT7455 (0. 01nM, 0. 1nM, 1nM, 10nM, 100nM, 1000nM, 24-72h). Then, floating cells which are detached from culture plate will be collected as late-stage

apoptotic cells. Along with adherent cells which are attached to plate surface, representing as early apoptotic cells. Afterwards, these harvest cells will centrifuge ($300\times g$, 5min), then wash with pbs. Next, resuspend cells in 100 μ L binding buffer (contains Ca^{2+} ions) and add Annexin V (5 μ L) and PI (5 μ L) to stain the cells. Thereafter, incubate the mixture for (15min, RT, dark). Subsequently, add 400 μ L binding buffer and analyze on flow cytometer within 1h. Excitation stage is performed using 488nm wavelength, Annexin V detection process will use 530/30nm wavelength and PI detection is run with 610/20nm wavelength. We will collect $\geq 10,000$ events per sample. Early apoptosis is illustrated with Annexin V+/PI- while late apoptosis is shown by Annexin V+/PI+. The total apoptosis can be calculated as (Early apoptosis Late apoptosis) %.

2.5. Statistical analysis

Each single experiment should be repeated for at least three independent biological replicates, defined as separate cell cultures treated and analyzed on different days. And for each biological replicate, technical triplicates will be included to account for intra-experiment variability. The data received from each experiment could be processed. The results of MTT viability could be concluded using $\text{Viability (\%)} = (\text{Absorbance treated} / \text{Absorbances/dmso}) \times 100$ and is measured by % negative control (pbs/dmso). The results (band intensity) from Western Blot could be processed by computer software's and presented as % control (pbs/dmso). The results from the two apoptosis assays could be shown as % of total cells.

3. Possible results table

Table 1. The performance of MTT assay, western blot, TUNNEL assay and Annexin V/PI assay of SU-DHL-4 cells treated with CFT7455

Combina tion Result# (CR#)	Increasing CFT7455 decreases viability by MTT	Increasing CFT7455 decreases BCL6 by western blot	Increasing CFT7455 increases apoptosis by TUNNEL	Increasing CFT7455 increases apoptosis by FACS for Annexin V/PI	Support of hypothesi s
1	+	+	+	+	Full
2	+	+	+	-	Partial
3	+	+	-	+	Partial
4	+	-	+	+	Partial
5	-	+	+	+	Partial
6	+	+	-	-	Partial
7	-	-	+	+	Partial
8	+	-	-	+	Partial
9	+	-	+	-	Partial
10	-	+	-	+	Partial
11	-	+	+	-	Partial
12	+	-	-	-	Partial
13	-	+	-	-	Partial
14	-	-	+	-	Partial
15	-	-	-	+	Partial
16	-	-	-	-	Fully Contradi cts

Table Legend: “+” indicates result supports hypothesis (viability decreases as CFT7455 increases, BCL6 presented decreases as CFT7455 increases and apoptosis increases as CFT7455 increases, according to the results when the cells are treated with negative control). “-” indicates result contradicts hypothesis, which involve the opposite trends or no significant change, according to the results when the cells are treated with negative control).

CR1: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis increases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR2: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis increases only in TUNNEL but decreases in Annexin V/PI with increasing [CFT7455].

CR3: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis increases only in Annexin V/PI but

decreases in TUNNEL with increasing [CFT7455].

CR4: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis increases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR5: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis increases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR6: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis decreases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR7: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis increases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR8: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis increases only in Annexin V/PI but decreases in TUNNEL with increasing [CFT7455].

CR9: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis increases only in TUNNEL but decreases in Annexin V/PI with increasing [CFT7455].

CR10: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis increases only in Annexin V/PI but decreases in TUNNEL with increasing [CFT7455].

CR11: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis increases only in TUNNEL but decreases in Annexin V/PI with increasing [CFT7455].

CR12: If I obtain this result, I would see viability decreases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis decreases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR13: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein decreases with increasing [CFT7455], apoptosis decreases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

CR14: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis increases only in TUNNEL but decreases in Annexin V/PI with increasing [CFT7455].

CR15: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis increases only in Annexin V/PI but decreases in TUNNEL with increasing [CFT7455].

CR16: If I obtain this result, I would see viability increases with increasing [CFT7455], level of BCL6 protein increases with increasing [CFT7455], apoptosis decreases in both TUNNEL and Annexin V/PI with increasing [CFT7455].

