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Combination of Umbelliprenin and Arsenic Trioxide Acts as an Effective Modality Against T-Cell Leukemia/Lymphoma Cells

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Abstract

Adult T-cell leukemia/lymphoma (ATLL) is a serious blood malignancy with distinct geographical distribution. ATLL patients have a short survival time because of intrinsic chemoresistance and severe immunosuppression. To introduce a novel treatment, we investigated whether umbelliprenin (UMB), a natural coumarin derivative, could improve the toxicity of arsenic trioxide (ATO) on ATLL cells. To determine the viability of MT-2 cells upon treatment with different concentrations of UMB and ATO, alamarBlue assay was applied. Cell cycle analysis was carried out by propidium iodide staining and the expression of candidate genes was assessed by quantitative reverse transcription-polymerase chain reaction. Our findings revealed that combination of UMB and ATO induced considerable cytotoxic effects on ATLL cells. Flow cytometry analysis indicated accumulation of MT-2 cells in the sub G_1 phase of the cell cycle after combinatorial treatment. In addition, significant downregulation in *BMI-1, CD44, c-MYC,* and *nuclear factor-κB (REL-A)* expression was observed after UMB + ATO administration. Agents with low side effects are potential candidates for novel cancer treatments. We demonstrated, for the first time, that combination of UMB and ATO might be regarded as an effective regimen for ATLL treatment.

Keywords

adult T-cell leukemia/lymphoma, umbelliprenin, arsenic trioxide, combination

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Introduction

Human cancers are major health concerns worldwide, as more than 18 million new cases and 9.6 million related deaths were reported globally in 2018.[1] Adult T-cell leukemia/lymphoma (ATLL) is a non-Hodgkin lymphoma caused by human T-cell leukemia virus type 1 (HTLV-1). This peripheral T-cell neoplasm shows distinct geographical distribution, as the main HTLV-1 endemic regions include Japan, sub-Saharan Africa, South America, the Caribbean area, and foci in the Middle East and Melanesia.[2] Current treatments for ATLL depend on the disease subtypes that are acute, lymphoma, chronic, and smoldering. Among chemotherapeutic regimens, interferon-α/zidovudine (IFN/AZT) is an antiviral drug that is used in the treatment of acute, chronic, and smoldering types.[3,4] Arsenic trioxide (ATO) is a chemical agent that is more effective when used in combination with IFN/AZT.[5] Despite the use of various anticancer drugs, ATLL has a poor prognosis because of intrinsic chemoresistance and

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severe immunosuppression. In addition, ATLL affects multiorgans such as lymph nodes, bone marrow, liver, and the central nervous system that all make the management and therapy of this malignancy more challenging.[3,6]

The viral regulatory protein, Tax, is thought to play indispensable roles in the oncogenic process of ATLL, such as activation of nuclear factor-κB (NF-κB) and Akt signaling pathways, induction of several anti-apoptotic proteins, and inhibition of *P53* expression.[7-9] In addition, Tax-mediated NF-κB activation induces the expression of cell migration factors, resulting in the infiltration of infected T-lymphocytes.[10,11] Elevated expression of *CD44* and *c-MYC*, which was determined in HTLV-1-infected cells, has been correlated with the poor prognosis and severity of ATLL.[12,13] Moreover, upregulation of *BMI-1* has been associated with drug resistance of hematologic malignancies such as chronic myeloid leukemia, acute myeloid leukemia, and lymphoma.[14]

