Comparison of 2-Methoxyestradiol and Methotrexate Effects on Non-Hodgkin’s B-Cell Lymphoma

ABSTRACT Purpose: Methotrexate (MTX) is the most commonly used chemotherapeutic agent to treat primary central nervous system lymphoma (PCNSL) and intraocular lymphoma (IOL). 2-Methoxyestradiol (2ME2) is a potent antitumor and anti-angiogenesis agent which, unlike other cytotoxic drugs, has minimal toxicity. In this study, anti-proliferative, apoptotic, and cell-cycle effects of 2ME2 and MTX were compared to evaluate 2ME2 efficacy in human lymphoma cells, models for non-Hodgkin B cell lymphomas. Methods: The cells were cultured and incubated with varying concentrations of 2ME2 or MTX. A tetrazolium-based colorimetric assay was used to quantify the anti-proliferative effects of 2ME2 and MTX using a microplate reader. To detect apoptotic and cell cycle distribution changes induced by 2ME2 and MTX, the cells were stained with Annexin V-FITC and/or propidium iodide (PI) and analyzed by flow cytometry. Results: Lymphoma cell proliferation was inhibited by 50% at concentrations ranging from 0.4 to 1 µM for 2ME2 and 0.06 to 0.2 µM for MTX. Induction of apoptosis by 2ME2 and MTX was observed in the tested cells. 2ME2 was a G2/M-phase specific blocker whereas MTX was an S-phase specific blocker in cell cycle analyses. At 1 µM concentration, 2ME2 and MTX showed similar anti-proliferative effect on the lymphoma cell lines. In previously reported studies, for normal endothelial cells, 1 µM 2ME2 showed no appreciable toxicity, while MTX at this same concentration exhibited significant cytotoxicity. 2ME2 at a therapeutic target concentration of 1 µM may be an effective and relatively non-toxic drug for the treatment of PCNSL with IOL. Conclusions: Our study of the effect of 2ME2 and MTX on anti-proliferation, apoptosis, and cell cycling suggests that 2ME2 is a potential agent for treating PCNSL and IOL.

KEYWORDS 2-methoxyestradiol (2ME2); methotrexate (MTX); primary central nervous system lymphoma (PCNSL); intraocular lymphoma (IOL); tissue culture

INTRODUCTION

Primary central nervous system lymphoma (PCNSL) is a B-cell non-Hodgkin’s lymphoma that is generally confined to the central nervous system.1,2 Intraocular lymphoma (IOL) occurs in approximately one quarter
of patients with PCNSL.\textsuperscript{3} Methotrexate (MTX) is the most commonly used chemotherapeutic agent to treat PCNSL\textsuperscript{4–6} and IOL.\textsuperscript{7–10} However, therapeutic doses of this medication are associated with side effects such as bone marrow depression and hair loss. Also, the clinical application of MTX is hindered by drug resistance and toxic interactions with many commonly used medications.\textsuperscript{11} In addition, the optimal dose of MTX to treat PCNSL or IOL has not been determined yet due to (1) the dose heterogeneity in prospective trials, and (2) combination of MTX with other drugs and/or radiotherapy in clinical studies.\textsuperscript{12}

2-methoxyestradiol (2ME\textsubscript{2}), a natural metabolic byproduct synthesized \textit{in vivo} by hydroxylation at the 2-position of estradiol and sequential O-methylation by catechol-O-methyltransferase,\textsuperscript{13,14} new potential chemotherapeutic agent for PCNSL and IOL. It has been shown to inhibit tumor growth in a variety of different cell lines and solid tumors as a result of anti-proliferative and anti-angiogenic activity.\textsuperscript{15–19} The anti-proliferative activity of 2ME\textsubscript{2} results mainly from induction of apoptosis and an arrest at the G2/M phase of the cell cycle that is potentially mediated by p53.\textsuperscript{20}

The hypothesis of this study is that 2ME\textsubscript{2}, like MTX, is an effective agent against IOL. To test the hypothesis, 2ME\textsubscript{2} and MTX effects on human lymphoma cell lines were evaluated with anti-proliferative, apoptotic and cell cycle analyses to obtain preliminary data that would justify as well as aid in the design of an \textit{in vivo} study for treatment of IOL.

**MATERIALS AND METHODS**

\textbf{Chemicals and Materials}: 2ME\textsubscript{2} was a gift from EntreMed Inc. (Rockville, MD, USA). MTX and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Diffuse large B-cell non-Hodgkin’s lymphoma cell lines (Farage and Pfeiffer), fetal bovine serum (FBS), and RPMI 1640 were supplied by the American Type Culture Collection (ATCC) (Rockville, MD, USA).

\textbf{Cell Culture}: Farage and Pfeiffer cell lines were grown in RPMI 1640 supplemented with 10\% FBS, 2mM L-glutamine, 1.0 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin (CM/10\% FBS). Cultures were maintained under standard conditions (37°C, 5\% CO\textsubscript{2} in a humidified atmosphere).

