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Melphalan improves toxicity of arsenic trioxide in adult T-cell leukemia/lymphoma cells

Faeze Khodadadi [a], Mohammad Hadi Sadeghian [a], Zahra Delbari [b], Mohadese Kazemi [b], Hamide Koohpaykar [c], Fatemeh B. Rassouli [d,e,*]

[a] Cancer Molecular Pathology Research Center, Department of Hematology and Blood Bank, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Immunology Research Center, Inflammation and Inflammatory Diseases Division, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Department of Hematology and Blood Banking, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran
[d] Novel Diagnostics and Therapeutics Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran
[e] Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATLL) is a hematologic neoplasm with poor prognosis. Melphalan is an alkylating anti-cancer agent, and arsenic trioxide (ATO) is routine chemotherapy drug for ATLL with low response rate. Due to the significant challenge that chemoresistance poses in treating ATLL, we aimed to investigate the potential of melphalan to enhance the effects of ATO as a combinatorial treatment approach for ATLL.

MT-2 cells were exposed to different concentrations of melphalan and ATO and viability was evaluated by alamarBlue assay. Upon IC_{50} determination, cells were treated with 0.5 μg/ml melphalan and 2 μM ATO for 72 h, and changes induced on the cell cycle were analyzed by PI staining and flow cytometry, while the expression of candidate genes was assessed by quantitative PCR. For *in silico* analysis, the expression of *ABCG2* was assessed in MT-2 cells and ATLL subtypes using GEO database, and molecular docking was performed to predict the interaction of melphalan with this drug transporter.

Melphalan enhanced the cytotoxicity of ATO up to 32.05 %, and caused accumulation of cells in the sub G_1 phase of the cell cycle. Besides, combination of melphalan and ATO induced considerable reduction in *c-MYC, BMI-1*, *CD44* and *NF-κB* (*REL-A*) expression. Volcano plots revealed the overexpression of *ABCG2* in MT-2 cells and acute and smoldering ATLL subtypes, and molecular docking indicated favorable affinity of melphalan with ABCG2. Current findings provide valuable insights into the mechanism of action of melphalan and highlight the importance of targeting drug transporters in improving chemotherapy efficacy in ATLL.

1. Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a type of lymphoproliferative malignancy linked to the human T-cell lymphotropic virus type 1 (HTLV-1). This disease is characterized by a poor prognosis, largely due to its aggressive nature and the involvement of multiple organs, which complicates treatment and management strategies [1,2]. ATLL exhibits a distinct geographical distribution, with HTLV-1 infection being endemic in tropical regions. Notable areas include South America, Central Africa, the Caribbean, southern Japan, and parts of the Middle East [3–6]. ATLL is categorized into four main subtypes: acute, chronic, smoldering, and lymphomatous, each exhibiting distinct prognoses. The chronic and smoldering forms generally have a median survival ranging from two to five years, whereas the acute and lymphomatous types are more aggressive, with median survival times less than a year. Acute ATLL represents less than half of all cases, and survival rates differ significantly among the subtypes, with smoldering ATLL showing the highest overall survival at 52 %, followed by chronic at 36 %, lymphomatous at 14 %, and acute at only 11 % [7].

HTLV-1-infected cells express the viral Tax protein, which is essential for the development of ATLL. The Tax oncoprotein interacts with numerous host cellular proteins, facilitating the regulation of viral gene

* Corresponding author. Novel Diagnostics and Therapeutics Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran
E-mail address: behnam3260@um.ac.ir (F.B. Rassouli).

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expression. Additionally, it promotes the abnormal activation of signaling pathways, particularly NF-κB, which drives the clonal proliferation and survival of T cells harboring the HTLV-1 provirus. Research has demonstrated that both canonical and noncanonical NF-κB pathways are persistently activated in cell lines transformed by HTLV-1, in cells expressing Tax, and in samples from patients with ATLL [8–10]. Additionally, Tax can directly interact with NF-κB proteins, such as RelA, by binding to the Rel homology domain. This interaction promotes dimerization of the proteins, leading to increased DNA binding and enhanced transcriptional activity (Mohanty and Harhaj, 2020). Tax also drives the expression of important proteins, including c-MYC and CD44. These proteins collectively support cell survival and proliferation, inhibit apoptosis, and assist the cells in evading detection by the immune system [11,12]. Elevated level of c-MYC is linked to enhanced growth of leukemic cells and is considered as a significant prognostic indicator in patients with ATLL [13,14]. Likewise, the overexpression of CD44 is associated with various clinicopathological characteristics and is correlated with a poor prognosis in ATLL, as well as in acute myeloid leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma, and both acute and chronic lymphocytic leukemia [15,16]. Furthermore, dysregulation of BMI-1 plays a significant role in the development of hematologic malignancies and is linked to poor prognosis in T-cell lymphoma, acute and chronic myeloid leukemia, multiple myeloma, as well as acute and chronic lymphoblastic leukemia [17].

