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The Antihelminthic Drug, Mebendazole, Induces Apoptosis in Adult T-Cell Leukemia/Lymphoma Cancer Cells: *In-Vitro* Trial

Amirhosein Maali[1,2,3], Elaheh Ferdosi-Shahandashti[1,2,4], Farzin Sadeghi[2], Ehsan Aali[5]

[1]Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
[2]Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
[3]Student Research Committee, Babol University of Medical Sciences, Babol, Iran
[4]Department of Medical Biotechnology, School of Medicine, Babol University of Medical Sciences, Babol, Iran
[5]Department of Pharmacology, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran

Corresponding Author: Elaheh Ferdosi-Shahandashti, Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
Email: elaheh.ferdosi@yahoo.com

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ABSTRACT

Background: Adult T-cell leukemia/lymphoma (ATLL) is a poor prognostic Hematopoietic malignancy with various therapeutic challenges, which had been classified as non-Hodgkin lymphoma. The Drug switching, as a novel, innovative and promising approach, is an opportunity to overcoming on therapeutic challenges of hard-treating disease, *e.g*. ATLL. Our aim is evaluating the antiproliferative and apoptotic effect of Mebendazole (MBZ) on ATLL cancer cells in *in-vitro* conditions.

Materials and Methods: We used Jurkat cell-line as ATLL cancer cells. After treatment of MBZ in different concentrations on jurkat cells, the cell viabilities were determined by MTT assay. After IC_{50} value determination, the 24-, 48- and 72-h treatments had been performed in IC_{50} concentration and control to evaluating the quantitative apoptosis rate by Annexin/PI Flowcytometry and qualitative apoptosis by DAPI Nuclear staining. Also, Glucose spectrophotometry were performed to evaluate the reduced amount of glucose uptake through MBZ treatment.

Results: MBZ inhibits proliferation of jurkat cells and IC_{50} value had been estimated 10 µM ($P< 0.01$). According to the flowcytometric results, increasing in drug concentration is associated with decrease cell viability and the percentage of full-apoptosis. However, it inversely correlates with percentage of early-apoptosis rate. Also, the microscopic captures of DAPI Nuclear staining confirms the flowcytometry results in qualitative manner. In addition, it was found that inhibition of glucose uptake was inversely correlated with increased MBZ concentration ($P< 0.05$).

Conclusion: MBZ potentially inhibits the proliferation of ATLL cancer cells in *in-vitro* condition. MBZ inhibits the growth of Jurkat cells by inducing apoptosis. Also, we suggest that indirectly inhibition of Glucose transporting occurs by MBZ, which could induce apoptosis in cancer cells.

Keywords: Adult T-cell leukemia/lymphoma; Mebendazole; Drug switching; Apoptosis

INTRODUCTION

Adult T-cell Leukemia/Lymphoma (ATLL) is an aggressive hematopoietic malignancy associated with Human Lymphotropic virus 1 (HTLV-1) with a poor prognosis [1,2]. Currently, treatments of ATLL are based on Azacytidine and Interferon-alpha (combined chemotherapy), zidovudine and Cyclophosphamide-Hydroxydaunorubicin-Oncovin-

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Prednisone (CHOP) regimen, Pralatrexate, thioredoxin binding protein-2 (TBP2), mogamulizumab, brentuximab, and allogenic Hematopoietic Stem Cell transplantation (HSCT) [3-5], while there is no standard therapy regimen for ATLL[6]. Also, the current ATLL therapy strategies are faced by many challenges, including long-term therapy, side effects of chemotherapy, and immunological rejection in grafted cases [7, 8].

Recently, the therapeutic switching of drugs has been considered to evaluate other diseases (including cancers), due to their characterized pharmacokinetic, pharmacodynamic and toxicity[9]. The "*Drug switching*" (also known as *Drug repurposing*) is a high-potent strategy to validating drugs to rapid development on another incidence. Currently, Mebendazole (MBZ) [$C_{16}H_{13}N_3O_3$], as a benznidazole-based antihelminthic agent, is an interested cancer switched drug [10].

