Overexpression of MCL-1 provides OCI-Ly1 with resistance to Venetocalx

Xinchi Zhang

Abstract

Overexpression of MCL-1 is an antiapoptotic member of the BCL-2 family and has been seen in various human tumors, correlating with the patient's poor prognosis. This study tested whether cancers overexpress MCL-1 to gain resistance to venetoclax. The expression of MCL-1 protein was measured by Western blot. Cell viability after venetoclax and MCL-1 inhibitor AZD5991 was added was measured by MTT. FP-based binding assays measured Venetoclax's binding affinity for MCL-1. The result of the study would give insights into venetoclax resistance and the overexpression of MCL-1 in NHL OCI-Ly1. Future studies should focus on new drugs that can circumvent this resistance if the hypothesis is correct.

Keywords-MCL-1, Venetoclax, OCI-Ly1

I. Introduction

About 10 million fatalities were caused by cancer in 2020, causing cancer to rank the highest on the list of global cause of death [1]. Cancer is a generic term for a large group of diseases. One distinguishing characteristic of it is the quick development of abnormal cells that expand beyond their normal bounds, infiltrate nearby body tissues, and spread to other organs [1]. The primary cause of death from cancer is the spread of dysfunctional cancer cells, which prevent healthy cells and systems from operating normally.

A type of cancer was Non-Hodgkin's lymphoma (NHL). NHL is a heterogeneous group of lymphoproliferative malignancies and is the sixth most common cause of death related to cancer in the USA [2]. It is a cancer that starts in lymphocytes, a component of the lymphatic system. More than 85% of Non-Hodgkin's lymphomas derive from B cells, while others derive from T cells or NK cells [3]. The affected lymphocytes lose their functions, rendering the patients vulnerable to infections. The most common symptom of NHL is a painless swelling in lymph nodes [4]. Usually, overcrowding of cells would trigger apoptosis. The term 'apoptosis' was first coined by Kerr, Wyllie, and Currie in 1972 [5]. It is used to describe the process of programmed cell death which get rid of cells that were damaged beyond repair [6]. Apoptosis is crucial because it allows tissues to maintain homeostasis [5].

The B cell lymphoma 2 (BCL-2) family –a set of proteins that control the integrity of the mitochondria – plays an important role in apoptosis [5]. The family is subdivided according to their functions and contributions to the progression of apoptosis. These sub-groups are proapoptotic proteins, antiapoptotic proteins, and BH3-only proteins [5]. Proapoptotic proteins include BAK and BAX. Antiapoptotic proteins include BCL-2, BCL-XL, BCL-B, MCL-1, etc. BH3-only proteins include BIM, BAD, BID, NOXA, and PUMA [7]. A cell's trajectory towards apoptosis is determined by the balance between these protein groups.

Through the hydrophobic groove, BCL-2 sequesters the proapoptotic proteins BAK/BAX to perform its antiapoptotic function [8]. In the antiapoptosis mode, BAK/BAX interacts with antiapoptotic proteins (Figure 1A). The interaction between the two proteins prevents BAK/BAX, the proapoptotic proteins, from executing the apoptotic program, therefore enabling the cells to maintain homeostasis [8]. In the proapoptotic mode, BH3-only proteins are acted upon by stress signals or other intrinsic pathways [8]. BH3-only proteins bind with antiapoptosis proteins, releasing BAK/BAX [8]. After dissociating from antiapoptotic proteins, BAK/BAX exposes its BH3 domain and forms an oligomer [8]. By rupturing the lipid bilayer which forms the outer membrane of the mitochondria, the oligomers cause mitochondrial herniation and DNA efflux. This is followed by the release of cytochrome c and activation of initiator caspases which dismantles the cell's components(Figure 1B) [8].

