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Original Research Article

Bioymifi suppresses Burkitt's lymphoma cell line proliferation via cell cycle arrest and apoptosis

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Abstract

Purpose: *To find a new treatment alternative for Burkitt's lymphoma, one of the B cell-derived non-Hodgkin's lymphomas that has an extremely aggressive growth profile with a high mortality rate.*
Methods: *Bioymifi (death receptor agonist) was administered at various doses to Human Burkitt's Lymphoma Raji cell lines, and cell proliferation was evaluated using XTT analysis. Apoptosis induction and cell cycle analysis were assessed using flow cytometry.*
Results: *Bioymifi exhibited good cytotoxicity in Raji cell lines with IC_{50} value of 29.5 µM. Furthermore, results of apoptosis induction and cell cycle analysis indicated that Bioymifi had potent anti-proliferative activity through sub-S cell cycle arrest and stimulation of apoptosis ($p < 0.05$).*
Conclusion: *Bioymifi has anticancer potentials against Human Burkitt's Lymphoma Raji cell lines. Further pharmacological studies will be required to establish the mechanism of action.*

Keywords: *Apoptosis, Bioymifi, Cell cycle, Death receptor, DNA damage*

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INTRODUCTION

Burkitt's Lymphoma (BL) is an extremely aggressive B-cell Non-Hodgkin's Lymphoma (NHL) that is capable of multi-organ involvement and is characterized by chromosome 8-MYC gene translocation and deregulation. One of the germinal center B-cells BL has three subtypes according to clinical presentation, risk factors, and epidemiology. They include sporadic, endemic, and immunodeficiency [1]. Chemotherapy is the basis for the treatment in BL, where mass growth occurs in a very short time due to the high proliferation rate. However, there are no known randomized controlled trials for chemotherapeutic options, therefore, there is still no definite treatment option for BL.

Cancer is caused by various mutations that activate the mechanisms that limit the survival and proliferation of cells. Programmed cell death (PCD) is essentially a compulsory procedure in cellular activities such as cell growth, wound healing, and tumor suppression [2]. The malfunction of PCD is closely associated with neurodegenerative diseases and some types of cancer. Apoptosis, which is an essential part of the cell life cycle and is also described as PCD, primarily takes part in the pathogenesis of numerous illnesses such as cancer. The evasion

of apoptosis by tumor cells is regarded as the most fundamental mechanism in the development of cancer.

One of the leading mechanisms mediating development of cancer in human beings is loss of apoptosis. The clarification of the mechanisms of apoptosis leads to the development of new agents that target these pathways. Members of the proinflammatory cytokine family, which can paradoxically induce apoptosis or cell survival, specifically cause extrinsic apoptosis (through death receptors) on the outer surface of cell membrane. The Apo2 ligand/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL), from the TNF superfamily, binds to five other members from this family in order to induce apoptosis [3]. Particularly, Death Receptor-4 (DR4, TRAIL-R1, TR1) and Death Receptor-5 (DR5, TRAIL-R2, TR2) receptors contain the cytoplasmic death domain and mediate apoptotic signal transduction only when they are bound by their specific ligand. When cell surface death receptors are induced by the Apo2L/TRAIL and DR agonists, this offers a remarkable antitumoral therapeutic strategy with activation of the extrinsic pathway during the apoptosis.

Bioymifi induces apoptosis through the death receptor DR5 independent of TRAIL and mediates activation of this receptor with high affinity by binding to its extracellular region. Bioymifi causes apoptosis and cytotoxicity in some human cancer cell lines [4]. Some studies have reported that DR5 agonist derivatives are among the targeted therapy options for new anticancer drug approaches. Furthermore, many researchers have focused on the PCD of small molecules in terms of how diverse their biological responses are [4]. In the light of these information, the aim of this study was to explore the anticancer activity of Bioymifi on Raji BL cells using flow cytometry.

EXPERIMENTAL

Cell culture techniques

Human BL Raji cell lines (CCL-86) was supplied from ATCC (Manassas, Virginia). The cells were incubated in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10 % (v/v) fetal bovine serum (FBS) (Biochrom, Berlin) and 1 % pen/strep (Thermo Fisher) until they reached approximately 80 - 90 % density in a humidified atmosphere with 5 % CO_2 at 37 °C. This study was carried out with the approval of the ethics committee of Sivas Cumhuriyet University (approval no. 2019-12/30).