MBZ inhibits the absorption of glucose in eukaryotic cells and cause its energy depletion by inhibiting the surficial expression of Glucose Transporters (GluT), specially GluT-4, through dysregulation in Microtubular polymerization [11,12]. The metabolism of cancer cells changes to anaerobic oxidative and hypoxia phenomenon, which leads to less ATP production. Thus, cancer cells need a higher amount of glucose uptake and is more sensitive to inhibiting glucose transporters. Therefore, MBZ has the anti-cancerous effects through indirectly inhibiting Glucose transporters. *In-vitro* anti-cancerous effects of MBZ are approved on lung cancer cells[13], glioblastoma multiforme [14], colon cancer cells [15], melanoma cells[16], *etc*. Significant antiproliferative results of MBZ are approved in some *in-vivo* studies, including metastatic adrenocortical cancer[17] and melanoma [16]. Also, anti-cancerous effect of MBZ are approved on High-Grade Glioma (NCT01729260, clinical trial-phase I) and Pediatric Low-Grade Gliomas (NCT01837862 clinical trial-phase I and II)[18]. Due to the therapeutic challenges of ATLL, we studied MBZ switching on ATLL. In this study, we established the anti-cancerous and anti-proliferative potential of MBZ on ATLL cell line (Jurkat) aimed to introducing as a novel anti-ATLL agent, in *in-vitro* condition.

MATERIALS AND METHODS

Cell Culture and Drug Preparation

The Jurkat cell-line Clone E6-1 (TIB-152™, Pasteur Institute of Iran) cultured in complete medium, including RPMI-1640 medium (Biowest, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Biowest, USA) and 100U/ml-100µg/ml Pen-strep (Sigma-Aldrich, USA), at humidified 37 ºC in atmosphere containing 5% CO_2. The viability cell count assay was done by Trypan-blue staining via hemocytometer.

MBZ had been suspended in Dimethyl sulfoxide (DMSO) to prepare a final concentration of 10 mM. Then, the serial dilution of MBZ prepared in DMSO to 1.25 mM (corresponding to the 25µM treatment) 500 µM (corresponding to the 10µM treatment), 250 µM (corresponding to the 5µM treatment), 50 µM (corresponding to the 1µM treatment) and 5 µM (corresponding to the 0.1µM treatment) concentrations.

Cell Viability by MTT Assay

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Alpha-Aesar, Thermofisher, USA) assay was conducted to measure the potential of MBZ in inhibiting the cell growth. For this purpose, 1×10^4 cells/100µl per well were seeded in U-form 96-well-containing microplates. To achieve the final concentration, 2 µl of related concentrations was treated in each well in triplicates. The plates incubated for 24, 48, and 72 hours. After incubation, the microplates centrifuged in 250*g* for 5 min. The supernatants were removed and 50 µl of MTT x1 was added to each well. After 3 hours incubation in 37 ºC, 150 µl of DMSO was added to each well. The OD measured in 540 *nm* by plate-reader, and Inhibitory Concentration-50 (IC_{50}) calculated for 24h-treated cells by *Excel 2019* and *SPSS ver.22.0*. MTT assays experimented in triplicates.

Quantitative Apoptosis Rate by Annexin/PI Flowcytometry

Annexin/PI flowcytometry prepared by Phosphatidyl Serine Detection Kit (IQ products, Netherland) to evaluating the apoptosis in time-depended

treatment of MBZ on Jurkat cancer cells. After 24-, 48- and 72-hours incubation at humidified 37 ºC in atmosphere containing 5% CO_2, the IC_{50}-treated cells were centrifuged in 500× *g* for 5 min. The cell pellets were suspended in 2 ml Phosphate-buffered saline (PBS) x1 and washed by centrifuging in 500× *g* for 5 min. The cell pellets were suspended in 500µl of Binding buffer x1 and incubated with 5 µl Annexin V-FITC, for 15 min at darkroom. One ml of Binding buffer x1 were added to tubes and centrifuged in 500× *g* for 5 min. After supernatant removal, the cells pellets were suspended in 250 µl of Binding buffer x1. Three µl of PI was added to each well. Immediately, the reactions were read by flowcytometer (BD FACSCalibur™, BD Biosciences, USA).