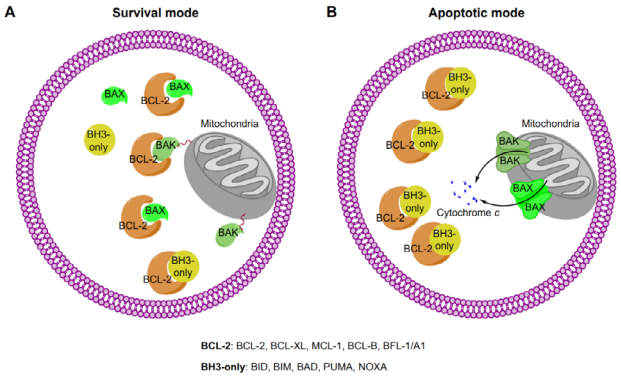

Figure 1. Proapoptotic and antiapoptotic protein interactions [8]

Great efforts have been directed toward developing small molecules or BH3 mimetics, molecules which mimic the functions of certain BH3-only proteins, to inhibit the interaction between BAX/BAK and antiapoptotic family proteins, thus promoting apoptosis in cancer cells [9]. These medicines regulate the apoptotic process and balance the proapoptotic and antiapoptotic proteins [8]. Take the inhibitor venetoclax for an example, it is a BH3 mimetic that can bind to the BH3-binding groove of BCL-2. The binding of venetoclax competitively inhibits the interaction between BCL-2 and BAX/BAK, therefore releasing BAX/BAK, and eventually leading to apoptosis in cancer cells [9]. Other drugs targeting BCL-2 include navitoclax, A-1155463, and A-1331852 [7].

However, all of the BCL-2 family inhibitors described above performed poorly on cancers with Myeloid Cell Leukemia 1 [7,10]. Myeloid Cell Leukemia 1(MCL-1) is also an antiapoptotic member of BCL-2 family proteins. It is extensively expressed in human tissues. The majority of its expression is found in the mitochondria of cells, where its hydrophobic tail penetrates the mitochondrial membrane [8]. Observations regarding the overexpression and amplification of MCL-1 in tumors such as lung cancer, pancreatic cancer, and breast cancer are countless. According to an investigation of 3,131 cancer specimens, MCL-1 expression soared in 36% of breast cancers and 54% of lung cancers [8].

Would MCL-1 be overexpressed in an environment full of BCL-2 inhibitors? If so, would the overexpression of antiapoptotic protein MCL-1 in these cancers be the cause of their resistance to venetoclax? To test the hypothesis that "Non-Hodgkins Leukemia OCI-Ly1 cancer cells will overexpress MCL-1 in response to increasing amounts of venetoclax treatment and MCL-1 would render the cells resistant to venetoclax.", venetoclax-resistant cancer cells were first cultured. Then, western blot was implemented to determine the MCL-1 protein expression. MCL-1 inhibitor AZD5991 with venetocalx was then added to replicates of the cultured cells. MTT was used to determine cell viability. Finally, fluorescence polarization-based binding assays were done to determine the binding affinity between venetoclax and MCL-1.

II. Methods

A. Material

Venetoclax (ABT-199/GDC-0199), NHL cell lines OCI-Ly1, IMDM (Invitrogen Corp., Grand Island, NY) supplemented with 10% human serum (Sigma), MCL-1 gene, MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MCL-1 inhibitor AZD5991, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488), Anti-MCL-1 antibody (ab28147), Human HeLa cells

B. Cell culture and cell-based assays

NHL cell line OCI-Ly1 were cultured in IMDM at 36.5°C with 6.5% CO_2 [11]. Cells' resistance to venetoclax was developed by exposing the them to venetoclax. The concentration of the drug starts at a sub-lethal level and gradually increases throughout the months [11]. A minimum venetoclax concentration of 2 μM was maintained to sustain selection pressure [11]. A negative control in which cells were cultured in PBS/DMSO was implemented. A positive control could not be added as there were no studies prior to this one, making it impossible to make sure that specific cancer would overexpress MCL-1 after being exposed to venetoclax.

C. MTT cell viability assays

venetoclax and MCL-1 inhibitor AZD5991 of lethal concentration were added to a replicate the cultured cells. Cell viability was measured for each replicate using MTT colorimetric assay. Cultured cancer cells were seeded in 96-well microplates at a density that enabled the cells to grow exponentially throughout the experiment. Cells were first incubated for 48 h. Then, they were incubated with 1mg/ml MTT for 4 h at 37°C. Lysis buffer (20% SDS) was added and absorbance was measured using a microplate (ELISA) reader at 540 nm after 18 hours. The averaged percentage of viable cells was then calculated. The experiment was repeated three times. The final average percentage was then calculated. A result was considered significant if its P-value was smaller than 0.05. A positive control where cell viability was tested by MTT after lethal amount of venetocalx was added to SCLC tumor cells was implemented. A negative control where PBS/DMSO was tested by MTT was implemented.