Umbelliprenin ($C_{24}H_{30}O_3$, UMB) is a natural 7-prenyloxycoumarin mainly found in *Ferula* species (Apiaceae).[15] Beside anti-inflammatory, immunomodulatory, antioxidant, and anti-leishmanial effects of UMB, the great anticancer activity of this agent has been shown in gastric, colorectal, and breast carcinomas.[16-18] Specifically, inhibitory effects on matrix metalloproteinases, signaling pathways, pro-inflammatory enzymes, and drug efflux pumps, as well as induction of apoptosis and cell cycle arrest, are main proved mechanisms of UMB anticancer actions.[16-18] Nevertheless, there are no reports on the toxic effects of UMB, either alone or in combination with other agents, on HTLV-1-infected human lymphocytes. Hence, this study was aimed to determine whether UMB has the potential to improve ATO efficacy in vitro. In this regard, the viability of MT-2 cells, a human ATLL cell line, was determined upon administration of UMB and ATO, alone and in combination. Moreover, changes induced in the cell cycle were analyzed by flow cytometry, and the expression of candidate genes was assessed by quantitative polymerase chain reaction (qPCR).

Materials and Methods

Preparation of UMB

UMB (M_W: 366.5 g/mol) was extracted from *Ferula szowitsiana*, as previously described.[19] Briefly, the roots were powdered and extracted thoroughly by maceration with acetone at room temperature. Then, the obtained extract was concentrated under vacuum and UMB was separated by silica gel layer chromatography. To prepare different concentrations of UMB, 2 mg of the crystal powder was dissolved in 100 μL of dimethyl sulfoxide (DMSO; Merck) and immediately diluted with complete culture medium before use. To note, equal amounts of DMSO in all UMB concentrations (0.2% and 0.1%, v/v) were considered as control treatments to eliminate the toxic effects of the solvent.

Cell Culture, Treatment, and Viability Assessment

MT-2 cells were obtained from the Pasteur Institute of Iran. The cells were grown in Rosewell Park Memorial Institute 1640 medium (Biosera) supplemented with 10% fetal bovine serum (Gibco) and incubated in a 5% CO_2 humidified atmosphere at 37 °C. To determine the half-maximal inhibitory concentration (IC_{50}) of UMB and ATO, cells were seeded at a density of 5×10^4 cells/well in 96-well cell culture plates (SPL) and treated with different concentrations of either UMB (54, 82, 110, and 136 μM) or ATO (Sigma; 2, 4, 8, and 16 μM) for 24, 48, and 72 h. Then, to investigate combinatorial effects, the cells were treated with non-toxic UMB (68 μM) + ATO (2 μM) for 72 h, while 0.1% DMSO + ATO was considered as a control.

For quantitative analysis of cell viability, alamarBlue assay was performed. In summary, alamarBlue dye (Sigma) was dissolved in phosphate-buffered saline (PBS; 0.1 mg/mL) and added to each well (20 μL/well) at the end of each time point. Afterwards, plates were incubated for 2 h at 37 °C in the dark, and optical density (OD) was measured at 600 nm using a microplate reader (Epoch). For calculation of cell

Table 1. The Sequences of Primers and Probes Used in This Study.

Genes	Sequence (5′→3′)	Products size (bp)
BMI-1	Forward: CTGCAGCTCGCTTCAAGATG	192
	Reverse: CACACACATCAGGTGGGGAT	
c-MYC	Forward: ACTCTGAGGAGGAACAAGAA	159
	Reverse: TGGAGACGTGGCACCTCTT	
CD44	Forward: CGGACACCATGGACAAGTTT	176
	Reverse: GAAAGCCTTGCAGAGGTCAG	
NF-κB (REL-A)	Forward: ACCCCTTCCAAGTTCCTATAGAAGAG	145
	Reverse: CGATTGTCAAAGATGGGATGAGAAAG	
	Probe: FAM-ACTACGACCTGAATGCTGTGCGGCTCT-BHQ-1	
β2M	Forward: CTTGTCTTTCAGCAAGGACTGG	127
	Reverse: CCACTTAACTATCTTGGGCTGTG	
	Probe: FAM-TCACATGGTTCACACGGCAGGCAT-BHQ-1	

Abbreviations: NF-κB, nuclear factor-κB; *β*2M, *β*2-microglobulin.