Qualitative Apoptosis Rate by DAPI Nuclear Staining

DAPI [4',6-diamidino-2-phenylindole] (Sigma-Aldrich, USA) was used to analysis of nuclei degradation through apoptosis. For this purpose, 5×10^5 cells were cultured in 6-well plates and treated by either MBZ (IC_{50}) and DMSO (control) for 24-, 48- and 72-hours. The treated cells centrifuged in 200× *g* for 3 min. The supernatant was removed and the cells were washed once in 1 µg/ml DAPI-methanol solution. The suspended cells were incubated at 37 ºC for 15 min. Further, the cells centrifuged in 200× *g* for 30 sec. After staining solution removal, the cells were suspended in PBS. Twenty µl of samples was loaded on slide and imaged by DAPI fluorescent filter, set on Fluorescence microscope (Leitz, Germany).

Glucose Measurement by Spectrophotometric Assay

To approve the inhibitory effect of MBZ on Glucose uptake in ATLL cells, the glucose quantified by Spectrophotometric Glucose Quantification kit (Pars Azmun, Iran). To this aim, $5x10^5$ Jurkat cells were treated by MBZ in different concentration of MBZ. After incubation at 37 ºC for 24h, the supernatant was cultivated to Glucometery. The ODs of photometric reactions were analyzed by Prestige 24i Biochemical Autoanalyzer (Japan) in 546*nm*. To eliminating the effect of cell viability, the results were normalized with MBZ 24h-treated MTT assay results. The Spectrophotometric Glucose Quantification were experimented in duplicated reactions.

RESULTS

MBZ Reduced the Cell Viability on ATLL Cancer Cells

Based on our results, the MTT assay results showed that MBZ inhibits the ATLL cells proliferation (figure 1). The IC_{50}-value of MBZ on jurkat cell-line was estimated 10 ± 0.09 µM. The statistical analysis showed that in 10 µM treatment of MBZ, there is a significant time-depended manner correlation (*P<0.01*). Also, we found that in 24-hours treatment of MBZ, there is a significant Dose-depended manner (*P<0.01*).