Cell viability assay

Stock solution for Bioymifi was prepared in dimethyl sulfoxide (DMSO) (Not exceeding 0.1 %) and also diluted in RPMI-1640 in order to obtain the desired concentrations. A 1.5×10^4 cells culture from Raji cells were incubated in a 96-well plate and handled with an increasing concentration of Bioymifi (3.125, 6.25, 12.5, 25, 50 and 100 µM). Afterwards, they were incubated for 48 h. The cytotoxicity of Bioymifi was determined using the XTT test kit (BIOTIUM, Inc). The XTT labeling mixture was supplemented to the wells to detect viable cells and incubated at 37 °C for 4 h. The tetrazolium salt was reduced to the orange formazon dye by the mitochondrial dehydrogenase enzyme. Formazon can be measured photometrically and is associated with metabolic activity and viable cell number. Absorbance was detected using an ELISA plate reader (Thermo, Germany) at 450 nm. All experimental tests were performed in three independent trials and cytotoxicity findings were recorded as a percentage of the control.

Apoptosis

Apoptosis assay was performed by flow cytometry based on the principle of externalization of phosphatidylserine in apoptotic cells, which is normally confined to the membrane internal layer in healthy cells. The Muse® Annexin V & Dead Cell kit (Guava® Muse® Cell Analyzer, Luminex) was used to carry out quantitative assay of live, early apoptotic, late apoptotic, and dead cells based on the supplier's instructions. The Raji cells were maintained in a 6-well plate at a density of 5×10^5 cells/2 mL per well. The cells were handled in Bioymifi at 12.5 µM (IC_{50}: half-maximal inhibitory effect), for 48 h. Once this period was completed, both treated cells and non-treated cells were centrifuged, resuspended with PBS:FBS mixture (99:1), and evaluated using the Muse® Annexin V & Dead Cell kit at room temperature for 20 min in the dark.

Cell cycle analysis

Muse® Cell Cycle Assay Kit (Luminex, India) was employed to detect how cells were distributed in cell cycle. Raji cells were inoculated in a 6-well cell culture plate and subjected to IC_{50} of Bioymifi. The cells were harvested and then fixed in cold 70 % ethanol for 3 h. The cells were stained with the Muse® Cell Cycle reagent containing the nuclear DNA intercalating stain propidium iodide (PI) and RNAse A and analyzed in Guava® Muse® Cell Analyzer.

Multi-color DNA damage analysis

Bioymifi damage of DNA in Raji cells was detected through the Muse® Multi-Color DNA Damage Kit by assessing the presence of ATM and H2A.X activation. This kit simultaneously detects the phosphorylation status of ATM and Histone-H2A.X via flow analysis. It reliably measures the extent of DNA damage. Bioymifi at IC_{50} concentration was applied to Raji cells and then incubated at 37 °C for 48 h. Afterwards, the cells incubated with the antibody cocktail solution were identified by the Guava® Muse® Cell Analyzer in four different populations, depending on whether or not they were activated with ATM/H2AX.

Statistical analysis

The data analysis was performed by Student's t-test or one-way analysis of variance (ANOVA) using GraphPad Prism 7 (GraphPad Software, Inc.) software. They are reported as mean ± SEM. Mann-Whitney U and Kruskal-Wallis, non-parametric tests, were used for unsuitable parametric test assumptions. Level of statistical significance was set at $p < 0.01$.

RESULTS

Bioymifi inhibited cell proliferation in Raji cells

The primary purpose of this study was to evaluate the proliferation inhibitory effect of Bioymifi on the Raji BL cells after 48 h. The cells were treated at concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 µM of Bioymifi, and the IC_{50} value was calculated. As seen in Figure 1, Bioymifi showed significant inhibitory effects on proliferation of treated cells for 48 h regardless of the studied concentrations when compared to untreated cells. The IC_{50} of Bioymifi was determined as 12.5 µM. A concentration-dependent pattern was observed in the proliferation inhibition of Raji cells.