D. Western blot analysis

Each of the 2 cell lines (one cultured cell line and its

parental cell line) was divided into three replicates, each with 500 thousand cancer cells. The expression of MCL-1 in each replicate was examined using western blot. Cell lysates were prepared in RIPA buffer (Sigma) plus protease inhibitor cocktail [11]. On Bis-Tris gels, 30 μg of protein was resolved. It was then transferred to PVDF membranes [11]. Rabbit polyclonal MCL-1 antibodies were used to probe the blots for 10 hours at 4° C, and the Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) were used for 1 hour [11]. The blots were imaged with LI-COR Biosciences Odyssey imager [11].

The grayscale of the fluorescence on the image was compared using ImageJ. The experiment was repeated three times. The average grayscale of the parental cell line and the cultured cell line was calculated. GraphPad Software was used for statistical analysis [12]. A result with P-value <0.05 was considered to be significant. Western blot was carried out using Human HeLa cells with the same antibodies as a positive control [13]. A negative control cannot be set because studies that had confirmed cells known not to express MCL-1 could not be found.

E. MCL-1 protein purification.

Human MCL-1 gene was incorporated into pET-28a using restriction enzymes. The modified pET-28a plasmid was then transformed into BL21(DE3)pLysS bacteria [12]. A single colony of the bacteria was translocated to a 5ml terrific broth which contains kanamycin and chloramphenicol at a concentration of 100μg/ml and 34μg/ml, respectively [12]. After 3 hours of growth at 37°C, the 5ml culture was used to inoculate 21 terrific broth containing the same antibiotics [12]. At an OD_{600} of 0.8, the temperature was reduced to 18 °C before inducting MCL-1 protein expression by the adding IPTG to a concentration of 1mM. Centrifugation was used to harvest the cells [12].

The harvested cells were resuspended in 3 volumes of 20mM Tris–HCl at pH 7.4, 200mM NaCl, 2mM EDTA, 1mM DTT, and lysed by passing three times through an emulsiflex-C5 [12]. Centrifugation was done at 40,000g at 4°C for 60min to clarify the lysate. The clarified product was then applied to a 5-ml MBPTrap column [12]. The MCL-1 protein was eluted in 20mM Tris-HCl pH 7.4, 200mM NaCl, 2mM EDTA, 1mM DTT, 10mM maltose. It was then purified by size exclusion chromatography in 20mM HEPES, 100mM NaCl and 1mM DTT [12].

F. Fluorescence Polarization Based Binding Assays

FP-based binding assays were deployed to determine the binding affinities between venetoclax and MCL-1. MCL-1 with a concentration of 90 nM and two fluorescent probes, Flu-BID and FAM-BID, with a concentration of 2 nM were added [14]. Then 5 μL of the tested compound in DMSO and 120 μL of protein-probe complex in the assay buffer (100 mM potassium phosphate, pH 7.5; 100 μg/mL bovine gamma globulin; 0.02% sodium azide) were added to assay plates [14]. They further went through 3 hours of incubation at room temperature. Their polarization values (mP) were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using the plate reader Synergy H1 Hybrid [14]. IC_{50} values were determined using GraphPad Prism 6.0 Software with a nonlinear regression fitting of the competition curves [14]. The experiment was repeated three times and the average IC_{50} value was calculated. Then, the average IC50 value was translated to Ki (Ki=IC50/(1+([L]/Kd)). A Ki value smaller than 0.5 was considered significant. The binding affinity between MCL-1 inhibitor AZD5991 and MCL-1 was tested as a positive control. FP-based binding assays were implemented on DMSO as a negative control.

