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Death receptor-dependent apoptosis and cell cycle delay induced by bioymifi in human cervical cancer cells

Ahmet Altun[1]*, Zeynep Deniz Şahin Inan[2] and Fatih Yulak[3]
[1]Department of Pharmacology, Sivas Cumhuriyet University, Sivas, Turkey
[2]Department of Histology and Embryology, Sivas Cumhuriyet University, Sivas, Turkey
[3]Department of Physiology, Sivas Cumhuriyet University, Sivas, Turkey

Abstract: In chemotherapy applied against cervical cancer, non-specific cytotoxicity and drug resistance that develops over time are trying to be overcome. Therefore, the development of effective and innovative chemotherapeutic drugs for the treatment is among the priority issues in the medical field. The anticancer activity of the Bioymifi, which can activate apoptosis by inducing DR-5 clustering and aggregation against the human cervical cancer cell line, was investigated in the current study. The cytotoxic activity of Bioymifi on the HeLa cell line was identified using XTT assay. The pathway of the cell death mechanism was analyzed through the cell cycle and Annexin V assays by the flow cytometry. DAPI staining assay was applied under fluorescence microscopy to examine the nuclear morphology. Bioymifi appeared to have a remarkable IC_{50} value (11.75µM) against HeLa cells. The cell cycle analysis demonstrated the increase of Bioymifi cured HeLa cells in the S phase. And also, 11.75µM of Bioymifi caused a significantly higher apoptotic effect compared to control. In addition, *in vitro* immunofluorescence experiments of this study represented that Bioymifi reduced Ki-67 localization in HeLa cells. Bioymifi has significantly anticancer actions in Human cervix cancer *in vitro* and can be combined with standard treatment.

Keywords: Apoptosis, bioymifi, cell cycle, cervical cancer, death receptor.

INTRODUCTION

Cervical cancer, which continues to be the second most common malignant tumor in women worldwide, contributes to more than 500,000 newly diagnosed cases, resulting in 250,000 deaths each year (Saenrueang *et al.*, 2019). To date, standard treatments include hysterectomy, radiotherapy and combination chemotherapy with cisplatin and 5-fluorouracil. While current treatment options provide the expected response in 60-90% of early diagnosed patients, the prognosis remains poor for patients suffering from advanced or recurrent cervical cancer. Therefore, a more specific and safer treatment is needed to increase survival rates, expand the spectrum of chemotherapy, and overcome possible drug resistance (Johnson *et al.*, 2019; Buskwofie *et al.*, 2020).

Apoptosis, which can be induced by two main signalling pathways, the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway, point to among the targeted anticancer treatment strategies in malignant cells. Tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL), which is associated with the TNF superfamily and has a poor cytotoxic effect on normal cells, activates the extrinsic apoptosis pathway through its five different receptors, especially DR-4 (TRAILR1) and DR-5 (TRAILR2) (Holbrook *et al.*, 2019). As a result of this activation, it takes Fas-associated protein with the death domain (FADD) and forms a series of caspase pathways (pro-caspase-8, pro-caspase-10, and caspase-3) and a signalling complex (DISC) that causes apoptotic death of cells (Aubrey *et al.*, 2018). While selective activation of the apoptotic pathway by death receptor-targeted agents provides enormous therapeutic potential for cancer therapy, findings from clinical studies suggest that targeting DR-4 and DR-5 with agonist antibodies or recombinant TRAIL selectively eliminates tumor cells while sparing normal cells (Yuan *et al.*, 2018). Moreover, expression of DR-4 and DR-5 in many cancer types, including cervical cancer cell lines, is induced by radiotherapy and chemotherapy, mediating the enhancement of TRAIL's effects (Gadducci and Cosio, 2020; Hassanzadeh, 2018).

As a result of high-throughput screening and medicinal chemistry and structure-activity relationship studies, Bioymifi, a small molecule agonist that directly targets the TRAIL receptor DR5 and induces the extrinsic apoptosis pathway, has been identified (Wang *et al.*, 2013). Bioymifi, which binds to the extracellular region of the DR5 receptor with high affinity independent of TRAIL and causes its oligomerization, has been shown to mediate the reduction of cell viability in various human cancer cell lines (Bai *et al.*, 2020). Here, we summarize our efforts to uncover the mechanisms that mediate the anticancer activity of Bioymifi on the HeLa cervical cancer cell line using flow cytometry analysis, histopathological and morphological observation assays.