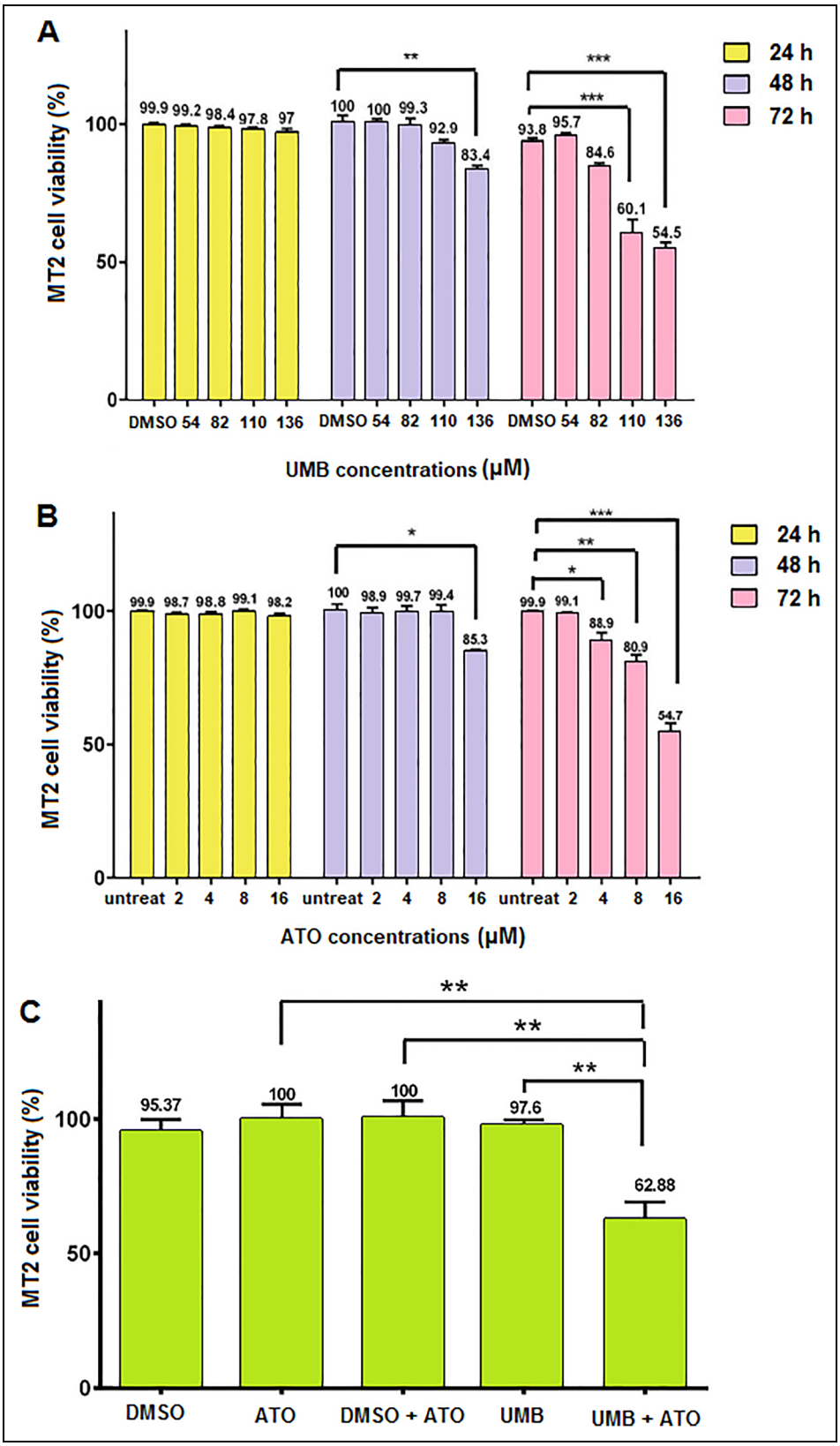

Figure 1. Viability assessment of MT-2 cells after single and combinatorial treatments with UMB and ATO. Effects of UMB (A) and ATO (B) were time- and dose-dependent; the IC_{50} of UMB and ATO was >136 μM and > 16 μM after 72 h, respectively. After cells were treated with 68 μM UMB, 2 μM ATO, and their combination for 72 h (C), the calculated viability of each treatment was 97.6%, 100%, and 62.8%, respectively. All values are represented as mean ± SEM and differences are shown as $*p < .05$, $**p < .01$, and $***p < .001$.
Abbreviations: ATO, arsenic trioxide; IC_{50}, half-maximal inhibitory concentration; SEM, standard error of the mean; UMB, umbelliprenin.