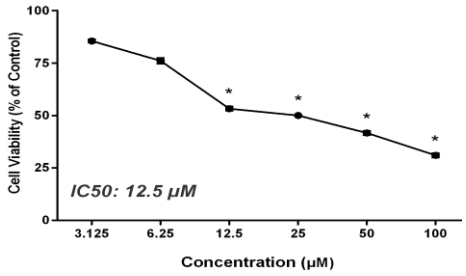

Figure 1: Results of the XTT assay showing that Bioymifi significantly inhibited proliferation of Raji cells. Data are presented as mean ± SEM of triplicate determination, *$p < 0.01$

Apoptosis of Raji cells was stimulated by Bioymifi

The apoptotic effects of 12.5 µM Bioymifi on the Raji cells were assessed through the Annexin V & Dead Cell binding assays using the flow cytometry technique. As shown in Figure 2, Bioymifi led to a significantly high apoptotic effect in 12.5 µM Bioymifi group compared to the control group ($p < 0.01$). The rate of the apoptotic cell obtained was 82.21 % in the Raji cells treated with Bioymifi 12.5 µM.

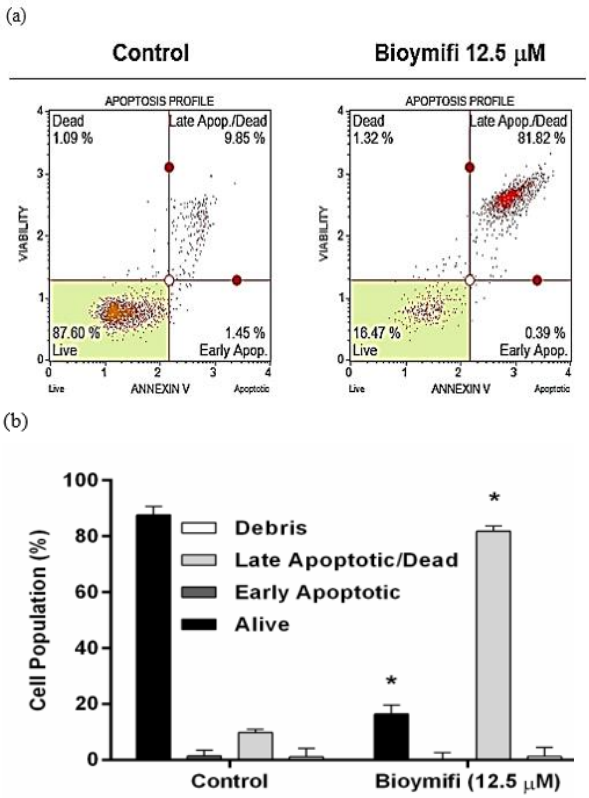

Figure 2: Flow cytometry assays showing (a) the apoptosis percentages of Raji cells by the quadrant graphs and (b) the bar graphs after treatment with Bioymifi at IC_{50}. Data are presented as mean ± SEM, *$P < 0.01$

Bioymifi induced S-phase cell cycle arrest

The effect of Bioymifi at IC_{50} (12.5 µM) on the cell cycle in Raji cells was determined via flow cytometric analysis. After 24 h of Bioymifi treatment, the rate of S-phase cells (21.0 ± 1.73 %) in Bioymifi-treated Raji cells significantly increased, when compared to untreated cells (15.8 ± 1.85 %, $p < 0.01$) (Figure 3 A and B). Thus, the antiproliferative effects of Bioymifi on Raji cells were caused by both apoptosis and cell cycle arrest.

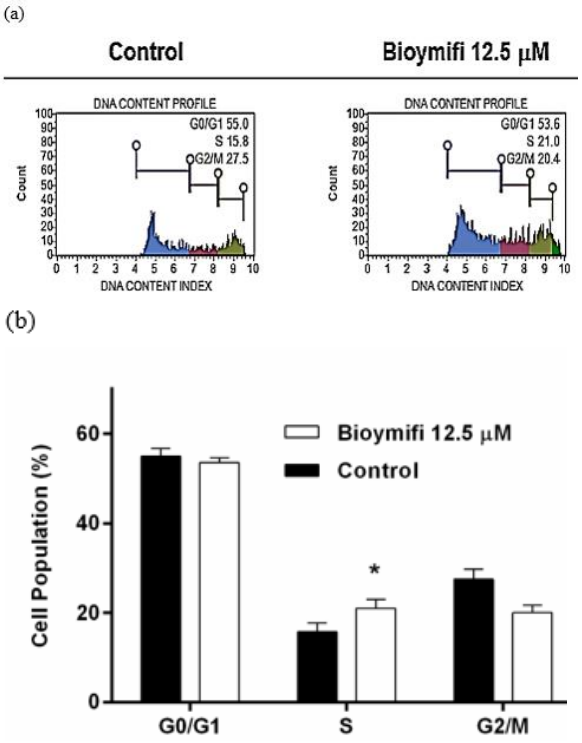

Figure 3: (a) The effect of Bioymifi on cell cycle stages in Raji cells. (b) Bar graph representation of quantitative values of cell cycle arrest data