For MTT assay, viability may decrease more with an increasing time period for CFT7455 treatment, and viability may decrease more results from increasing concentration of treatment. For Western Blot, BCL6 protein level may decrease more because of increasing concentration of treatment, but it may not decrease more significantly if the duration of CFT7455 treatment is over 24h. For TUNNEL assay, apoptosis may increase more effectively as the concentration of the treatment increases. And as the duration of the treatment increases to over 48h, the apoptosis might

increase significantly. For Annexin V/PI assay, apoptosis may increase more effectively as the concentration of the treatment increases. And as the duration of the treatment increases to over 48h, the apoptosis might increase significantly.

4. Discussion

CR1: This result will suggest that an increasing concentration and treatment of CFT7455 will have an effect on inhibiting the viability of SU-DHL-4 cells, avoiding the overexpression of BCL6 protein and increasing apoptosis process due to the successful function carried out by the degrader CFT7455. An increasing concentration of CFT7455 bind with BCL6 and VHL, promoting the degradation of BCL6 protein to achieve the refuction of apoptosis process controlled by the pro-apoptotic genes (e. g. p53, PUMA). So the proportion of living SU-DHL-4 cells (viability) decreases as a result of the treatment, along with the decreasing present of BCL6 protein. Finally, apoptosis takes place more frequently and effectively, giving positive results in both TUNNEL assay and Annexin V/PI assay. This result fully supports the hypothesis, however, to make it more trustworthy, future experiments should be carried out to test other cell lines involve function of BCL6 protein presented other than SU-DHL-4.

CR2: This result will suggest that an increase in apoptosis caused by an increasing concentration and treatment of CFT7455 will be clearly indicated by TUNNEL assay but Annexin V/PI assay doesn't support the hypothesis. While other two experiments also support the hypothesis. This combination result partially supports the hypothesis. The possible causes of this result might be the necroptosis via RIPK3/MLKL activation which causes DNA fragmentation but there is a lack of phosphatidylserine (PS) exposure [12]. Future experiments could be carried out in order to eliminate the possible causes of this result and test the hypothesis further. We could carry out MLKL Phosphorylation Western Blot to detect p-MLKL (phospho-S358) in CFT7455-treated cells in order to verify necroptosis activation [13].

CR3: This result will suggest that an increase in apoptosis caused by an increasing concentration and treatment of CFT7455 will be clearly indicated by Annexin V/PI assay but TUNNEL assay doesn't illustrate the result that supports the hypothesis. While other two experiments also support the hypothesis. This combination result partially supports the hypothesis. The possible causes of this result might include Caspase-independent apoptosis driven by AIF (apoptosis-inducing factor) or cathepsins, which causes PS exposure without DNA fragmentation [14]. Future experiments like Mitochondrial Membrane Potential assay could be carried out to confirm caspase-independent apoptosis [15].

CR4: This result will suggest that the presence of BCL6 protein won't go down as a result of an increasing concentration and treatment of CFT7455, but the apoptosis does increase which leads to a decrease in viability of the cells. This combination result partially supports the hypothesis. The possible causes of this combination result might include off-target toxicity of CFT7455, it may degrade other non-BCL6 proteins like BCL2 or MYC [16]. Several future experiments could be carried out test the hypothesis further. We could carry out Global Proteomics (TMT-MS) to measure quantitative mass spectrometry of CFT7455-treated vs. control cells to identify off-target degraded proteins.

CR5: This result will suggest that though apoptosis is increased effectively by decreasing the presence of BCL6 proteins, activating pro-apoptotic genes which is achieved with an increasing concentration and treatment of CFT7455. However, the viability of the cell increases. This combination result supports the hypothesis partially. There are some possible causes of this situation. Compensatory proliferation might happen via Wnt/ β -catenin pathway, the surviving cells

among all the cells might divide faster which cover the rapid dying of SU-DHL-4 cells triggered successfully by apoptosis [17]. Future experiments could be made to check the hypothesis further. We could use EdU incorporation to measure proliferation rates, which verifies the effect of an increasing concentration and treatment of CFT7455 on cell viability [18].

CR6: This result will suggest that though the viability of the cells decreases as a result of the increasing concentration and treatment of CFT7455 and CFT7455 successfully degrades BCL6 proteins, the apoptosis does not increase indeed during the experiments. This combination result partially supports the hypothesis. The possible causes of this result might be non-apoptotic death (autophagy/necrosis) instead of apoptosis. Or the BCL6 degradation will take more than 72 hours to trigger apoptosis of the cells. Future experiments could be carried out to check the hypothesis further. We could utilize LDH release assay to quantify necrosis or repeat the same experiments but with extended time course (96, 120h).