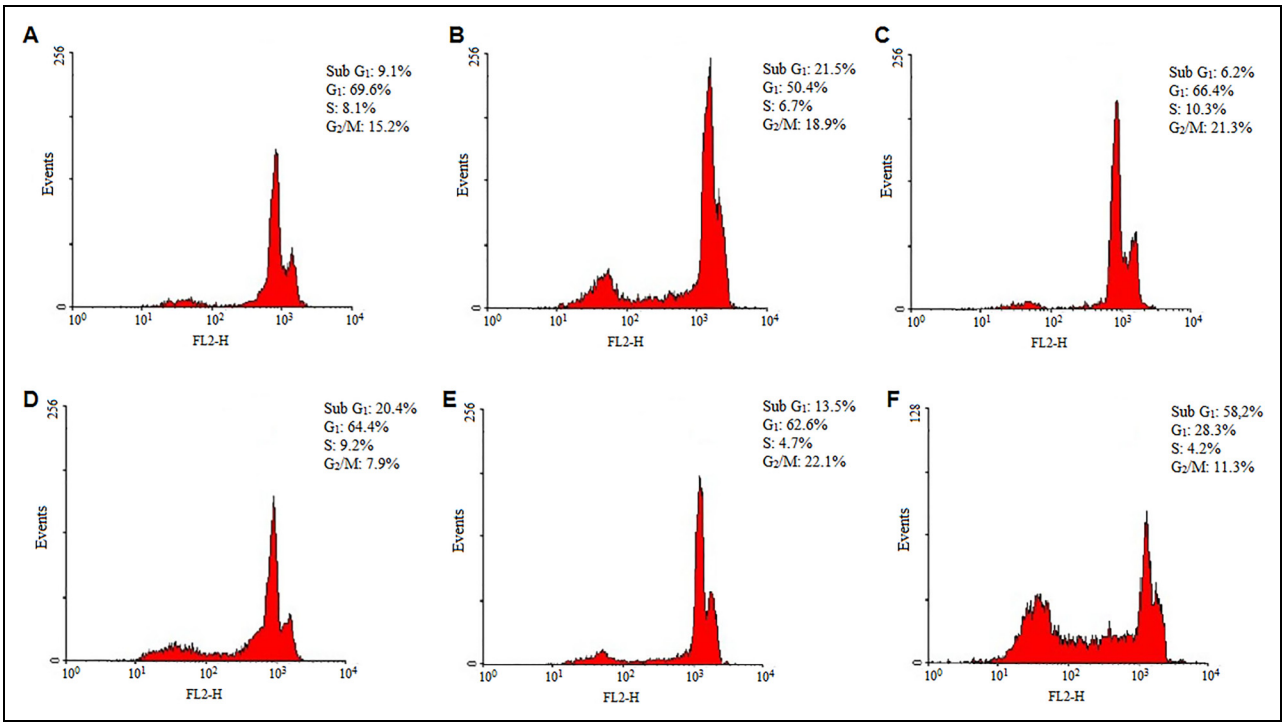

Figure 2. Effects of UMB, ATO, and their combination on MT-2 cell cycle progression after PI staining. Percentage of cells in the sub G_1, G_1, S, and G_2/M phases of the cell cycle with no treatment (A) and upon 72 h treatment with 2 μM ATO (B), 0.2% DMSO (C), 68 μM UMB (D), 0.1% DMSO + 2 μM ATO (E), and 68 μM UMB + 2 μM ATO (F).
Abbreviations: ATO, arsenic trioxide; DMSO, dimethyl sulfoxide; PI, propidium iodide; UMB, umbelliprenin.

viability (%), the following formula was used: $100 \times (100 - [OD_T - OD_U]/[OD_B - OD_U])$, in which OD_T, OD_U, and OD_B were the OD of treated cells, untreated cells, and blank control, respectively.