Melphalan ($C_{13}H_{18}Cl_2N_2O_2$) is an alkylating agent and a well-known chemotherapy drug that inhibits DNA and RNA syntheses. Currently, melphalan is used for treatment of several cancers including ovarian and breast cancers, multiple myeloma, neuroblastoma, lymphomas and acute leukemia ([18–20]; Bayraktar et al., 2012). Chemotherapeutic drugs including arsenic trioxide (ATO), zidovudine, cyclophosphamide, adriamycin and prednisolone are used as first-line treatments for ATLL [21–23]. Despite this treatment approach, the prognosis for ATLL remains poor. The primary factors contributing to these unfavorable clinical outcomes are intrinsic chemoresistance and significant immunosuppression [24]. Multiple regulatory mechanisms underlie the chemoresistance of lymphoblastic leukemia, including activation of oncogenic signaling pathways, inhibition of tumor suppressor functions, and alterations in drug transport and metabolism. These factors collectively lead to inadequate activation of apoptotic pathways in response to chemotherapy [25]. ATP binding cassette (ABC) proteins constitute a large family of membrane transporters that mediate the export of a diverse range of anticancer drugs. Among them, ABC subfamily G member 2 (ABCG2), also known as breast cancer resistance protein (BCRP), is involved in the extrusion of arsenic [26,27]. Given the significant challenge that chemoresistance poses in treating ATLL, where effective reversal agents are lacking, this study embarks on an exciting exploration. We aimed to investigate the potential of melphalan to enhance the effects of ATO as a combinatorial treatment approach for ATLL.

2. Methods and materials

2.1. Cell culture, treatment and viability assay

ATLL cells, MT-2 cell line, were purchased from Pasteur Institute (Tehran, Iran) and cultured in Roswell Park Memorial Institute-1640 medium (Sigma) supplemented with 10 % fetal bovine serum (Gibco), 1 % penicillin/streptomycin and 0.1 % L-glutamine (Gibco). Cells were incubated at 37 °C in the presence of 5 % CO_2.

To prepare different concentrations of melphalan (MW: 305.2 g/mol, Merck), at first a stock solution (10 mg/ml) was prepared using dimethyl sulfoxide (DMSO, Merck) as solvent, and then, final concentrations (0.5, 5, 10, 15 and 20 μg/ml) were freshly made using complete medium just before each experiment. In addition, MT-2 cell were treated with 2, 4, 8 and 16 μM ATO (Sigma) for 24, 48 and 72 h. To evaluate combinatorial effects, cells were treated with 0.5 μg/ml melphalan and 2 μM ATO, concentrations below the IC_{50} of each agent.

To assess viability of cells at the end of each time point, 0.1 mg/ml alamarBlue (Sigma) was added to each well and upon 4 h incubation, the absorbance (A) was determined at 600 nm by a spectrophotometer (Epoch). The percentage of cell viability was calculated using the following formula: $[100 - (A_t–A_c)/(A_b-A_c)] \times 100$, in which A_t, A_c and A_b were the absorbance of treated cells, control cells and blank control, respectively. Cell viability assessments were performed in triplicate and repeated four times independently.

2.2. Cell cycle analysis

To further evaluate effects of melphalan and ATO, alone and in combination, Propidium Iodide (PI, Sigma) staining was applied. In summary, MT-2 cells (5 × 10^4 cells per well in 96-well plate) were incubated with 0.5 μg/ml melphalan, 2 μM ATO and their combination for 72 h. Noteworthy, combination of 0.4 % DMSO and 2 μM ATO was considered as control treatment. Then, cells were collected and centrifuged at 200 g for 5 min, and resuspended in phosphate buffered saline containing 5 % FBS, 100 μg/ml PI, 0.1 % Triton X-100 and 0.1 % sodium citrate. After 30 min incubation at 37 °C in the dark, cells were analyzed by flow cytometry (BD FACSCalibur) using FL2-H filter. The assay was performed two times to ensure the reproducibility of the results.

2.3. Quantitative PCR

qPCR was applied to evaluate single and combinatorial effects of melphalan and ATO on the expression of *CD44, c-MYC, BMI-1* and *NF-κB* (*REL-A*) genes. In brief, RNA was extracted from MT-2 cells using Tri-Pure Isolation Reagent Kit (Roche) and the purity of the extracted RNA was determined by UV spectroscopy at 260 and 280 nm. Then, cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (Thermo Scientific) according to the manufacturer's instruction. To confirm the quality of cDNA, PCR was carried out on all samples using β-2 microglobulin (β2M) primers (Table 1), and the PCR products were loaded onto 2 % agarose gel. For qPCR, SYBR green kit (TaKaRa) and TaqMan probes were used. PCR conditions for CD44, c-MYC and BMI-1 primers were defined as holding at 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 NF-κB (REL-A) probe, PCR conditions were as 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 30s. Data were analyzed using standard curve relative method, and *β2M* was considered as the reference gene. qPCR was performed in triplicate and repeated two times independently.