III. Result

TABLE I. Possible result table

	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6	Result 7	Result 8
MCL-1 protein increases by WB	+	+	+	-	-	-	+	-
Venetoclax binding to MCL-1 is high by FP	+	+	-	+	-	+	-	-
Venetoclax kill OCI-Ly1 cells by MTT	-	-	-	-	-	-	-	-
Venetolax and MCL-1 inhibitor AZD5991 together kill OCI-Ly1 cells by MTT	+	-	+	+	+	-	-	-

Note. "+" represents the result is positive. "-" represent the result is not negative.

Possible result 1: There was overexpression of MCL-1 protein in the cultured cells, BCL-2 inhibitor venetoclax had high affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together killed cultured OCI-Ly1 cells.
Possible result 2: There was overexpression of MCL-1 protein in the cultured cells, BCL-2 inhibitor venetoclax had high affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together did not kill cultured OCI-Ly1 cells.
Possible result 3: There was overexpression of MCL-1 protein in the cultured cells, BCL-2 inhibitor venetoclax had low affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together killed cultured OCI-Ly1 cells.
Possible result 4: The level of MCL-1 proteins was relatively the same in both the cultured cells and the parental cells, BCL-2 inhibitor venetoclax had high affinity for MCL-1, and MCL-1 inhibitor AZD5991 together killed cultured OCI-Ly1 cells.
Possible result 5: The level of MCL-1 proteins was relatively the same in both the cultured cells and the parental cells, BCL-2 inhibitor venetoclax had low affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together killed cultured OCI-Ly1 cells.
Possible result 6: The level of MCL-1 proteins was relatively the same in both the cultured cells and the parental cells, BCL-2 inhibitor venetoclax had high affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together did not kill cultured OCI-Ly1 cells.
Possible result 7: There was overexpression of MCL-1 protein in the cultured cells, BCL-2 inhibitor venetoclax had low affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together did not kill cultured OCI-Ly1 cells.
Possible result 8: The level of MCL-1 proteins was relatively the same in both the cultured cells and the parental cells, BCL-2 inhibitor venetoclax had low affinity for MCL-1, and venetolax and MCL-1 inhibitor AZD5991 together did not kill cultured OCI-Ly1 cells.

IV. Discussion

Rigorous studies regarding MCL-1 inhibitors has been done since the twenty-first century. However, little has been done to reveal the relationship behind the overexpression of MCL-1 and cancers' resistance to venetoclax. In this study, we focused on the relationship between overexpression of MCL-1 and cancer cells' resistance to venetoclax.

Possible result 1 and 2 partially supported the hypothesis. Table 1 showed that MCL-1 increased in cultured cells and venetoclax had high affinity for MCL-1 and BCL-2 and inhibited both of them. In result 1, venetoclax and AZD5991 killed the cultured cells. One explanation was that the binding of venetoclax did not inhibit the interaction between BAX and MCL-1. Another explanation might be erroneous FP-based binding assay. Therefore, its positive and negative control should be examined. In result 2, venetolax and AZD5991 did not kill cultured cells. This indicated that the overexpression of MCL-1 protein was merely a byproduct of the mechanism behind the resistance of venetoclax.

Possible result 3 fully supported the hypothesis while possible result 7 partially supported the hypothesis. In both results, increase in MCL-1 protein in cultured cell and small binding affinity between venetoclax and MCL-1 indicated a strong correlation between venetoclax resistance and the expression of MCL-1. In result 3, venetolax and AZD5991 killed cultured cells, implying that MCL-1 was vital for the survival of cancer cells in venetoclax. In result 7, venetolax and AZD5991 did not kill cultured cells, suggesting that other mechanisms contributed to the resistance.

Possible result 4 and 6 partially supported the hypothesis. In both results, the level of MCL-1 protein was relatively the same in the two cell lines, and venetoclax had a high affinity for MCL-1. These two observations indicated that MCL-1 was irrelevant to venetoclax resistance. In result 4, venetolax and AZD5991 killed cultured cells. One explanation was that the binding between venetoclax and MCL-1 did not inhibit the interaction between BAX and MCL-1. Another explanation might be incorrect MTT assay. Therefore, the positive and negative control should be examined. In result 6, venetolax and AZD5991 did not kill cultured cells, denoting that mechanisms other than overexpression of MCL-1 accounted for the resistance in cultured cells.