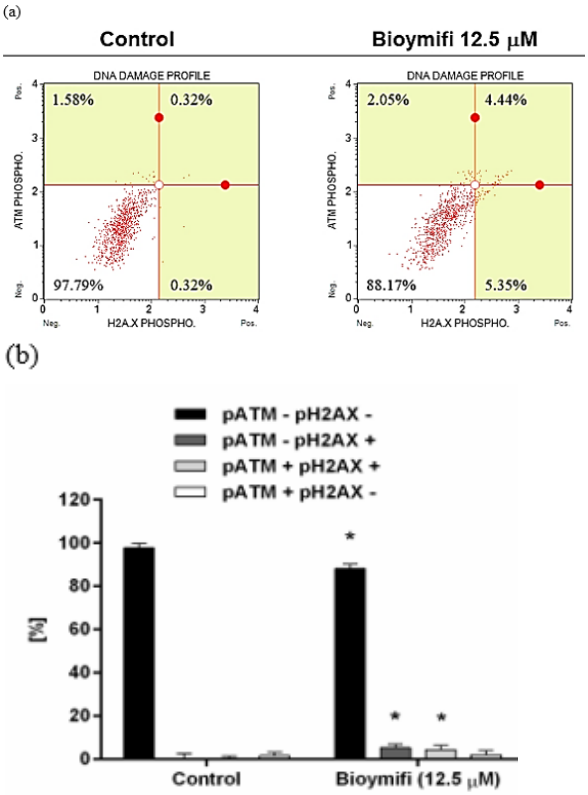

Figure 4: Flow cytometry assays showing (a) the DNA damage percentages of Raji cells by the quadrant graphs and (b) the bar graphs after treatment of Bioymifi at IC_{50}. Data are presented as the mean ± SEM, *$p < 0.01$

Bioymifi induced DNA damage in Raji cells

The induction of the DNA damage, as noted in the expression of γH2AX, was observed upon treatment of 12.5 µM Bioymifi in the Raji cells. The results suggested that Bioymifi stimulated the DNA damage in the Raji cells. As seen in Figure 4, the rate of cells phosphorylated with H2AX/ATM was 0.64 ± 1.57 % in untreated cells and 9.79 ± 1.34 % in treated cells, showing a significant increase in phosphorylated cell rate in cells treated with Bioymifi compared to control cells ($p < 0.01$). Furthermore, Bioymifi had a genotoxic effect on the Raji cells.

DISCUSSION

Burkitt's Lymphoma (BL) is the most invasive and aggressive subtype of B-cell Non-Hodgkin's Lymphomas (NHL) and accounts for approximately 1 - 3 % of all malignant NHL cases. Although it is more common in childhood, it accounts for approximately 30 - 50 % of childhood lymphomas, as well [5]. Tumor size may increase even in hours due to a very high proliferation rate and a short doubling time [6]. Chemotherapy is still the most important treatment modality for BL, although some NHL patients receiving routine treatment respond after a long time. However, more than 60 % of patients do not respond to routine treatment or their condition relapses while in remission.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which is a member of the TNF superfamily, is bound to death receptors (DR4 and DR5) found in many normal cells and tumor cells, causing apoptosis [7]. However, Apo2L/TRAIL induces apoptosis of tumor cells but has no toxicity or lethal effect on normal cells. Binding of Apo2L/TRAIL or agonist antibodies to death receptors triggers a caspase-mediated apoptosis pathway, leading to cell death. Recombinant forms of TRAIL, TRAIL receptor agonists, and other therapeutic agents are leading to the emergence of TRAIL-mediated chemotherapy studies through tumor-specific apoptosis induction mechanisms. So far, several agonists have been developed targeting first-generation TRAIL receptors, including agonistic monoclonal antibody against TRAIL-R1 or TRAIL-R2 for the treatment of cancer in clinical trials (phase ½) [8]. The DR4 and DR5 receptors need to be expressed in tumor tissue to achieve the anti-cancer effect of monoclonal antibodies for DR4 and DR5 receptor agonists. Previous studies have reported that DR4/DR5 causes leukemias, non-small cell lung cancer, breast cancer, ovarian cancer, and various

gastrointestinal malignancies at a high rate [9,10]. Although, the expression of DR4/DR5 from tumor tissues rather than normal tissues seem to be very promising in anti-cancer treatment, clinical trials using first-generation TRAIL agonists and anti-DR4/DR5 agonistic monoclonal antibodies have not produced satisfactory results [11]. However, the combination of Apo2L/TRAIL receptor agonist antibodies with other drugs can be considered as an alternative treatment protocol for anticancer effects [12].