2.4. Microarray data retrieval

Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.

Table 1
List of primers and probes used for qPCR analysis in the current study.

Name of gene	Length (bp)	Primer sequence 5'→3'
B_2MG	127	**Forward:** AATTGAAAAAGTGGAGCATTCAGA **Reverse:** GGCTGTGACAAAGTCACATGGTT
c-MYC	159	**Forward:** ACTCTGAGGAGGAGGAACAAGAA **Reverse:** TGGAGACGTGGCACCTCTT
BMI-1	192	**Forward:** CTGCAGCTCGCTTCAAGATG **Reverse:** CACACACATCAGGTGGGGAT
CD44	176	**Forward:** CGGACACCATGGACAAGTTT **Reverse:** GAAAGCCTTGCAGAGGTCAG
NF-κB	145	**Forward:** ACCCCTTCCAAGTTCCTATAGAAGAG **Reverse:** CGATTGTCAAAGATGGGATGAGAAAG **Probe:** ACTACGACCTGAATGCTGTGCGGCTCT
B2MG	127	**Forward**: TTGTCTTTCAGCAAGGACTGG **Reverse:** CCACTTAACTATCTTGGGCTGTG **Probe:** TCACATGGTTCACACGGCAGGCAT

gov/geo) was utilized as a public repository of high-throughput functional genomic data, to identify gene expression datasets for MT-2 cells and patient samples representing smoldering, acute, and chronic ATLL subtypes. Dataset GSE6034 was selected to analyze ABCG2 expression in MT-2 cells and normal lymphocytes. Dataset GSE55851 was used to assess the expression of *ABCG2* and *NF-κB* (*REL-A*) in samples from 6 smoldering, 3 chronic, and 3 acute ATLL patients, as well as 3 normal PBMC samples. Data analysis was conducted by GEO2R, and p values were adjusted using the Benjamini & Hochberg method to control the false discovery rate, with a cut-off set at 0.05. Volcano plots were generated using the "ggplot2" package in R, applying criteria of p value < 0.05 and log2 fold change ($\log_2$FC) > 1.

2.5. Molecular docking

To predict the interaction of melphalan with the drug transporter ABCG2, molecular docking was performed using a systematic approach. The three-dimensional (3D) structure of melphalan (CID: 460612) was sourced from PubChem (www.pubchem.ncbi.nlm.nih.gov), while the crystal structure of ABCG2 (PDB ID: 8BHT) was obtained from Protein Data Bank (https://www.rcsb.org/). Utilizing SwissDock (https://www.swissdock.ch/), a web-based platform designed for molecular docking, we optimized the molecular structures of both melphalan and ABCG2. SwissDock identifies potential binding sites by analyzing the protein's surface and predicting how ligands interact with these sites. The docking results were evaluated based on the free energy of binding, which indicates the stability of the interaction. Finally, the outcomes were visualized using 3D diagrams to provide a comprehensive understanding of the binding interactions and conformational changes that may occur upon melphalan binding to ABCG2.

2.6. Statistical analyses

The statistical significance for viability assay and qPCR was analyzed by one-way ANOVA and Tukey tests using GraphPad Prism. All data were reported as mean ± SD, and p values less than 0.05, 0.01, 0.001, and 0.0001 were considered statistically significant.

3. Results

To evaluate toxic effects of melphalan and ATO, alone and in combination, MT-2 cells were treated with different concentrations during 24, 48 and 72 h. As shown in Fig. 1, alamarBlue assay revealed that melphalan and ATO induced their cytotoxic effects in a time- and dose-dependent manner. After 72 h treatment, the highest concentration of melphalan (20 μ/ml) significantly ($p < 0.001$) reduced cell viability down to 53.8 %, and 16 μM ATO significantly ($p < 0.001$) decreased cell viability down to 54.7 %. Accordingly, 0.5 μg/ml melphalan and 2 μM ATO, concentrations with low toxicity, were selected for combinatorial treatment. As presented in Fig. 2-A, combinatorial treatment significantly ($p < 0.001$) reduced cell viability (64.10 %) in comparison with single use of each agent.

To determine whether combination of melphalan and ATO was associated with cell cycle changes, DNA content of cells was analyzed by flow cytometry (Fig. 2-B-C). Results revealed that 6 %, 76.8 %, 5.9 % and 11.6 % of DMSO treated cells were detected in sub G_1, G_1, S, and G_2/M phases of the cell cycle, respectively. After single use of melphalan, 8.3 %, 65.9 %, 5.5 % and 21.4 % of cells were detected in sub G_1, G_1, S, and G_2/M phases, respectively. Likewise, 8.7 %, 61.9 %, 9 % and 21.3 % of cells were detected in sub G_1, G_1, S, and G_2/M phases after treatment with DMSO and ATO, respectively. Interestingly, combinatorial use of melphalan and ATO increased the sub G_1 population up to 19.5 %, and 48.2 %, 4.3 % and 29 % of cells were detected in G_1, S, and G_2/M phases, respectively.