CR7: This result will suggest that only apoptosis increases as a result of an increasing concentration and treatment of CFT7455. But the viability does not decrease and also the BCL6 proteins' presence. This combination result partially supports the hypothesis. This could be caused by ferroptosis-induced membrane damage via lipid peroxidation [19]. Future experiments could be used to test that further. C11-BODIPY Lipid Peroxidation assay to measure fluorescence shift in order to detect ferroptosis.

CR8: This result will suggest that viability of the cells decrease as a result of an increasing concentration and treatment of CFT7455 but BCL6 proteins does not degrade. And the apoptosis only increases in Annexin V/PI assay. This combination result partially supports the hypothesis. There are several possible causes of this result. C-MYC and BIM proteins may cause background apoptosis [20]. Future experiments could be carried out to test the hypothesis further. C-MYC Knockdown might be carried out to test if background apoptosis is MYC-driven [21]. We could transfect siRNA-MYC and then repeat apoptosis assays to obtain that.

CR9: The result will suggest that the viability decreases as a result of an increasing concentration and treatment of CFT7455. And only the apoptosis measured by TUNNEL assay has increased. This combination result partially supports the hypothesis. There are several possible causes, including Topoisomerase II inhibition which would lead to DNA damage without BCL6 involvement [22]. Future experiments could be carried out to eliminate the possible causes. γ H2AX staining could be carried out to validate DNA damage independent of apoptosis [23].

CR10: The result will suggest that the presence of BCL6 proteins decreases and apoptosis measured by Annexin V/PI assay increases which support the hypothesis. The other two results of the experiments contradict. This combination results partially support the hypothesis. There are several possible causes like reversible apoptosis, meaning that there might be PS exposure but without commitment to death via FLIP-mediated caspase-8 inhibition [24]. Also, BCL6 degradation in subsets may elicit heterogeneous cell response, avoiding completion of the result from the apoptosis assays and so the MTT assay. Future experiments could be carried out to eliminate those possible causes. Caspase-8 Activity assay could be used use IETD-sfc substrate and then measure fluorescence, which can be used to test for FLIP-mediated caspase-8 inhibition [25]. And single-cell Western blot could be used to measure BCL6 in Annexin V positive cells.

CR11: The result will suggest that the presence of BCL6 proteins decreases and apoptosis measured by TUNNEL assay increases as a result of an increasing concentration and treatment of CFT7455. However, the viability doesn't decrease and apoptosis measured by Annexin V/PI assay. This combination result partially supports the hypothesis. There are several possible causes of this situation. DNA fragmentation may occur via RIPK3/MLKL pathway which leads to necroptosis.

Future experiments could be carried out to eliminate those possible causes, including RIPK3/MLKL Co-localization which is used to confirm necroses formation [26].

CR12: The result will suggest that only viability of the cells decrease as a result of an increasing concentration and treatment of CFT7455 while the presence of BCL6 proteins being not decreased and apoptosis is not increased. This combination result partially supports the hypothesis. This suggests that CFT7455 may have p-27-mediated cytostatic arrest effect on SU-DHL-4 cells but no cell death is triggered [27]. Future experiments could be carried out to test it further. P27 Western Blot could be carried out to measure p27 protein levels so to detect the cytostatic effect had on the cells.

CR13: This result will suggest that only presence of BCL6 proteins decreases as a result of an increasing in concentration and treatment of CFT7455 while apoptosis is not increased and viability of the cells is not decreased. This combination result partially supports the hypothesis. The possible cause of this situation might be functional redundancy caused by compensatory proteins (e. g. BCL2) [28]. Otherwise, the degradation of BCL6 proteins might be ineffective which retains oncogenic function after they become fragments. So, the function of BCL6 to promote pro-apoptotic genes might fail to work. Future experiments could be used to eliminate those possible causes and test the hypothesis further. Co-IP/MS assay could be used to identify BCL6-interacting proteins and BCL2 Western Blot can be used to identify compensatory survival pathways by quantifying anti-apoptotic protein expression.