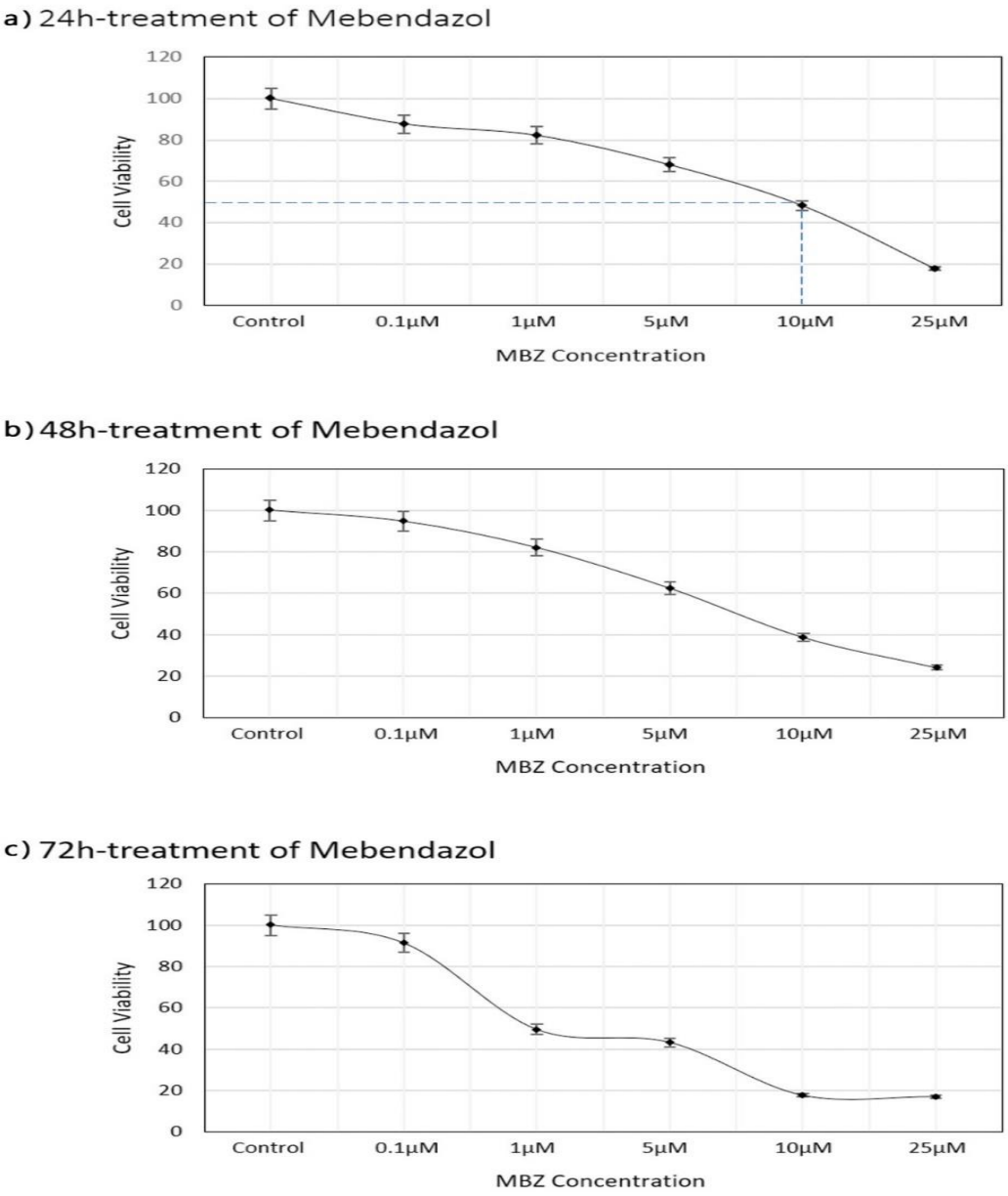

Figure 1. MTT results shows antiproliferative effect of Mebendazole on ATLL cancer cells. a) MTT result of 24h-treated jurkat cells established that decreasing in relative viable cell count starts from Mebendazole treatment in low concentration. IC_{50} value estimated 10µM in a Dose-depended manner ($P<0.01$). b) MTT result of 48h-treated jurkat cells approved the Time-dependence of viability in Mebendazole treatment on cancer cells. c) In 72h-treatment of Mebendazole, the result showed that maximum reducing in cell viability occurs in 10µM after 72h-treatment.

MBZ Induces Apoptosis on ATLL Cancer Cells

Due to the MTT results, the apoptosis induction assays were performed in 10 µM treatment. Annexin/PI flowcytometry assay shows the quantified apoptosis rate in a time-depended manner (*figure 2a*). In control, the cell viability ($Annexin^-/PI^-$) is 93.8%. In 24-h treatment of MBZ on jurkat cells, the cell viability ($Annexin^-/PI^-$) is 48.1%. It approves the accuracy of IC_{50} value that measured by MTT assay. $Annexin^-/PI^-$ cells percentage is 31.3% and 30.1% in 48h-treated and 72h-treated cells, respectively. Also, the rate of early apoptosis-induced cells ($Annexin^+/PI^-$) is estimated about 26.7% in 24h treatment, and 14.6% and 12.3% in 48h-treatment and 72h-treatment, respectively. The full apoptotic cells ($Annexin^+/PI^+$) contains 20.6% of 24h-

treated cells. Through increasing in the time of MBZ treatment, the treated cells lead to more full apoptosis rate. Also, the early-apoptosis percentage were reducing through time passes (*Figure 2c*).
DAPI Nuclear staining approved the Annexin/PI flowcytometry results, qualitatively (*figure 2b*). In control sample (untreated cells), the nuclei save its membrane integrity. In MBZ 24h-treated cells in IC_{50}, the result shows the early-apoptosis induction through nuclei membrane fading. In MBZ 48h-treated cells in IC_{50}, the chromosomal fragmentation and degradation have been appeared, qualitatively. In MBZ 72h-treated cells in IC_{50}, there is an increase in count of chromosomal full-fragmented cells.