Nowadays, the second-generation TRAIL agonists and single chain monoclonal antibodies targeting death receptors have been made biocompatible by nanoparticles or linkers. Clinical trials have been advanced to enhance agonistic activities and effectiveness for cancer treatment [13]. In the study by Trarbach *et al* [14], 38 refractory colorectal cancer patients received TRAIL receptor 1 agonist human monoclonal antibody; mapatumumab (loading dose of 20 mg/kg for the first two days, followed by a maintenance treatment of 10 mg/kg every 14 days). A stable disease response was achieved in 32 % (21 patients) of participants. They concluded that mapatumumab was not effective in monotherapy but may be effective in combination therapy [14]. In the other study, conatumumab was used in combination with standard chemotherapy in 172 patients suffering from non-small cell lung cancer, with satisfactory results compared to those who received only standard therapy [13-15]. In other clinical trials, specific or targeted agents and DR5 agonists have been used in patients suffering from advanced malignancies from different histological types. It was concluded that these agents could be safely combined because they are non-toxic and that the combinations can have good results [16,17].

In the literature, there are many *in vitro* and *in vivo* studies in which DR5 agonists are applied in many malignancies such as colon, pancreas, breast, lung cancer, multiple myeloma, non-hodgkin lymphoma, and glioma. In these studies, positive responses were generally obtained regarding the anti-cancer activity of DR5 agonists [18]. Bioymifi induces apoptosis by showing an agonistic effect via death receptors. The apoptotic effect has been reported in various cancer cell lines such as human colon adenocarcinoma and osteosarcoma. Bioymifi concentration at the micromolar level also increases the susceptibility of cancer cells to apoptosis by increasing DR5 receptor aggregation [4].

Many *in vitro*, *in vivo*, and clinical studies have investigated the anti-cancer efficacy of TRAIL agonists and monoclonal antibodies [14,16,17,19]. However, there are few studies investigating the anti-cancer efficacy of specific agonists of death receptors on hematologic malignancies. This study investigated how DR5 agonist Bioymifi affected cell proliferation in BL Raji cell lines. A significant inhibitory effect on cell proliferation was observed at IC_{50} (29.5 µM). Based on the IC_{50} value, the mechanism of anti-cancer activity was elucidated by using apoptosis, cell cycle, and DNA damage processes. It was found that 12.5 µM Bioymifi induced apoptosis by significantly increasing the rate of apoptotic cells from 11.3 to 82.21 % in Raji cells after 48 h compared to the untreated cells. When examining the effect on the cell cycle using the Guava® Muse® Cell Analyzer, it was found that cell development ceased in phase S. In a recent study, Guo *et al* examined the synergistic antiproliferative and apoptotic effect of the combined use of TRAIL and chlorambucil on the Raji BL cell line [19]. They administered TRAIL 80 ng/mL and chlorambucil to the Raji cells both alone and in combinations (10 µM) and then observed cell proliferation and apoptosis. The study revealed that the combined use of TRAIL and chlorambucil produced a synergistic effect. However, the molecule reported under the name TRAIL is not an agonist or monoclonal antibody, and no IC_{50} is stated. In this study, a molecule called Bioymifi, which is a death receptor-5 agonist was applied to Raji BL cells, and its antiproliferative, apoptotic, and cell cycle arrest have been reported. Accordingly, it can be utilized alone or in combination with other agents to treat cancer without any significant toxicity.

CONCLUSION

Bioymifi, a DR-specific agonist, significantly reduces concentration-dependent proliferation in the BL cell lines, induces apoptosis and DNA damage through death receptors. Furthermore, DR5 expression is higher in BL. However, further studies would be required in order to ascertain whether death receptors are overexpressed in Raji cells compared to healthy cells and to identify the *in vivo* pathways of the Bioymifi.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this study was conducted by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hatice TERZİ contributed to the interpretation of the data from a clinical perspective, literature review, and manuscript writing. Merve İNANIR contributed to the design of the experimental study, interpretation of the data, and manuscript writing.

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