**Corresponding author:* e-mail: md.ahmetaltun@gmail.com

MATERIALS AND METHODS

Cell culture conditions and reagents

Human cervix cancer (HeLa; CCL-2) cell line was obtained from the American Type Culture Collection (ATCC, UK). The cells were originated in DMEM (Dulbecco's Modified Eagle Medium) including 10% heat-inactivated FBS (Foetal Bovine Serum) and 1% antibiotics (penicillin and streptomycin) at 37°C in 5% CO_2.

Cell proliferation assay

The proliferation and number of cells were determined using a hemocytometer after using the trypan blue staining method. Bioymifi was dissolved in DMSO (DMSO final administration did not overrun 0.1%) and diluted in phenol red-free DMEM before handling. Growing cells were cultured in 96-well microplates (2×10^4 cells per well) in 50µL of phenol red-free DMEM culture media, incubated with various concentrations of 50µL Bioymifi (6.25, 12.5, 25, 50 and 100µM) for 24h. The cell viability was assigned by the XTT assay kit (Sigma Aldrich). Briefly, 50µL XTT labeling mixture (To prepare the activated XTT kit solution, the activation reagent and the XTT solution were mixed in a 5:1 ratio.) was suffixed to each well for the detection of alive cells and afterward, the plates were maintained at culture conditions for 4h. The absorbance of XTT formazone dye was evaluated by a spectrophotometer (ELISA reader; Thermo, Germany) at 450nm with reference at 630nm. The experiments were applied with triplicates and the results of the inhibition of cell proliferation results were presented as a % of control.

Apoptosis assay

Apoptosis was assigned by flow cytometry analysis of phosphatidylserine externalization. The Muse® Annexin V & Dead Cell kit (the Guava® Muse® Cell Analyzer, Luminex) was used for quantitative analysis of apoptotic and non-apoptotic cells following the user guideline. Briefly, the HeLa cells were seeded at a density of 6×10^5 cells/well in a six-well plate. The cells were incubated with Bioymifi at concentrations of 50% inhibition of cell growth (IC_{50}) 11.75µM, for 24h. After handling with the compound, the cells were gathered, resuspended with PBS including 1% FBS and maintained with the Muse® Apoptosis Assay kit reagent for 20min at 20-22°C in the dark.

Cell cycle distribution analysis

The cell cycle measurement was detected by the Guava® Muse® Cell Analyzer via Muse® Cell Cycle Assay Kit (Luminex, India) in accordance with the user's guide. After incubation with the IC_{50} of Bioymifi at culture conditions for 24h, the cells were centrifuged and fixed for 3hours at -20°C. The fixed cells were then stained with cell cycle assay reagent and incubated at 20-22°C for half an hour. After staining, different stages in the cell cycle were presented as a percentage by using the Guava® Muse® Cell Analyzer.

Immunofluorescent analysis

After induction by conditioned culture medium as indicated, the HeLa cells were fixed by 4% cold paraformaldehyde (pH: 7.4) for 10min and penetrated by 0.1% Triton X-100 for 30min (Thermo, USA) for immunofluorescence. The cells were incubated with Anti-Caspase-3 antibody (Abcam, USA) and Anti-Ki-67 antibody (Abcam, USA) for 30min following the user's guide, followed by labelling with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488, Abcam, USA) for 1h at 20-22°C. Then, 4′, 6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, Germany) staining was enforced on cells for 5min in the dark conditions. The cells were observed under a fluorescent microscope (Olympus BX51, Japan).

STATISTICAL ANALYSIS

All statistical analysis was run on GraphPad Prism 7 (GraphPad Software). The statistical differences between the two groups were analyzed by the Tukey test and the Student's t-test. Besides, Kruskal-Wallis and Mann-Whitney U tests were applied for nonparametric tests. All data are expressed as mean ±SD. $p<0.01$ was regarded as statistically significant.

RESULTS

Bioymifi inhibits cell proliferation in HeLa cells

In the current study, our first purpose was to appraise the inhibitory effects of Bioymifi on the HeLa cells for 24h. The cells were exposed with an increased concentration of 6.25, 12.5, 25, 50 and 100µM of Bioymifi and computed IC_{50} value. As seen in fig. 1, the proliferation of cells was markedly inhibited for 24hours with Bioymifi at concentrations of 12.5µM and higher. The IC_{50} value of Bioymifi was calculated as 11.75µM.