To investigate the molecular mechanisms underlying combinatorial effects of melphalan and ATO, the expression of candidate genes was studied by qPCR. As shown in Fig. 3, melphalan significantly ($p < 0.001$) down regulated the expression of *BMI-1, c-MYC* and *CD44* in single use and in combination with ATO when compared with the relevant controls. Regarding *NF-κB* (*REL-A*), melphalan alone exerted a significant ($p < 0.05$) inhibitory effect.

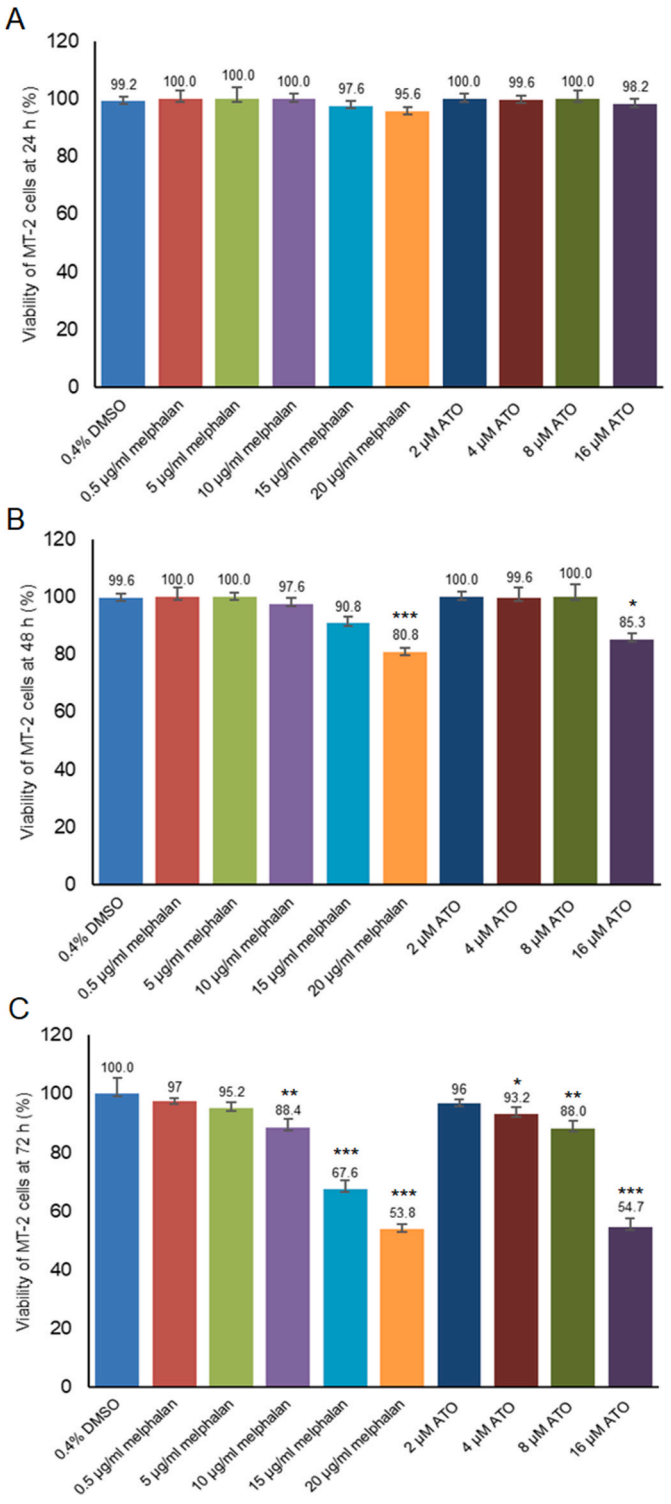

Fig. 1. Viability of the MT-2 cells upon treatment with the various concentration of melphalan and ATO for 24 (A), 48 (B) and 72 (C) h. The experiment was carried out in triplicate and three independent times. Values are reported as mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with DMSO control.