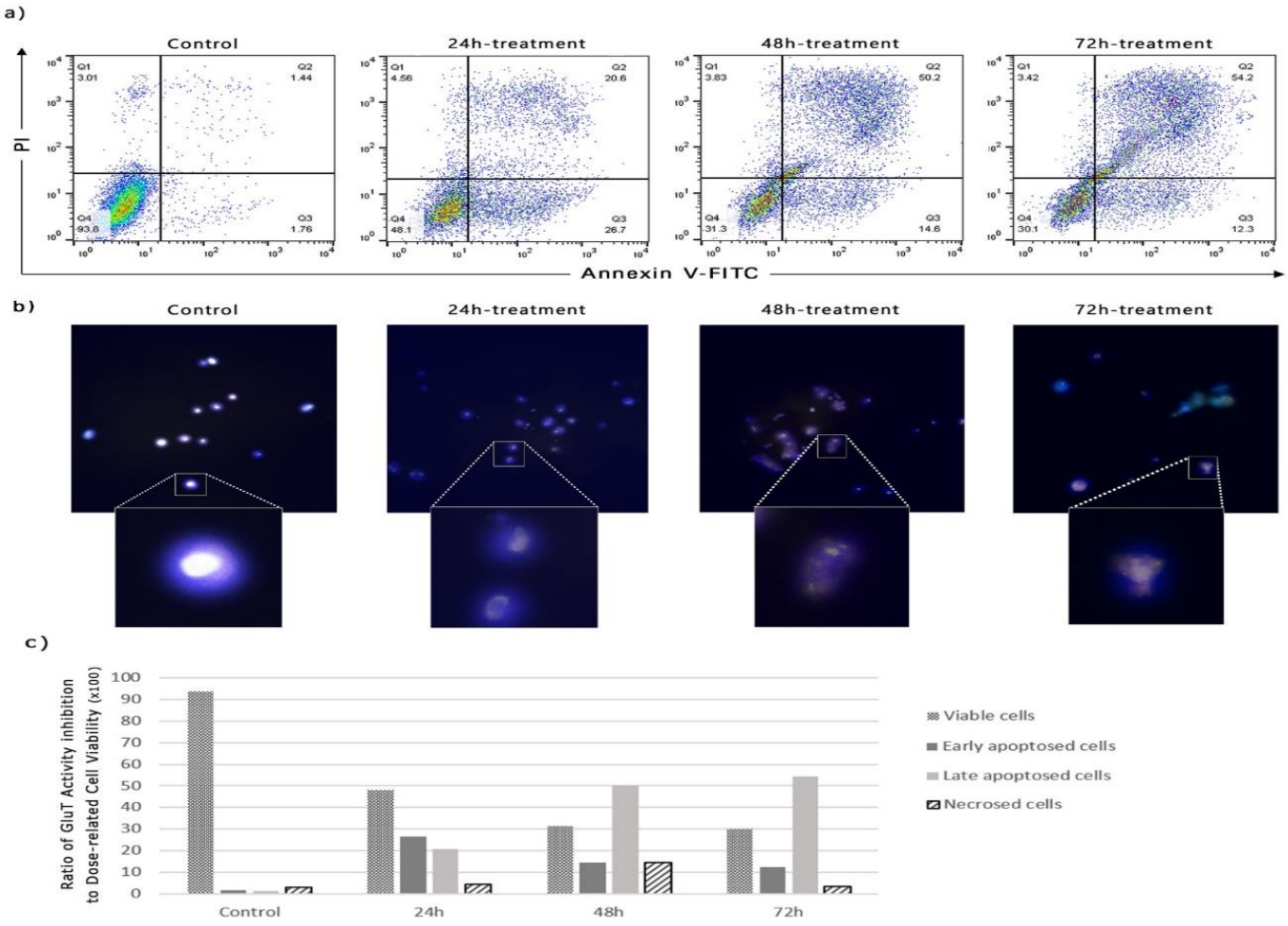

Figure 2: Mebendazole induces apoptosis in ATLL cancer cells. a) The result of Annexin/PI flowcytometry approved IC50 value (48.1% of cell viability compared to 93.8% in control). Total apoptosis percentage increases through time-pass. b) The spectral results of DAPI nuclei immunostaining shows the loosing of nuclear membrane integrity in Time-passed MBZ-treatment. In 72h-treated cancer cells, most population of cancer cells shows chromosomal fragmentation and nuclear membrane degradation. c) In MBZ-treated cancer cells, the ratio of Early-apoptosis to full-apoptosis decreases through time-pass (0.77 in 24h-treatment, 3.44 in 48h-treatment, 4.41 in 72h-treatment).

MBZ Inhibits the Glucose Transporters Activity in a Dose-depended Manner

The normalized spectrophotometric Glucometery results stablished the direct correlation of MBZ dosage and inhibition of glucose absorption (*figure 3*) (*P< 0.05)*. IC_{50}-treated glucometery show 13.91% less Glucose absorption activity than Control. In other words, a higher concentration of MBZ indirectly inhibits the activity of GluT more severely.