Cell Cycle Analysis

To study the cell cycle changes induced by our treatments, MT-2 cells were stained with propidium iodide (PI; Sigma). Briefly, treated cells and their relevant controls were harvested and washed with PBS twice. Then, cells were incubated in a hypotonic buffer containing 100 μg/mL PI in 0.1% sodium citrate and 0.1% Triton X-100 for 30 min at 37 °C in the dark, and finally analyzed by flow cytometry (BD FACSCalibur) using an FL2 filter.

Gene Expression Analysis

To determine whether the combination of UMB and ATO altered the expression pattern of our candidate genes, qPCR was applied. In summary, the total cellular RNA was extracted from treated cells, and the relevant controls using TriPure (Roche), and complementary DNAs (cDNAs) were synthesized by a RevertAid first-strand cDNA synthesis kit, according to the manufacturer's protocol (Thermo Scientific). The fidelity of amplified cDNAs was then confirmed by polymerase chain reaction (PCR) using hose-keeping primers, and amplicons were separated and viewed on 2% agarose gel (Invitrogen). qPCR was conducted in a Rotor-Gene Q real-time PCR machine (Qiagen) using either specific primers or probes for each gene (Table 1). The expression level of *BMI-1*, *CD44*, and *c-MYC* was assessed using SYBR green master mix (Takara) and PCR conditions were 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s For analysis of NF-κB (REL-A) expression, TaqMan probe (Takara) was used and PCR conditions were 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. To note, *β2-microglobulin* was used as internal control, and data were analyzed by a standard curve relative method.

Statistical Analysis

One-way analysis of variance and Tukey test were performed using SPSS software to assess the statistical difference between groups. The results are expressed as mean ± standard error of the mean (SEM), and *p*-values <.05, <.01, <.001, and <.0001 were considered to be statistically significant.

Results

To investigate the combinatorial effects of UMB and ATO, at first, the IC_{50} of each agent was determined after 72 h. As