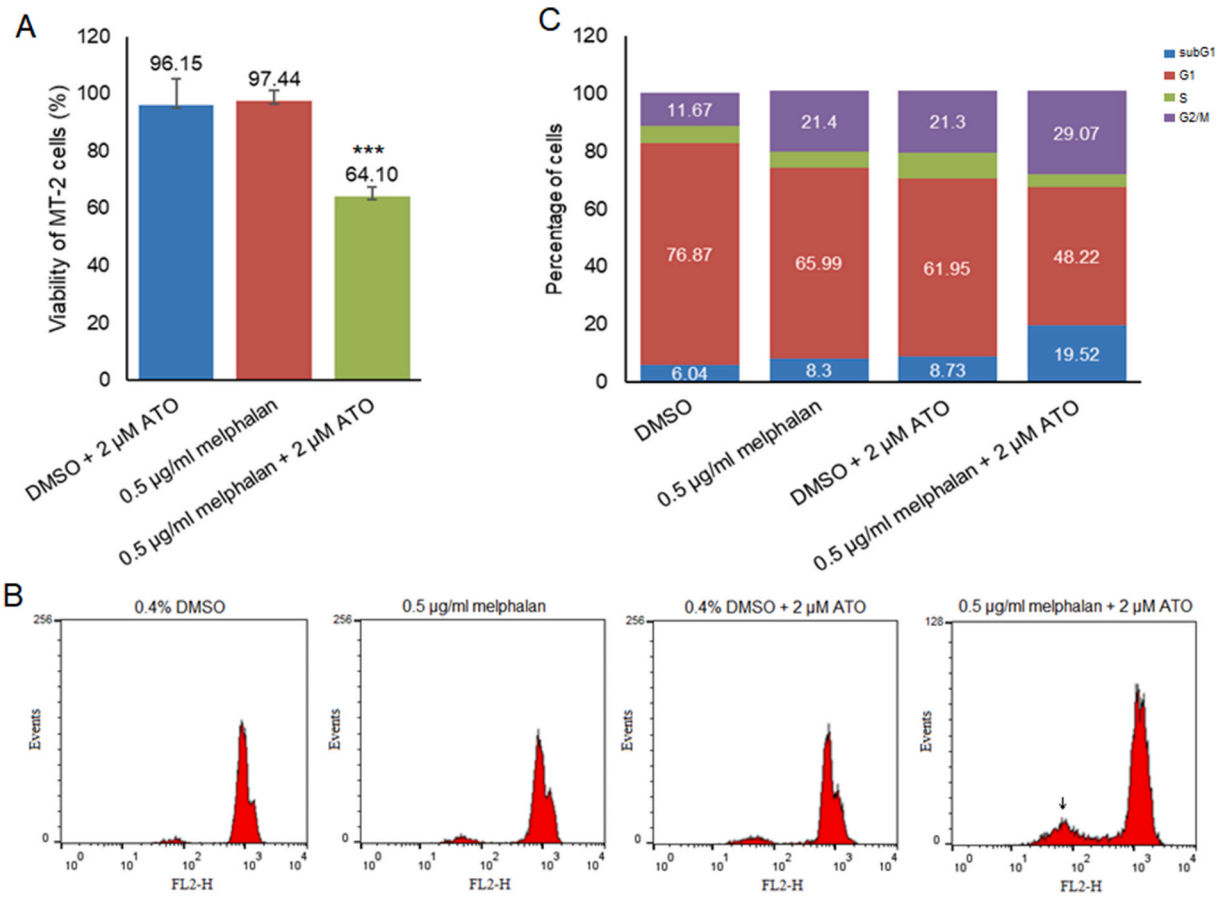

Fig. 2. Viability of MT-2 cells after 72 h treatment with melphalan and ATO, alone and in combination (A). Flow cytometry histograms (B) and quantitative analysis (C) of the cell cycle upon single and combinatorial use of melphalan and ATO. The arrow in the cell cycle histogram indicates the sub G_1 phase. ***$p < 0.001$ compared with DMSO control.

Expression profiles for MT-2 cells and patient samples with smoldering, acute, and chronic ATLL subtypes were obtained from GEO, and quantitively analyzed by R. As presented in Fig. 4, *ABCG2* was significantly ($p < 0.05$) upregulated in MT-2 cells compared to normal lymphocytes. Additionally, *ABCG2* expression was significantly ($p < 0.05$) increased in acute and smoldering ATLL subtypes. Moreover, significant ($p < 0.05$) upregulation of *NF-κB* (*REL-A*) was detected in acute and chronic ATLL samples (Supplementary File).

To determine whether observed combinatorial effects were due to the interaction of melphalan with ABCG, molecular docking was performed. As shown in Fig. 5, melphalan interacted with NPADF motif of ABCG2 with a favorable SwissParam score of −6.6 and AC score of 33.77. The interaction involved two hydrogen bonds with Asp292, one hydrogen bond with Asp298, and two hydrophobic interactions with Gln244 and Arg256.

4. Discussion

ATLL is a non-Hodgkin lymphoid malignancy characterized by a poor prognosis. Despite the availability of various combination therapies, these treatments often fall short in improving survival rates, primarily due to the challenge of chemoresistance. Melphalan has been widely used for the treatment of solid tumors and hematological malignancies such as multiple myeloma [28]. As an alkylating agent, melphalan induces cytotoxicity by interfering with DNA and RNA synthesis [29]. Previous studies have demonstrated effects of melphalan in combination with anticancer drugs such as thalidomide, lenalidomide, and pomalidomid as well as monoclonal antibodies and histone deacetylase inhibitors [30,31]. ATO is an approved and effective drug for clinical treatment of hematological malignancies, including acute promyelocytic leukemia and multiple myeloma, with its mechanism of action actively investigated (Paul et al., 20222). For instance, research on Burkitt lymphoma cells revealed that ATO induces autophagic cell death [32]. Moreover, the combination of ATO with all-trans retinoic acid has transformed diseases like acute promyelocytic leukemia from being highly lethal to having significantly improved treatment outcomes [33]. The current study aimed to investigate effects of melphalan in combination with ATO on ATLL cells for the first time. Our findings revealed that melphalan improved toxicity of ATO, induced accumulation of cells in the sub G_1 phase of the cell cycle, and down regulated the expression of *CD44, BMI-1*, *c-MYC* and *NF-κB* (*REL-A*). Similarly, Campbell and colleagues demonstrated synergistic activity of melphalan and ATO in multiple myeloma cells [31]. Studying combinatorial effects of melphalan and ATO in murine models of human myeloma indicated improved efficacy (Berenson t al., 2006). In addition, a clinical study on combinatorial effects of melphalan and ATO was performed on patients with relapsed/refractory multiple myeloma who had autologous stem cell transplantation. Results revealed that the combination therapy was effective and well tolerated as a preparative regimen [34].

