Emerging Effects of Sepantronium Bromide (YM155) on MOLT-4 Cell Line Apoptosis Induction and Expression of Critical Genes Involved in Apoptotic Pathways

Kobra Shojaei Moghadam¹,², Majid Farshdousti Hagh³, Mohammad Reza Alivand⁴, Masoumeh Fardi⁵,², Ali Akbar Movassaghpour⁵, Ali Mohammadii, Maryam Moghadasi¹, Saeed Solali⁷,⁸*

¹Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.
²Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
³Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
⁴Department of Medical Genetic, Tabriz University of Medical Sciences, Tabriz, Iran.
⁵Hematology and Oncology Research Center, Tabriz University of medical Sciences, Tabriz, Iran.
⁶Departments of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark.
⁷Molecular Medicine Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
⁸Division of Hematology and Transfusion Medicine, Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz.

Abstract

Purpose: Sepantronium bromide (YM155) is a Survivin inhibitor which recently advanced as an anticancer agent in phase II clinical trials. Survivin belongs to IAP (inhibitor of apoptosis) gene family and is a pivotal target for treatment due to its overexpression and oncogenic function in many malignancies, including acute lymphoblastic leukemia (ALL). Although survivin is a specific target for YM155, recent reports have shown that it has many other crucial targets that regulate its anti-apoptotic effects. The aim of this study was to investigate whether YM155 could have an effect on cell death-inducing genes as well as inducing apoptosis in T-ALL MOLT-4 cell line.

Methods: We treated MOLT-4 cells with increasing concentrations of YM155 and then cell viability was determined using MTT (methyl thiazolyl tetrazolium) assay. Also, the rate of induction of apoptosis in MOLT-4 cells and the target genes expression levels were evaluated by Annexin V/PI and real-time PCR, respectively.

Results: YM155 inhibited cell growth in MOLT-4 cells. This outcome is achieved by inducing apoptosis and a significant increase in the expression level of P53, MiR-9, caspase 3 and decreasing the mRNA expression levels of survivin, Sirtuin1(SIRT1), member of anti-apoptotic proteins family (Bcl-2), and epithelial-to-mesenchymal transition (EMT) initiating factors Snail1and Zeb2.

Conclusion: The results showed that use of YM155 can be a potential drug therapy in T-ALL patients with promising effects on apoptosis induction.

Keywords:
- Apoptosis
- Cancer
- Drug
- Survivin
- MOLT-4
- YM155

Introduction

Acute lymphoblastic leukemia (ALL) is one of the most common variants of leukemia, which affects all ages, with high costs and treatment failure.¹ Although the diagnosis and treatment of these malignancies nowadays have greatly improved, the overall survival rate of patients is still low.² Therefore, in order to reduce the therapeutic burden, target therapy in ALL patients seems essential. For this reason, in recent clinical and preclinical studies, many targets have been tested for effective treatment.³ YM155 with the original name of 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d] is a pioneer chemical compound with significant therapeutic effects, which has been shown to induce apoptosis, inhibit invasion and suppress drug resistance in numerous cancer cell lines.⁴-⁶ YM155 has been shown to be tolerable in phase II clinical trials in patients with melanoma⁷ and advanced refractory NSCLS.⁸ It has been reported that YM155 applies its anti-tumor property through direct suppression of survivin expression by binding to its promoter.³ Survivin, which is the smallest member of the IAP
family, is overexpressed during the development of human cancer and is associated with poor survival.\textsuperscript{10} In cancer cells, upregulation of survivin leads to the inhibition of apoptosis via interaction with caspase3 and 7 to inhibit their enzymatic activity.\textsuperscript{11} In addition, it has been demonstrated that overexpression of survivin in primary ALL plays a critical role in drug resistance and its down-regulation in combination with chemotheraphy leads to the eradication of ALL leukemia cells.\textsuperscript{10}

Studies have shown that YM155 causes cell death by different mechanisms other than suppression of survivin; for instance, it is able to arrest the cell cycle in the S phase and as a result it can evoke DNA damagecascades.\textsuperscript{12} Also, it was documented that YM155 reduced the resistance to treatment through reversing a process called epithelial-to-mesenchymal transition (EMT) by inhibiting the STAT3 pathway.\textsuperscript{13}