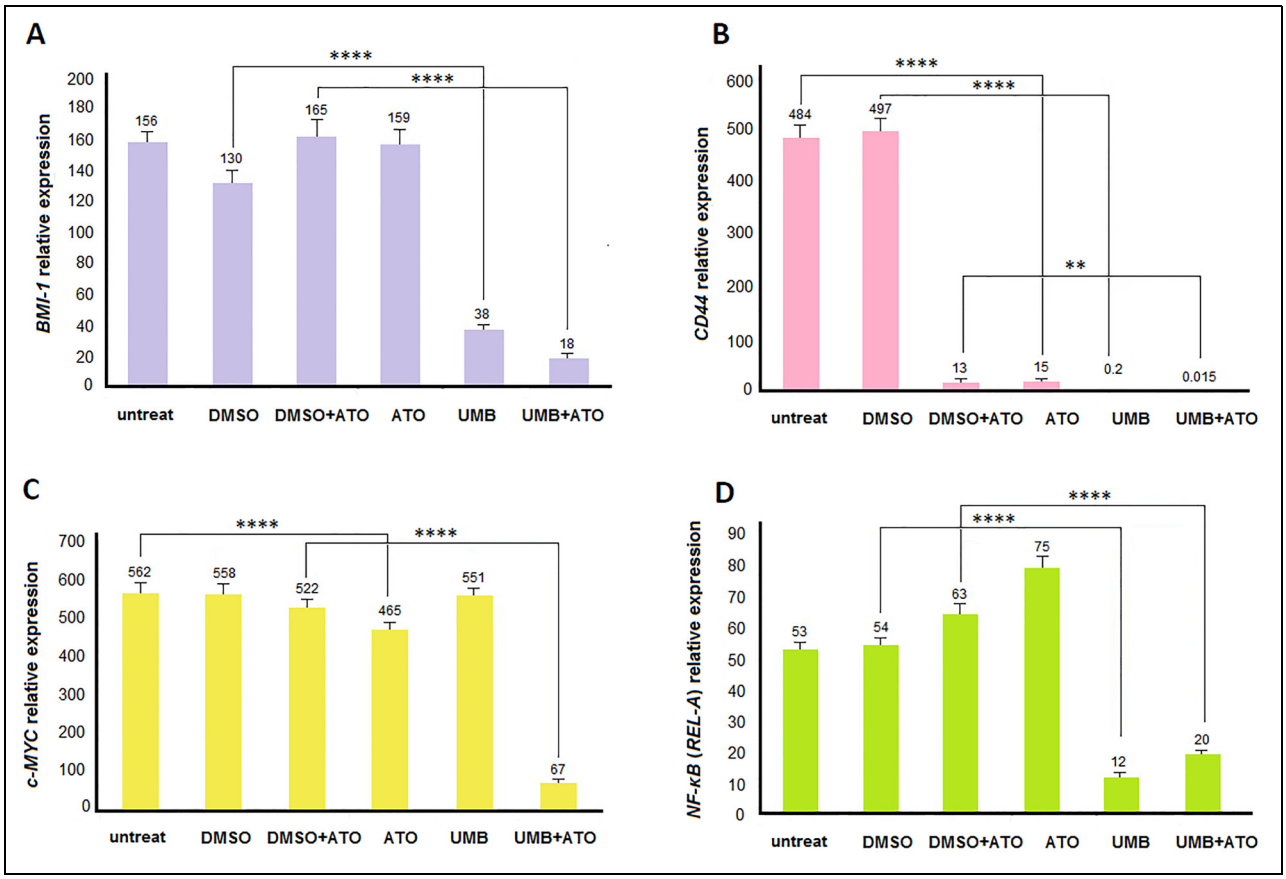

Figure 3. The expression pattern of *BMI-1*, *CD44*, *c-MYC*, and *NF-κB* (*REL-A*) upon single and combinatorial treatments with UMB (68 μM) and ATO (2 μM) for 72 h. UMB and UMB + ATO treatments significantly decreased *BMI-1* expression compared to their relevant controls (A). UMB, ATO, and UMB + ATO treatments significantly down-regulated *CD44* expression compared to their relevant controls (B). Treatment of MT-2 cells with ATO and UMB + ATO significantly decreased *c-MYC* expression compared to their relevant controls (C). *NF-κB* (*REL-A*) expression in UMB and UMB + ATO treated groups was significantly lower than that of their relevant controls (D). All gene expression values are represented as mean ± SEM and differences are shown as $^{**}p < .01$ and $^{****}p < .0001$.
Abbreviations: ATO, arsenic trioxide; NF-κB, nuclear factor-κB; SEM, standard error of the mean; UMB, umbelliprenin.

shown in Figure 1A and B, the IC_{50} of UMB and ATO was > 136 and >16 μM on MT-2 cells, respectively. Then, viability assessment of cells revealed that upon treatment with 68 μM UMB + 2 μM ATO, concentrations lower than their IC_{50}, UMB improved cytotoxicity of ATO up to 37.1% (Figure 1C).

To determine whether the observed effects of UMB + ATO were associated with cell cycle changes, the DNA content of cells was assessed by flow cytometry. As presented in Figure 2, 21.5%, 6.27%, and 20.4% of MT-2 cells were detected in the sub G_1 phase of the cell cycle after 72 h treatment with DMSO, ATO, or UMB alone, respectively. In DMSO + ATO treatment, 13.5%, 62.68%, and 22.17% of cells were present in the sub G_1, G_1, and G_2/M phases, respectively. Intriguingly, the combination of UMB + ATO led to the accumulation of cells in the sub G_1 phase, as 58.24%, 28.38%, and 11.31% of cells were detected in the sub G_1, G_1, and G_2/M peaks on DNA content histograms, respectively.