HTLV-1 infected cells express Tax protein which activates *NF-κB* signaling pathway [9]. *NF-κB* is a pro-inflammatory transcription factor that plays important roles in tumor cell proliferation, survival, metastasis and angiogenesis [35]. In the current study, overexpression of *NF-κB* (*REL-A*) was detected in acute and chronic ATLL samples. Additionally, our findings indicated down regulation of *NF-κB* upon single and combinatorial use of melphalan. In line with our results, it has been reported that down regulation of *NF-κB* is crucial for the enhancement of melphalan sensitivity and/or for declining melphalan resistance in the

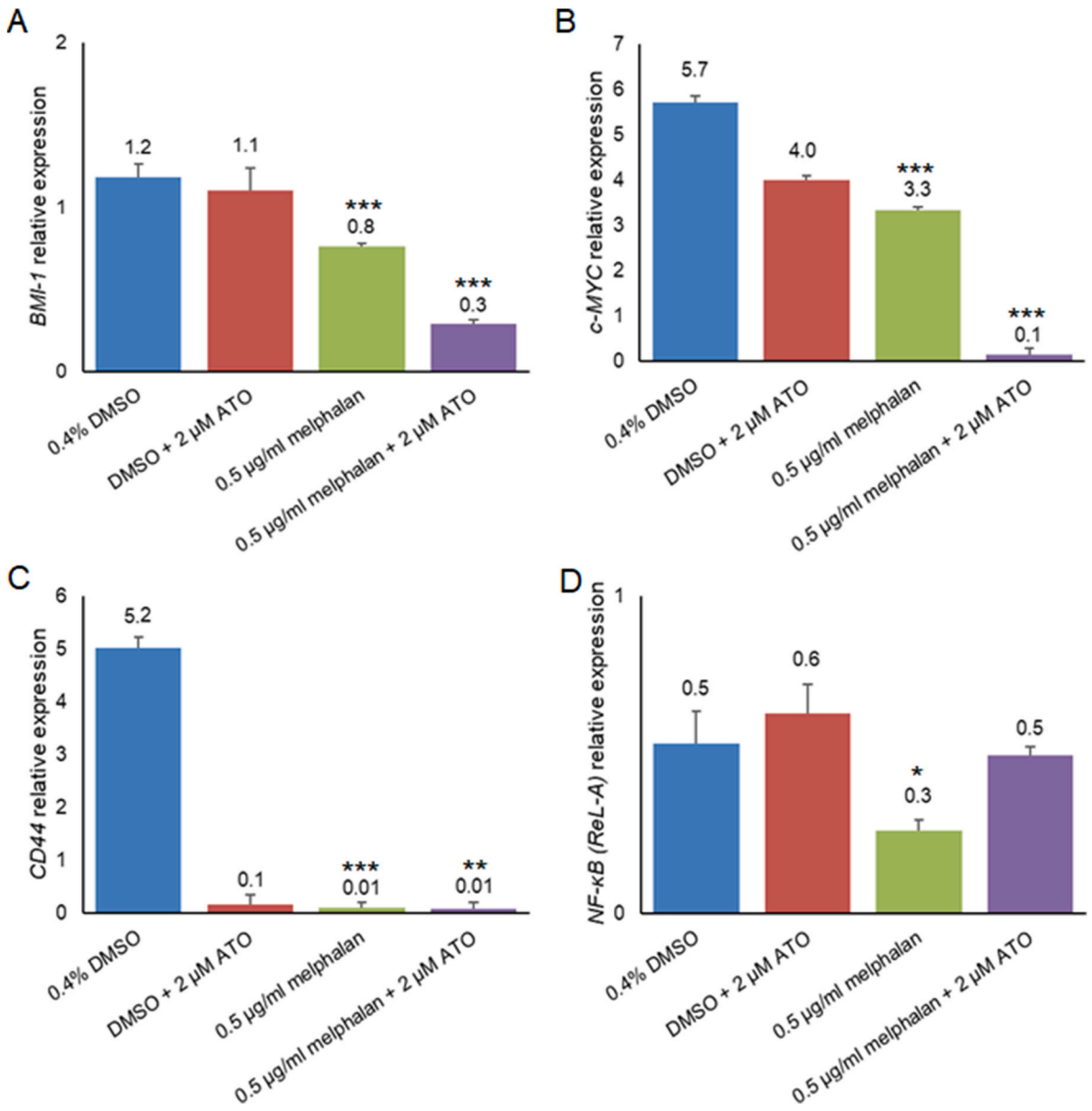

Fig. 3. Expression of *BMI-1, c-MYC, CD44* and *NF-κB* (*REL-A*) in MT-2 cells after 72 h treatment with melphalan and ATO, alone and in combination. Values are reported as mean ± SD. *$p < 0.05$ and ***$p < 0.001$ compared with the relevant controls; DMSO for melphalan, DMSO and ATO for combination of melphalan and ATO.

patients with multiple myeloma [36]. Overexpression of *CD44* and *c-MYC* in HTLV-1-infected cells is correlated with poor prognosis and severity of the disease [16,37]. Current findings revealed that melphalan significantly decreased the expression of *c-MYC* and *CD44* in single use and in combination with ATO. This is in agreement with previous reports, indicating that ATO induced cell death in leukemia cell lines via down regulation of *c-MYC* [38]. Furthermore, treatment of human chronic myeloid leukemia cells with ATO inhibited cell proliferation and induced cell cycle arrest in G_0/G_1 phase via down regulation of *CD44* [39]. BMI-1 is a member of polycomb group family with