EMT is a crucial mechanism during cancer progression by changing the epithelial state of the cells to mobile and mesenchymal-like state. This process is accompanied by acquiring many oncogenic properties for the tumor cells, including apoptosis resistance, proliferation, differentiation arrest and invasion of cancer cells.\textsuperscript{14-16} A study found that the use of YM155 reduces the EMT-inducing transcription factor called Zeb1 (zinc-finger E-box-binding) via the STAT3 pathway.\textsuperscript{17} However, the effect of this agent on other EMT-inducing factors, including Zeb2 and Snai1, has not yet been studied.

It has also been shown that the use of YM155 is associated with a significant reduction in the expression of member of anti-apoptotic proteins family (Bcl-2) in the HL-60 cell line, but its effects on the U937 cell line was not significant. In addition, this study examines the role of YM155 in the induction of caspases. The results indicate that the use of this inhibitor, in contrast to the U937 cell line, is able to increase caspases expression in the HL-60 cell line.\textsuperscript{18} We also investigated the expression levels of Bcl2 and caspase3 in MOLT-4 cell line treated with YM155.

Furthermore, a whole transcription study on MDA-MB-231 cells after YM155 treatment documented that this drug also changed the expression pattern of micro-RNAs such as miR-125b-1.\textsuperscript{19} In the present study, we studied the role of YM155 on the induction of apoptosis and gene expression associated with the cell death and apoptosis; we also defined the expression of miR-9 as an important regulator of gene expression in T-ALL MOLT4 cell line.

**Materials and Methods**

**Cell culture**

We used the MOLT-4 cell line, purchased from the cell bank of the Pasteur Institute of Tehran (Iran). The cells were then grown under standard conditions suitable for their growth, including culture media, RPMI-1640, plus 10% fetal bovine serum (FBS) and 100 mg/mL of penicillin and streptomycin antibiotics (all purchased from Gibco Inc.). Then the cells were incubated in 5% carbon dioxide (CO\textsubscript{2}) at 37°C.

**Evaluation of cytotoxic effect of YM155 on MOLT4 cells**

YM155 was purchased from Sigma Aldrich (Cat Number 781661-94-7), dissolved in dimethyl sulfoxide (DMSO) with 225mM stock solution and stored at 20°C.

This test is based on the activity of the mitochondrial succinate dehydrogenase enzyme in living cells by measuring their optical absorption. The cells were cultured with an initial number of 1x10\textsuperscript{4} cells/well in each well of a 96-well plate. After 24 hours, the cultured cells were treated with different concentrations of YM155 (0–6 mM). Twenty-four and 48 hours after treatment, 25 μL of MTT solution was added to the cells and the cells were incubated for 4 hours. We then centrifuged the plate to re-precipitate the cells. Then we dispersed the supernatant. To each well, 200 μL of MTT solubilizing solution (containing DMSO and Sorenson buffer) were added. After 30 minutes of incubation of the cells under the above conditions, optical densities of the wells were read at 570 nm with an ELISA reader.

**Apoptosis study using flowcytometry**

In order to determine the percentage of apoptotic cells treated with YM155 and compare with the control cells’ population, staining of cells with two stains, Annexin V and propidium iodide (PI), was performed using the Apoptotest\textsuperscript{TM} FITC kit (EXbio). First MOLT4 cells were treated for 24 and 48 hours with the IC50 concentration of YM155; subsequently, the cells were multiple-washed with PBS buffer and the pellets were re-suspended in 100 μL of the binding buffer.

The cells were incubated with 20 μL of PI and 10 μL of Annexin V labeled with FITC at room temperature in a dark environment for 10 minutes. Finally, the study of apoptosis in MOLT-4 cells treated with YM155 was evaluated by FACSCalibur (BD Biosciences, Franklin Lakes, NJ) and FlowJo software version 10.4.1 (Treestar, FlowJo).

**RNA extraction**

Extraction of total mRNA from MOLT-4 cells was carried out 24 hours after treatment with YM155 by using RiboEx reagent (Gene All, South Korea), according to the manufacturer’s instructions.