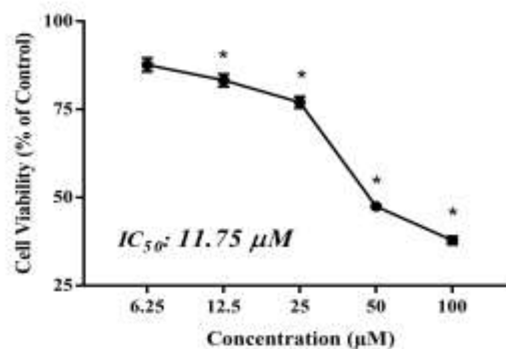

Fig. 1: Antiproliferative activity of Bioymifi on the HeLa cells for 24 h. The cell proliferation was determined by XTT assay. All data are indicated as mean ± SD in triplicate. The differences are defined as * from control $p<0.01$.

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Bioymifi increases apoptosis in HeLa cells

The assessment of the apoptotic effects of 11.75µM Bioymifi on the HeLa cell line was administered by the Annexin V assays through flow cytometry. As seen in fig. 2a and 2b, when compared to control and 11.75µM of Bioymifi reasoned a notably increased apoptotic effect ($p<0.01$). The total apoptotic cell population percentage was determined as 45.65% on the HeLa cells treated with Bioymifi 11.75µM.

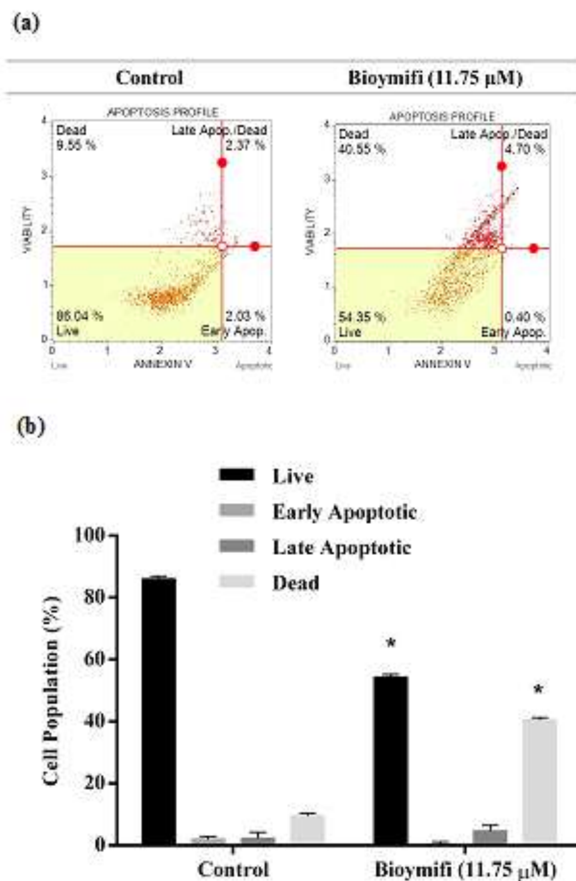

Fig. 2: (a) The quadrant graphs show the apoptotic effects of Bioymifi on the HeLa cells for 24h. (b) The percentage of non-apoptotic cells, early apoptotic cells, late-stage apoptotic cells, and necrotic cells.

Bioymifi leads to cell cycle arrest in hela cells

Our previous experiments disclosed that Bioymifi restricted cell viability and stimulated apoptotic cell death considerably when compared to the control group. In this step, the effect of the compound on cell cycle arrest was assigned by using the cell cycle assay kit to search whether or not the Bioymifi stimulated proliferation inhibition of cells was caused by changes in the cell cycle. The HeLa cells were applied with 11.75µM of Bioymifi and the results displayed a slight increment in the cell percentage during the S phase in the Bioymifi handled group (22.0±1.25%) when compared to control (17.6±1.08%) ($p<0.01$; fig. 3a, 3b).