essential roles in self-renewal and differentiation. Upregulation of *BMI-1* has been reported in various hematological malignancies including acute and chronic myeloid leukemia [40]. In acute lymphoblastic leukemia patients who had fully recovered, *BMI-1* level was obviously reduced [41]. Interestingly, present findings demonstrated that enhanced toxicity of ATO in ATLL cells was correlated with significant down regulation of *BMI-1*.

Since our findings indicated, for the first time, that combination of melphalan and ATO increased toxicity on ATLL cells, we further investigated whether melphalan could interact with an ABC transporter and reduce ATO efflux. ABCG2, which consists of a nucleotide (ATP) binding domain (NBD) and a transmembrane domain (TMD), requires dimerization to gain full functionality [42]. Disrupting the interaction between two NBDs in the NPADF motifs (N289–F293) negatively impacts ABCG2 trafficking [43,44]. In the present study, molecular docking analyses revealed favorable binding affinities of melphalan with the NPADF motif in ABCG2, suggesting a significant probability that melphalan may effectively target this transporter. This interaction could help explain the observed combinatorial effects of melphalan and ATO in ATLL cells. Additionally, volcano plots generated from the expression profile of MT-2 cells and ATLL subtypes highlighted the overexpression of ABCG2, underscoring its potential role as a critical transporter in ATLL. These findings collectively indicate that targeting ABCG2 with melphalan could be a promising strategy for enhancing therapeutic efficacy in ATLL, warranting further experimental validation to confirm this hypothesis.