**Quantitative real-time PCR (qPCR) for miRNA and mRNA expression**

All the primers used in this study were designed by Oligo\textsuperscript{7} software and then blasted with the NCBI site by Primer-Blast (Table 1). Briefly, 1 μg in 20 μL of total volume RiboEx extracted RNA was reversely transcribed into cDNA system-loop primer or oligo (dT) using the Script\textsuperscript{TM} First-μ Strand Synthesis System (Invitrogen) and incubated for 60 minutes at 42°C and 5 minutes at 70°C,
Sepantronium bromide (YM155) induced apoptosis in MOLT4 cell line

Table 1. List of primers used in this research

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Survivin</td>
<td>Forward: ATGGGTGGCCCGACGTTGCC</td>
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<tr>
<td></td>
<td>Reverse: GCTCCGGCCAGAGGCTCAA</td>
</tr>
<tr>
<td>miR9</td>
<td>Forward: ATTCTGGCAGGCCGCTTAC</td>
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<tr>
<td></td>
<td>Reverse: ATCCGCAAATCTGCTCCTT</td>
</tr>
<tr>
<td>MDR1</td>
<td>Forward: TCACATGTTTCTGAGCCCTT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTTGCGAAATGTAACCCC</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Forward: GCCTCACATGCAAGCTGTGCAC</td>
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<tr>
<td></td>
<td>Reverse: TGCGAGATCTGTGCAACTCAA</td>
</tr>
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<td>Snail1</td>
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<tr>
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<tr>
<td>P53</td>
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<tr>
<td></td>
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<tr>
<td>Caspase3</td>
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<td></td>
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<td>Bcl-2</td>
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<td></td>
<td>Reverse: TGCCAAATCTGAGACGACAC</td>
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<td>Apex1</td>
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<td>Zeb2</td>
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<td></td>
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<tr>
<td>β-actin</td>
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</tr>
<tr>
<td></td>
<td>Reverse: CTGAGCACTGCACTGCACTGCA</td>
</tr>
</tbody>
</table>

followed by enzyme heat-inactivation.

Synthesis of cDNA was performed using a Thermo Scientific Revert Aid First Strand cDNA Synthesis kit and 500 ng of extracted RNA.

Evaluation of the expression of genes was performed by qRT-PCR using SYBR Green master mix (Exiqon, Vedbaek, Denmark) in the Roche Light Cycler 96 system (Roche, Germany). β-actin was used as an internal control for the evaluation of mRNAs expression. In addition, we used U6 as a reference gene for the evaluation of miRNA expression.

Statistical analysis

Data were analyzed with GraphPad Prism version 8.0.2 for Windows. Data were presented as M ± SEM (mean ± standard error of the mean). P < 0.05 (*) and P < 0.01 (**) were considered statistically significant. Also, flowcytometry data were analyzed using FlowJo® software (Treestar, Inc).

Results and Discussion

YM155 has a cytotoxic effect on MOLT4 cells

To assess the cytotoxic effect of YM155 on the viability of MOLT-4 cells, they were treated with different increasing concentrations of YM155 (0 to 6 mM for 24 and 48 hours) and evaluated using MTT test. The mean IC50 was 1.82 mM for 24 hours (Figure 1).

YM155 induced cell death through apoptosis in MOLT4 cells

To study the effect of YM155 on apoptosis in MOLT4 cells in vitro, we prepared MOLT4 cells for apoptosis analysis via Annexin V-FITC, following treatment with YM155 (1.82 mM) for 24 and 48 hours. The results showed that YM155 was able to augment apoptosis in MOLT4 cells in a time-dependent manner compared to the untreated group (Figure 2).
YM155 increased miR-9 and decreased survivin, Bcl-2 and genes related to EMT and DNA repair expression level

We used a qRT-PCR to evaluate survivin and several other candidate genes along with miR-9 expression in MOLT-4 cells treated with YM155 (1.82 mM) for 24 hours. It was observed that YM155 decreased the expression of genes associated with apoptotic inhibition such as survivin, Zeb2, Snail1, SIRT1, MDR1, Apex1 and Bcl-2 significantly. It also caused an increase in the expression of caspase3, P53, and miR-9. To determine differentially expressed miR-9, survivin, Bcl-2, caspase3 and other genes, the ANOVA results of normalized data generated (at P<0.05) between the group with YM155 treatment and the control group (no treatment) are shown in Figure 3.