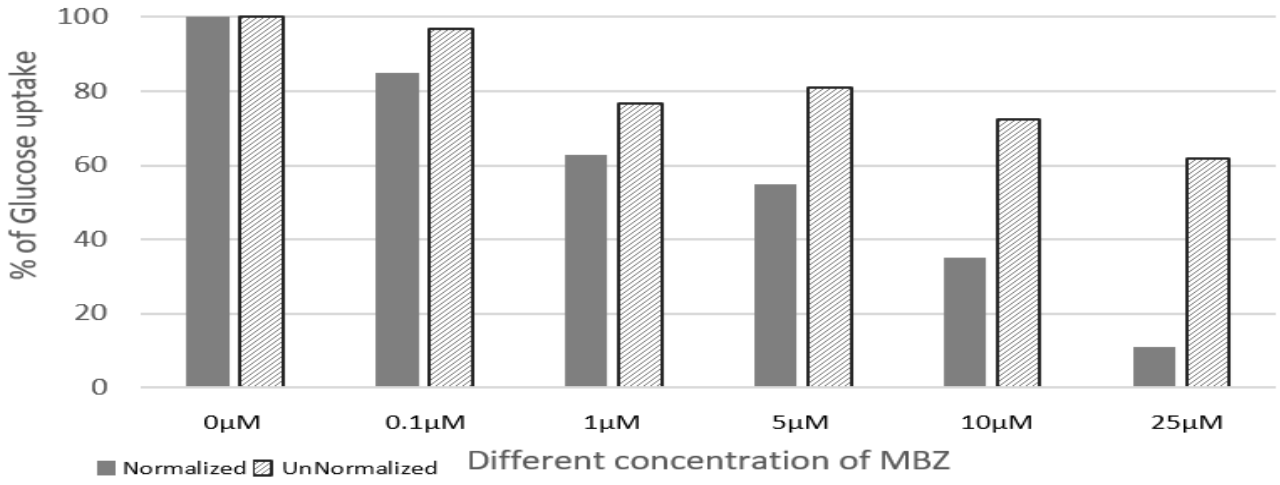

Figure 3. Normalized dose-depended glucose uptake (in 24h-treatment**)**. The results of glucometery in IC_{50}-treated cancer cells show 13.91% less Glucose absorption than control cells (untreated).

DISCUSSION

Adult T-cell Leukemia/Lymphoma (ATLL) is a poor prognostic hematopoietic malignancy classified as non-Hodgkin Lymphoma and faced with a huge therapeutic challenge. The change in drug indication to other incidence, known as "*Drug Switching*" (also *Drug Repurposing*), has been highly respected in recent years to overcoming on treatment of hard therapeutic indicated malignancies, *e.*g. ATLL. Mebendazole, applied as an antihelminthic agent, is a benzothiazole-based compound which has been the focus of much attention in the drug switch approach on cancer therapy. The wide range of therapeutic window, safety, cost-effectiveness and accessibility of MBZ are the potentials of this drug for introducing to drug switching.