CR14: This result will suggest that an increasing concentration and treatment of CFT7455 will not result in a decrease in viability and level of BCL6 proteins presented. Only the apoptosis measured in the TUNNEL assay increases. This combination result partially supports the hypothesis. The possible causes of this situation might include replication stress elicited by non-apoptotic DNA breaks via ATR/Chk1 activation [29]. Future experiment could be carried out to eliminate the impacts of those possible causes and test the hypothesis further. ATR/Ch1 inhibition could be carried out to confirm replication stress by treating cells with ATRi or Chk1i and repeating TUNNEL assay.

CR15: This result will suggest that an increasing concentration and treatment of CFT7455 won't result in a decrease in viability and level of BCL6 proteins presented. Only the apoptosis measured in the Annexin V/PI assay increases. This combination result partially supports the hypothesis. The possible causes of this result might include TMEM16F-mediated phospholipid scrambling which mimics apoptosis [30]. Future experiments could be carried out to eliminate these possible causes and test the hypothesis further. TMEM16F Knockout could be carried out to test phospholipid scrambling dependency by using CRISPR-Cas9 to validate with DNA sequencing.

CR16: This result will suggest that none of the experiments illustrate the ideal results. This combination result fully contradicts the hypothesis. This might be caused by the HSP90 stabilization of BCL6 [31]. Also, SU-DHL-4 may have defective ubiquitin-proteasome system which might be PSMB5 mutation. Future experiments could be carried out to test the hypothesis further. Proteasome Activity assay could be used to check proteasome function by using Suc-LLVY-AMC substrate to measure fluorescence [32].

We interpret that an increasing concentration of CFT7455 would have a positive impact on showing the ideal results of the experiments as CFT7455 acts directly on BCL6 degradation and effects at low concentration ($\leq 10\text{nM}$) suggest high target selectivity. Future experiments could be done to check the impact of concentration on hypothesis. For example, TPP (Thermal Proteome Profiling) could identify off-target engagement at high doses ($>100\text{nM}$) and determine EC₅₀ ($\approx 10\text{nM}$) by fitting dose-response curves for BCL6 degradation (Western blot) and apoptosis (TUNNEL assay) could validate its impact if the curves are in sigmoidal shapes. As for treatment

duration, effects intensify with an increase in time since that BCL6 degradation happens early and apoptosis then occurs at a later stage. It can validate PROTAC mechanism if a decrease in level of BCL6 proteins occurs before the decrease in viability and the increase in apoptosis. Future experiments including time-resolved RNA-seq to track pro-apoptotic gene expression hourly after treatment and pulse-chase assays to wash out CFT7455 after treatment to measure BCL6 recovery and apoptosis reversal.

However, negative results could be attained as the dose of treatment goes above the threshold. In this case, cellular uptake assay could be done to quantify intracellular CFT7455 and proteasome inhibition could be used to test if chemicals (MG-132) would block BCL6 degradation at threshold doses and treatment durations.

5. Conclusions

The results of the research may help us to come up with a better way of PROTACs to treat DLBCL, translating CFT7455 treatment into a method of DLBCL therapy.

This research tests the hypothesis that an increasing concentration and treatment of CFT7455 will reduce the viability and BCL6 protein levels so increase the apoptosis in SU-DHL-4 cells. The results of this research are collected from four experiments, consisting of MTT assay, Western blot, TUNNEL assay and Annexin V/PI assay, which might confirm that BCL6 degradation will drive the reactivate of pro-apoptotic genes and promote the increase in apoptosis. And catalytic PROTAC efficiency enables sub-stoichiometric activity at nanomolar doses. What's more, the result may validate the kinetic progression of the whole mechanism. Despite the limitations of CFT7455 the result of the experiments might show, these findings might establish CFT7455 as a superior alternative to BCL6 inhibitors and other current treatments of DLBCL since it eliminates BCL6 oncogenic functions without blocking it totally while overcoming resistance in R-CHOP-refractory DLBCL models. These findings may provide an better alternative of therapy for treating DLBCL and explore more potentials in the PROTACs technology field. More future work still needs to be done like toxicity profiling and in vivo experiments of CFT7455 to check the function of it further.

In conclusion, this work pioneers a PROTAC-based strategy for aggressive lymphomas, potentially extending to other BCL6-driven cancers. If this could be done successfully, the number of patients suffer from aggressive lymphomas, specially DLBCL could be greatly reduced. And by quantitatively linking target degradation to phenotypic outcomes, we provide a blueprint for precision oncology.

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