To elucidate the molecular mechanism of our combinatorial approach, the expression pattern of candidate genes was examined after UMB + ATO treatment. Figure 3A to D shows relative quantitation in *BMI-1, CD44, c-MYC,* and *NF-κB (REL-A)* expression compared to the corresponding control groups. As indicated, UMB alone and in combination with ATO induced a significant ($p < .0001$) decrease in *BMI-1* expression when compared with relevant controls. Meanwhile, single-use of UMB and ATO significantly ($p < 0.0001$) down-regulated *CD44* expression and such decrease was also observed when both agents were used in combination. In addition, *c-MYC* expression significantly ($p < .0001$) decreased after treatment with ATO and UMB + ATO in comparison with their relevant controls, while UMB alone did not induce significant effects. Finally, significant ($p < .0001$) downregulation in *NF-κB* (*REL-A*) expression was detected upon single and combinatorial use of UMB, but not ATO alone.

Discussion and Conclusion

ATLL is a malignancy of adult T lymphocytes with a weaker prognosis compared to other non-Hodgkin's invasive lymphomas. Excessive expression of drug efflux pumps and anti-apoptotic proteins in ATLL cells are among the reasons that impair the efficacy of chemotherapy.[3]

Although anti-neoplastic and differentiation-inducing effects of natural agents have been reported on human leukemia and lymphoma cells,[20,21] finding new and more effective therapeutic approaches that could improve clinical outcomes is still a critical demand. ATO is a routine chemotherapy drug prescribed for ATLL, and UMB is a natural coumarin with valuable pharmaceutical effects. In the present attempt, we examined whether combination of UMB and ATO could enhance cytotoxicity against HTLV-1 transformed human T-cell leukemia cells. The obtained findings revealed that treatment of MT-2 cells with 68 μM UMB + 2 μM ATO significantly decreased cell viability, which was confirmed by the accumulation of cells in the sub G_1 phase of the cell cycle. Similarly, it has been previously reported that 12.5 μM UMB inhibited the growth of M4Beu cells via G_1 arrest, and at higher concentrations (≥25 μM), UMB-induced accumulation of cells in the sub G_1 phase.[22]

Examining the expression of *BMI-1, CD44, c-MYC,* and *NF-κB (REL-A)* revealed, to some extent, mechanisms underlying the observed combinatorial effects. The overexpression of *BMI-1*, a member of the polycomb transcription repressors, has been reported in different types of human cancers, such as leukemia, lung, gastric, and breast carcinomas.[23] In addition, upregulation of *BMI-1* has been associated with drug resistance and treatment failure, and thus, *BMI-1* is considered as a useful biomarker for the prognosis of cancer patients.[24] *CD44* expression, which is linked to malignant cellular activities such as metastasis and drug resistance, has also been detected on the surface of T-cell acute lymphoblastic leukaemia cells.[25] The present study indicated significant downregulation of *BMI-1* and *CD44* by UMB alone and in combination with ATO. Upregulation of *c-MYC*, a transcriptional factor that plays key roles in cell proliferation and apoptosis, was demonstrated in lymphoma and acute ATLL.[26] NF-κB (REL-A) interferes with cellular functions that promote oncogenesis and chemoresistance.[27] Since HTLV-1-Tax protein is a potent activator of *NF-κB*, expression of this gene is altered in HTLV-1-infected patients.[28] In this regard, several agents that can inhibit NF-κB function are used as chemotherapeutic drugs, including ATO.[27] The current study, which revealed significant downregulation of *c-MYC* and *NF-κB* (*REL-A*) by UMB + ATO, demonstrates that UMB could be considered as a potent natural coumarin to combat drug resistance of ATLL cells.

In conclusion, our study indicated, for the first time, that UMB has the potential to improve the effectiveness of ATO in ATLL cells. Future investigations, however, are necessary to define more precisely the mechanisms of such combination in ATLL and other lymphomas.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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