Anti-proliferative effect of MBZ has been proven in various cancers, including different types of leukemia and lymphoma, but not ATLL. For this reason, in this study we examined the control of cancer within 200 mg daily in oral administration (17). Time-depended Apoptosis Assay were conducted by Annexin/PI and DAPI nuclei immunostaining. The result of MBZ-24h-treated jurkat cell-line which conducted by flowcytometry approves IC_{50} value (Viable cell percentage of MBZ-24h-treated jurkat cell-line estimated 48.1% in 10 μM). DAPI nuclei immunostaining confirmed the antiproliferative and apoptosis induction potential of MBZ on jurkat cell-line (utilized as ATLL cancer cells) by different assays including MTT, Annexin/PI flowcytometry and DAPI nuclei immunostaining. Our study confirmed *in-vitro* anti-cancerous effect of MBZ on ATLL cancer cells, as a Time- and Dose-depended manner. Results of MTT assay approves that IC_{50} value of MBZ on Jurkat cell-line is 10 μM, as many reports by other scientists on other cancer cell-lines (10), *e.g.* Nygren *et al.* experiment that reported IC_{50} of MBZ on Colon cancer as 10 μM (15). However, in 2011, Bai *et al.* reports IC_{50} of MBZ on Glioblastoma multiform as 0.1 to 0.3 μM (19). Also, Skibinski *et al.* showed IC_{50} of MBZ on Intracranial Rodent Model of Malignant Meningioma as 0.26 to 0.42 μM in combination with Radiation therapy (12). In study of Dobrosotskaya *et al.* on an end-staged 48-year-old man with Metastatic Adrenocortical Carcinoma, MBZ showed a beneficial effect on

nuclei degradation and losing the integrity of nuclear membrane in MBZ-treatment-passed Jurkat cells, which is hallmark of apoptosis. DAPI spectral results shows that the degradation of nuclear membrane is time-depended manner.

Anti-proliferative effect of MBZ on jurkat is based on inhibition of Glucose absorption through dysregulation in intracellular cytoskeleton. Glucose

measurement in supernatant of cultured cells shows that increase in MBZ concentration reduces glucose uptake. Thus, cancer cells face by energy depletion. Thus, we show the mechanism of MBZ in growth inhibition of jurkat cancer cell is dependent on the reduction of cellular glucose levels, while it is suggested that the signaling pathway and other factors involved in cellular growth should be investigated.

According to the molecular weight (295 g/mol), elimination half-life (3 to 6 hours), and pharmacodynamic of MBZ approved by FDA, we predict that MBZ dosage for ATLL therapy is lower than the constant dosage in the body for parasite therapy. Therefore, the administrable dosage of MBZ on ATLL patients are in therapeutic window and falls below the lethal dose. Thus, MBZ, potentially, is safe and cost-effective agent for ATLL patients. Also, we suggest *in-vivo* study on anti-cancerous effect of MBZ on ATLL in animal models. After approval in animal trail, it can be examined on clinical trial.

There are some limitations in our study in methodology section. For example, it was better to including Matrigel cell migration assay, evaluation of apoptosis-related gene expression level before and after MBZ treatment, TUNEL assay to determining the changes of telomerase activity level in MBZ treatment, and Cell-cycle arrest analysis by flowcytometry, in our experiment that was ignored because of financial problems.

CONCLUSION

This study demonstrated that Mebendazole is a high-potent proliferation inhibitor of ATLL cancer cells (Jurkat cell-line) with 10 µM IC_{50} value in *in-vitro* conditions. According to our results, Mebendazole is an apoptosis inducer agent on ATLL cancer cells and would be a valuable addition in chemotherapeutic field of ATLL due to its selectivity in inhibition of cancer cells.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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