Possible result 5 and 8 partially supported the hypothesis. In both possible results, there were no overexpression of MCL-1 protein, implying that MCL-1 had little to do with the resistance of venetoclax. However, the low binding affinity between MCL-1 and venetoclax suggested MCL-1's potential to be a key factor in cancers' resistance. Experiment should be repeated with new kinds of cancers and cell lines to get a compelling conclusion. In result 5, the addition of AZD5991 killed cultured cells as shown in Table 1. One possible explanation was a faulty western blot analysis. The positive control and negative control should be examined. Then, the gel should be check for overlapping of proteins. In result 8, venetolax and AZD5991 did not kill cultured cells, indicating that other mechanisms contributed to the resistance.

One hypothesis for the mechanism mentioned in possible result 2,6,7, and 8 was that some other molecule would bind to BAX and inhibit them. Another hypothesis was

that the binding site of BAX on the mitochondrial surface was inhibited, thus blocking apoptosis. Further studies regarding apoptosis pathways in cultured OCI-Ly1 cells should to be made.

V. Conclusion

In conclusion, this study explored the expression of MCL-1 in cultured venetoclax- resistant cancer cells, the viability of the cultured cells after venetoclax with AZD5991 was added, and the binding affinity between MCL-1 and venetoclax. The results of the study have given insights into the resistance to venetoclax and overexpression of MCL-1 in OCI-Ly1 cells. Hopefully, the study would provide a basis for further investigation of the mechanism behind cancer's resistance to venetoclax due to overexpression of MCL-1 and promote the development of new therapeutics which could circumvent this resistance.

References

[1] *Cancer*, <https://www.who.int/news-room/fact-sheets/detail/cancer> (2022).

[2] Singh, R. *et al.* Non-Hodgkin's lymphoma: A review. *J Family Med Prim Care* **9**, 1834-1840, doi:10.4103/jfmpc.jfmpc_1037_19 (2020).

[3] Armitage, J. O., Gascoyne, R. D., Lunning, M. A. & Cavalli, F. Non-Hodgkin lymphoma. *Lancet* **390**, 298-310, doi:10.1016/S0140-6736(16)32407-2 (2017).

[4] *Non-Hodgkin lymphoma*, <https://www.nhs.uk/conditions/non-hodgkin-lymphoma/symptoms/> (2022).

[5] Thomas, L. W., Lam, C. & Edwards, S. W. Mcl-1; the molecular regulation of protein function. *FEBS Lett* **584**, 2981-2989, doi:10.1016/j.febslet.2010.05.061 (2010).

[6] *Apoptosis*, <https://www.genome.gov/genetics-glossary/apoptosis> (2022).

[7] Leverson, J. D. *et al.* Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). *Cell Death Dis* **6**, e1590, doi:10.1038/cddis.2014.561 (2015).

[8] Xiang, W., Yang, C. Y. & Bai, L. MCL-1 inhibition in cancer treatment. *Onco Targets Ther* **11**, 7301-7314, doi:10.2147/OTT.S146228 (2018).

[9] Montero, J. & Letai, A. Why do BCL-2 inhibitors work and where should we use them in the clinic? *Cell Death Differ* **25**, 56-64, doi:10.1038/cdd.2017.183 (2018).

[10] Xu, C. *et al.* The prognostic significance of MCL1 copy number gain in esophageal squamous cell carcinoma. *Oncotarget* **8**, 87699-87709, doi:10.18632/oncotarget.21181 (2017).

[11] Tahir, S. K. *et al.* Potential mechanisms of resistance to venetoclax and strategies to circumvent it. *BMC Cancer* **17**, 399, doi:10.1186/s12885-017-3383-5 (2017).

[12] Kotschy, A. *et al.* The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature* **538**, 477-482, doi:10.1038/nature19830 (2016).

[13] *Anti-MCL1 antibody (ab28147)*, <https://www.abcam.com/mcl1-antibody-ab28147.html>

[14] Abulwerdi, F. A. *et al.* 3-Substituted-N-(4-hydroxynaphthalen-1-yl)arylsulfonamides as a novel class of selective Mcl-1 inhibitors: structure-based design, synthesis, SAR, and biological evaluation. *J Med Chem* **57**, 4111-4133, doi:10.1021/jm500010b (2014).