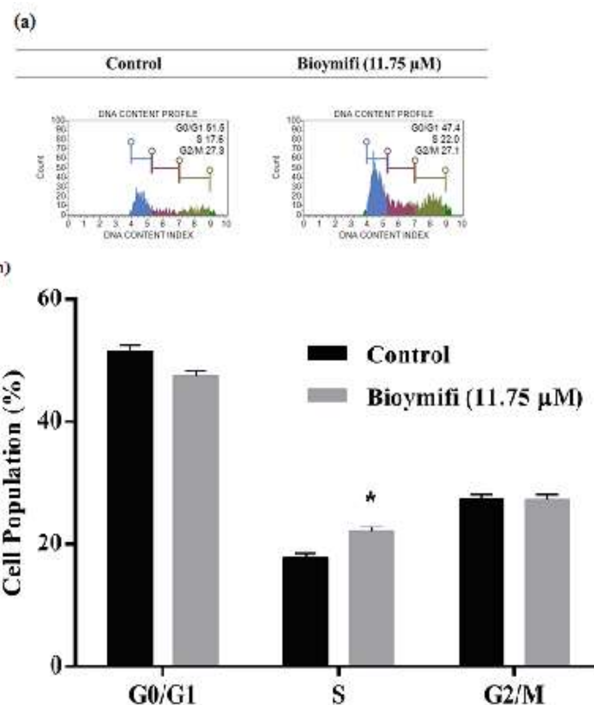

Fig. 3: (a) Effect of Bioymifi on cell cycle phases (G0/G1, G2/M and S) on the HeLa cells for 48 h. (b) The bar graphs indicating quantified values of the Guava® Muse® Cell Analyzer data.

Bioymifi leads to an increase in caspase-3 localization and decreased ki-67 localization

In immunofluorescent labeling using Caspase-3 and Ki-67 antibodies, an increase in the localization of Caspase 3 (fig. 4), which is used as an apoptosis marker, and a decrease in the localization of Ki-67 (fig. 5), which is used as a proliferation marker, was observed compared to the Bioymifi-induced group control group.

DISCUSSION

Our knowledge about the mechanisms underlying cancer, its progression, diagnosis, and treatment options is increasing day by day tremendously, but the incidence and mortality rates continue to increase, unfortunately (Kumar *et al*., 2018). Despite the existence of constantly developing early screening tests, cervical cancer is one of the most common malignancies encountered in women worldwide. Although chemotherapy continues to maintain its importance as a very important approach in the treatment of the disease, new target-specific treatment strategies are needed to overcome the chemoresistance that develops in advanced and recurrent cases (Xu *et al*., 2021). In this context, we summarize for the first time that Bioymifi, which induces the extrinsic apoptosis pathway by death receptor agonism, suppresses proliferation, stimulates apoptosis and cell cycle arrest in human cervical cancer *in vitro*.

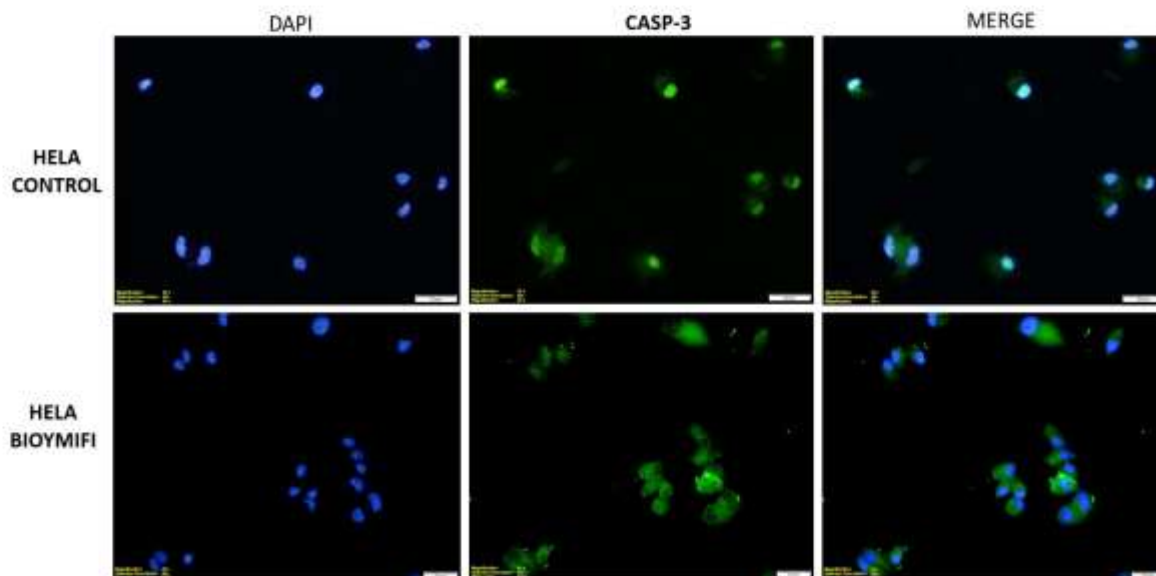

Fig. 4: In the HELA cell line, cell nuclei were imaged in blue by DAPI staining and green with Alexa Fluor 488, the secondary antibody used to make the CASP-3 primary antibody visible in the cell cytoplasm, and the images were merged. It was determined that CASP-3 localization increased in the experimental group.