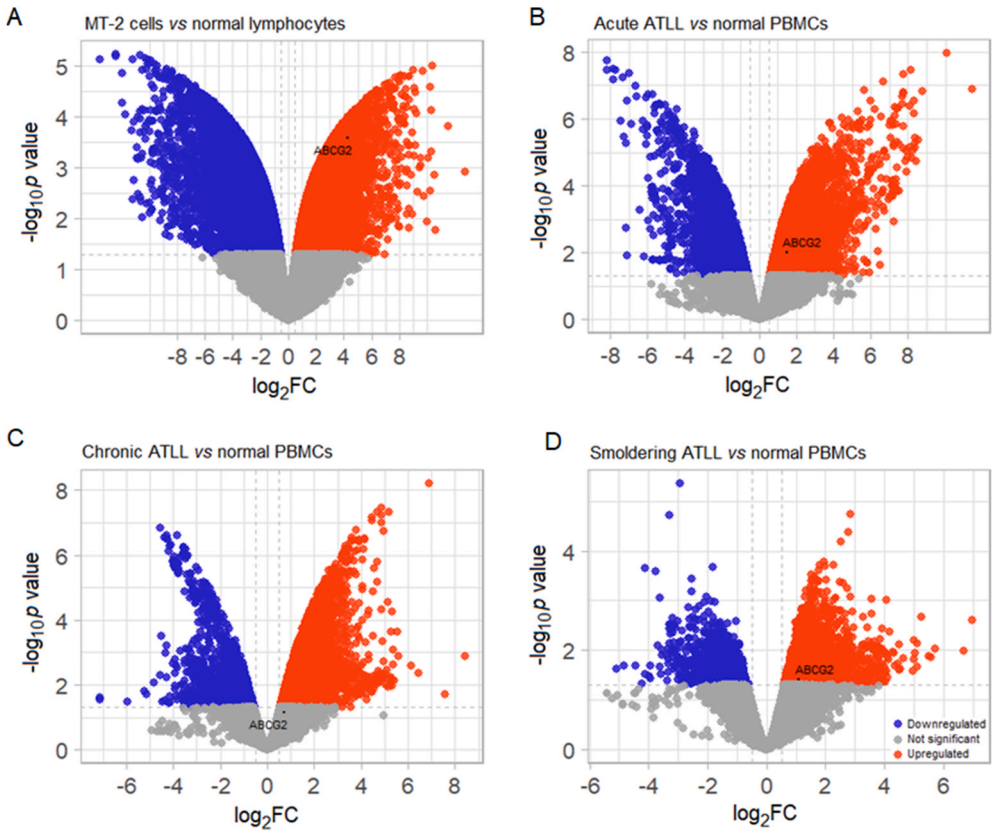

Fig. 4. Volcano plots display *ABCG2* expression in MT-2 cells (A) and ATLL patient samples with acute (B), chronic (C) and smoldering (D) subtypes. The plots use Log_{10} *p* value and log_2fold-change (FC) to discriminate genes of interest.

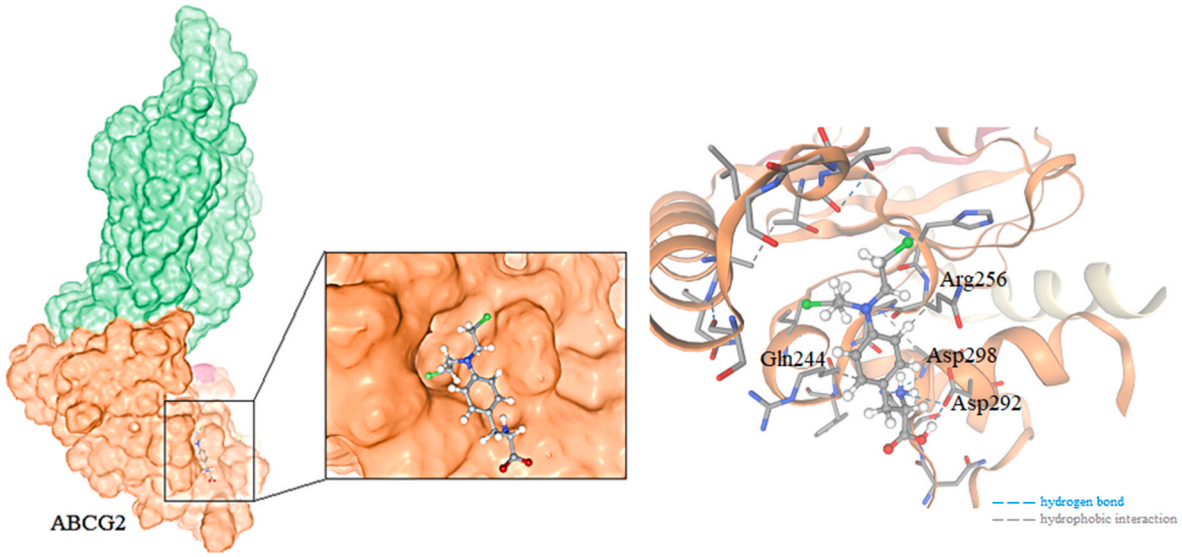

Fig. 5. The 3D diagram of molecular docking for melphalan and ABCG2 indicate three stable hydrogen bonds and two hydrophobic interactions. Images were generated by SwissDock.

The present study has certain limitations that warrant further investigation. While our findings provide valuable insights into the interaction between melphalan and ABCG2 in MT-2 cells, it is crucial to recognize how these results may translate to actual ATLL cells in patients. Establishing the direct interaction between ABCG2 and melphalan is also critical; implementing negative control cell lines that do not express ABCG2 or utilizing ABCG2 knockdown strategies would offer significant insights into this interaction. Addressing these aspects will not only enhance our understanding of the underlying mechanisms but also strengthen the evidence supporting the potential of melphalan as a reversal agent in ATLL treatment.

5. Conclusion

The present study provides compelling evidence for the first time that the combinatorial use of melphalan and ATO significantly reduces

cell viability and induces cell cycle arrest more effectively than the individual application of either agent. Our findings also suggest that melphalan may enhance the cytotoxicity of ATO by targeting ABCG2, a drug transporter that is overexpressed in ATLL. This interaction underscores the probability that melphalan could serve as a potent agent in combination therapy for ATLL, potentially improving treatment outcomes. Given these promising results, further investigation into the mechanisms underlying this synergistic effect is warranted to fully realize the therapeutic potential of this combination.

CRediT authorship contribution statement

Faeze Khodadadi: Investigation. **Mohammad Hadi Sadeghian:** Conceptualization. **Zahra Delbari:** Investigation. **Mohadese Kazemi:** Investigation. **Hamide Koohpaykar:** Investigation. **Fatemeh B. Rassouli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Conceptualization.

Ethical approval

Not applicable.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.102109.

Data availability

Data will be made available on request.

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