In this study, we showed that YM155 inhibits MOLT-4 cell growth. Treatment of MOLT4 cells with different concentrations of YM155 led to the inhibition of proliferation and induction of apoptosis. In fact, we showed that in addition to survivin, YM155 down-regulated Bcl-2, which consequently resulted in elevated expression of caspase3 as an apoptosis inducer.

YM155 is a dioxonaphthoimidazolium analog that is the first drug reported to inhibit survivin expression.20 Survivin as an inhibitor of apoptosis is encoded by the BIRC5 gene with crucial functions in the regulation of apoptosis, cell division and cellular stress responses.21 Survivin is markedly expressed in most malignancies, including leukemia, and is associated with poor prognosis.22 Previous studies have demonstrated that survivin can promote drug resistance through both the stabilization of microtubule organization and co-working with other drug resistance molecules. Wang et al showed that patients with AML that exhibit overexpression of survivin and MDR1 have poor therapeutic response and survival.23 Survivin is also associated with apoptosis inhibition such as Apex1 and results in the activation of p53, leading to cell death through the activation of a number of genes.

Figure 3. Changes in the expression of survivin, Snail1, Zeb2, P53, SIRT1, Apex1, Bcl-2, caspase3, miR-9 and MDR1 in MOLT-4 cell line treated with the IC50 concentration of YM155 compared to a control group (untreated cell line) (*P<0.05, **P<0.001).
involved in apoptosis.30

This study showed that after 24 hours of treatment with YM155, the miR-9 expression levels increased significantly compared to control cells; in addition, the expression of two target genes, including MDR1 and SIRT1, decreased significantly. It is suggested that one of the effects of YM155 on inhibiting the growth and DNA damage response of leukemic cells can be via the miR-9/ SIRT1/p53 pathway (Figure 4). Furthermore, a decrease in the expression of MDR1 can indicate the effectiveness of YM155 in increasing the sensitivity of cancer cells to chemotherapy drugs and reducing drug resistance.

EMT can be defined by the loss of cell–cell adhesion by down-regulation of the adhesion molecule E-cadherin and up-regulation of mesenchymal markers, such as N-cadherin. Upon this process, cancer cells are able to increase their mobility and invasiveness during cancer progression.39,40

Different transcription factors, including the Snail1 and Zeb2, serve as molecular regulators of the EMT process. Several studies have demonstrated that low levels of Zeb2 and Snail1 contribute to apoptosis induction. While increased expression of these genes contributes to tumor progression by protecting cancer cells from apoptosis via various processes such as repressing the expression of caspase3; therefore, one of the features of EMT in cancer cells is the induction of a program of tumor-associated genes, including Snail and Zeb2.13,41 This study showed that YM155, in addition to survivin inhibition, reduces the expression of Zeb2 and Snail1, possibly, indicating that one of the major ways that YM155 induces apoptosis is through suppressing EMT-TFs like Snail1 and Zeb2.

Schematic pathways of YM155 effects on the expression of selected genes in MOLT-4 cell line with q-RT PCR analysis. In this study, we found that survivin and Bcl2 genes were inhibited by YM155, and as a result, the expression of Caspase3 increased. It was also found that MiR-9 expression was induced with this agent. The result is inhibition of the expression of SIRT1 and MDR1. In addition, YM155 inhibited the expression of EMT-inducing factors (Zeb2, Snail1).

Figure 4. Schematic pathways of YM155 effects on the expression of selected genes in MOLT-4 cell line with q-RT PCR analysis. In this study, we found that survivin and Bcl2 genes were inhibited by YM155, and as a result, the expression of Caspase3 increased. It was also found that MiR-9 expression was induced with this agent. The result is inhibition of the expression of SIRT1 and MDR1. In addition, YM155 inhibited the expression of EMT-inducing factors (Zeb2, Snail1).