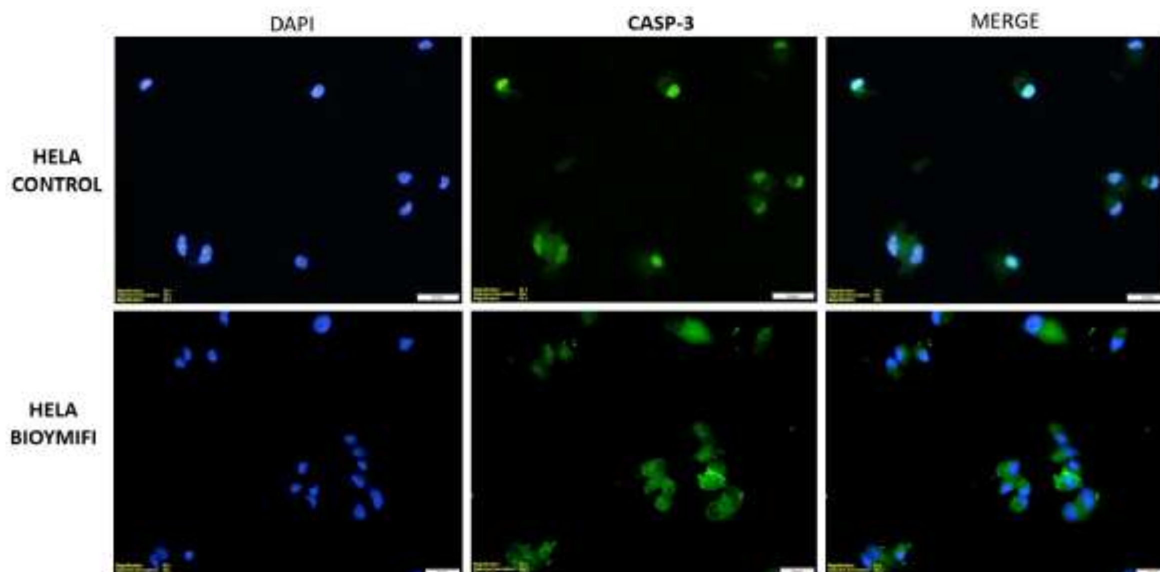

Fig. 5: In the HELA cell line, cell nuclei were visualized in blue by DAPI staining and green with the Alexa Fluor 488 secondary antibody used to highlight the KI-67 antibody localized in the cell nucleus and the images were merged. It was determined that KI-67 localization decreased in the experimental group.

Death receptors, which are among the members of the tumor necrosis growth factor (TNF) receptor gene superfamily, mediate many physiological functions such as cell differentiation, survival, cell death and regulation of the immune system. TRAIL is a member of the TNF-protein superfamily, which has the mechanism of activation of caspases via the extrinsic apoptosis pathway and selective apoptosis induction in malignant cells by binding to the death receptors on the cell membrane, especially DR-4 and DR-5 receptors from its outer surface. Normal cells are protected from TRAIL-induced death signaling complex via DR-1 and DR-2 decoy receptors and thus, TRAIL only induces tumor cells to apoptosis via DR-4/DR-5. In addition to its gorgeous mechanism of action, the induction of apoptosis it causes can be increased by combinations of radiotherapy and chemotherapy, making it a very promising candidate for the development of targeted anticancer agents (Oh and Sun, 2021). It has been shown that TRAIL has an active role in many malignancies, both solid (breast, bladder, prostate, cervix, etc.) and hematological (leukemia, lymphoma, multiple myeloma), and significantly reduces

cell viability in these cancer cells (Hassanzadeh, 2018; Yin *et al*., 2019; Ye *et al*., 2017).

While the recognition of TRAIL as a selective inducer of apoptosis against cancer cells has been met with grand excitement, it led to the improvement of new TRAIL pathway agonists, including the TRAIL ligand, antibodies against DR-4, and DR-5, as new strategies in cancer therapy. Bioymifi, a selective DR-5 (TRAIL-receptor) receptor agonist, induces the intracellular apoptosis signaling cascade by binding to the extracellular death domain of the DR-5 receptor. Initial studies have been showing that it induces apoptosis even in cancer cells that do not respond to TRAIL. Furthermore, it is also stated that the critical playmaker in the induction of apoptosis induced by Bioymifi is the concentration at which Bioymifi is administered, rather than DR-5 receptor expression. This effect of Bioymifi is mediated by its ability to aggregate DR-5 receptors, making it a valuable candidate for the treatment of many types of cancer (Wang *et al*., 2013). Based on this proposal, we first prosperously determined the cytotoxic activity of Bioymifi, the synthetic chemical compound we know to induce apoptosis by directly targeting the TRAIL receptor DR-5 in HeLa cells. To study the cytotoxic effect of Bioymifi, we treated HeLa cells with increasing concentrations of this DR-5 agonist compound, resulting in significant cell growth inhibition. As expected, HeLa cells treated with increasing concentrations of Bioymifi showed weaker proliferation than control HeLa cells. The IC_{50} value for Bioymifi after 24hours of application was calculated as 11.75µM. Wang *et al* investigated the effect of Bioymifi on cell proliferation in a variety of cancer cell lines (Non-small-cell lung cells; H460, H1155, osteosarcoma cells; U2OS, colorectal adenocarcinoma cells; HT29 and pancreas carcinoma Miapaca-2 cells), including the HeLa cervical cancer cell line. With the IC_{50} value varying between 3-10µM, Bioymifi has attracted attention for DR-5-mediated apoptosis induction and anticancer effect (Wang *et al*., 2013).

Growing evidence has indicated that the extrinsic apoptosis pathway is induced in cancer cells as a result of agonist-mediated stimulation of the DR-5 receptor. Based on the IC_{50} value, we aimed to elucidate the mechanism of anti-cancer activity by using apoptosis, cell cycle, histopathological and morphological methods. Bioymifi was found to significantly induce apoptosis on the HeLa cells at a rate of 45.65% apoptotic cells after 24hours compared to control. In our tests to investigate the effects of Bioymifi with DR-5 targeted agonist effect on cell life cycle *in vitro*, TRAIL-mimetic effect has enabled us to obtain satisfactory results in the treatment of cervical cancer. When we investigated the effect on the cell cycle with the Guava® Muse® Cell Analyzer, we found that a significant increase in the S phase was observed in 11.7µM Bioymifi treated cells compared to the control.

Antigen KI-67 is a protein that in humans is encoded by the MKI67 gene. Antigen KI-67, a protein encoded by the MKI67 gene in humans, is a great marker for determining the growth fraction of a cell population because it is a nuclear protein associated with cell proliferation (Menon, 2019). Herein, we examined the Ki-67 levels of the HeLa cells treated with IC_{50} concentration of Bioymifi to identify the Bioymifi effect on cell growth. *In vitro*, immunofluorescence experiments of this study displayed that Bioymifi reduced Ki-67 localization in HeLa cells.

Undoubtedly, the most important role in maintaining the balance between cell proliferation and cell death is in apoptosis signaling mechanisms. Drug candidates used to induce cell apoptosis *in vitro* have been recognized as an effective means for targeted cancer therapy. Stimulation of the DR-5 receptor leads to increased caspase-3 activity through the intracellular apoptosis signaling cascade (Wu *et al*., 2016). Activated caspase-3 plays a critical role in the extrinsic and intrinsic pathways of apoptosis (Xu, 2019). In this study, we also proved that Bioymifi promoted cell apoptosis with higher caspase-3 activity.

Further researches should focus on *in vivo* and clinical efficacy and safety studies based on the findings presented here from the DR-5 receptor agonist Biomifi, which could lead to the identification of novel compounds with higher activity, providing a basis for the future development of the TRAIL pathway and DR-5-mediated cancer therapies.

CONCLUSION

Due to TRAIL can selectively affect cancer cells, the TRAIL signaling pathway is a very attractive therapeutic target. Unfortunately, many cancer cells become resistant to TRAIL signaling over time. Therefore, small molecules capable of sensitizing cancer cells to TRAIL are needed for cancer therapy. Considering the studies in the literature, we think that death receptor agonists can be used alone or in combination with other chemotherapeutic agents in the treatment of cancer without significant toxicity. There are currently no studies showing the possible effects of Bioymifi on proliferation, apoptosis and cell cycle in the human cervical cancer cell line HeLa. We conclude that the results obtained from studies in the literature are a probably higher expression of DR-5 in cervical cancer. However, further studies are needed to determine whether cell surface death receptors are expressed more in HeLa cells than in normal cells and to determine the *in vivo* and clinical effects of the Bioymifi molecule.

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