\textbf{Proliferation Studies}: Tetrazolium-based colorimetric assay was used to quantify the anti-proliferative effects of 2ME\textsubscript{2} and MTX using a microplate reader. Cells were seeded into 96-well plates at a concentration of 2.5 × 10\textsuperscript{4} cells per well in a 50 µl volume of CM/5\% FBS. Stock solutions of 10 µM 2ME\textsubscript{2} or MTX were made in DMSO and diluted with CM/5\% FBS to yield concentrations of 0.02 µM, 0.2 µM, 0.6 µM, 1.4 µM, 2 µM, and 20 µM of 2ME\textsubscript{2} or MTX. The different drug concentration solutions (50 µl) were added to each set of wells in quintuplicate. Final concentrations of 2ME\textsubscript{2} and MTX were 0.01, 0.1, 0.3, 0.7, 1, and 10 µM. The control culture contained 0.1\% DMSO, equivalent to the 10 µM 2ME\textsubscript{2} and MTX cultures. The cells were allowed to incubate at standard conditions for 24 hr, 48 hr, and 72 hr, after which 20 µL per well CellTiter 96 Aqueous One (Promega, MTS tetrazolium compound) was added. The absorbance of each well was determined using a ELX800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Analytical Diagnostic Products, Weltevreden, SA) at a wavelength of 490 nm. The absorbance of each well corresponds to the number of living cells in that well. Data from the experiment were normalized against the absorbance of the control well, and data were plotted as a percentage of...
the control (mean ± standard deviation). Each proliferation assay was performed in triplicate for each cell line and drug in three independent experiments.

**Apoptosis Studies:** To detect early stages of apoptosis, approximately 5 × 10^5 cells were seeded in 60 mm tissue culture dish in CM/5% FBS containing 2ME2 or MTX at concentrations of 0 µM (0.1% DMSO vehicle), 0.1 µM, 1 µM, and 10 µM. The cells were incubated in triplicate for an additional 48 hr. Following incubation, the cells were washed twice with cold HBSS and resuspended in 1× binding buffer (Invitrogen, Chicago, IL, USA). Approximately 1 × 10^5 cells in 100 µL of 1× binding buffer were incubated for 15 min in the dark with 10 µL of Annexin V-FITC solution (Invitrogen, Chicago, IL, USA) and 10 µL of 10 µg/mL propidium iodide (PI) solution (Invitrogen, Chicago, IL, USA). The samples were diluted with 400 µL of 1× binding buffer and analyzed by flow cytometry (Coulter FlowCentre, Miami, FL, USA) and WinList software (Verity Software House Inc. Topsham, Maine, USA). To conduct time-dependant apoptosis studies, approximately 5 × 10^6 Farage cells were seeded in a 60-mm tissue culture dish in CM/5% FBS containing 2ME2 or MTX for concentrations of 1 µM, as described previously. The cells were incubated in triplicate for an additional 4 hr, 12 hr, 24 hr, 36 hr, and 48 hr. Following incubation, Farage cells were analyzed with flow cytometry as described above.

**Cell Cycle Analysis:** Cells exposed to 2ME2, MTX, or DMSO vehicle for 36 hr were analyzed by flow cytometry to study changes in cell cycle progression. Approximately 2 × 10^6 cells were seeded in 60 mm tissue culture dishes in CM/5% FBS containing 2ME2 or MTX at concentrations of 0 µM (0.1% DMSO vehicle), 1 µM, and 10 µM as previously described. Cells were centrifuged at 100 × g for 10 min and the pellets washed with PBS. The cells were resuspended in 100 µL of 0.85% NaCl, and fixed in 70% ethanol and stored at 4°C for at least 2 hr before the cells were stained with PI. Subsequently, the fixed cells were centrifuged and washed with PBS. The cells were resuspended in 0.5 ml PBS containing 50 µg/ml PI and 50 µg/ml RNase A and incubated for at least 10 min at room temperature. The samples were analyzed with flow cytometry. At least 10,000 events were registered for each determination. The data were analyzed with ModFit software (Verity Software House Inc. Topsham, Maine, USA).

**Statistical Analysis:** The data obtained in the studies of proliferation, apoptosis, and cell cycle analysis were analyzed for statistical significance of detected differences between groups using the unpaired Student’s t-test. The 2-sided P value was determined, testing the null hypothesis that the 2 population medians are equal. P values less than 0.05 were considered to be statistically significant.

**FIGURE 1** Dose-dependant cell proliferation after treatment with 2ME2 and MTX for 72 hr. Tetrazolium-based colorimetric assay was used to quantify the relative viable cell number using a microplate reader. Cells were seeded into 96-well plates at a concentration of 2.5 × 10^4 cells per well at 2ME2 and MTX concentrations of 0.01, 0.1, 0.3, 0.7, 1, 10 µM in CM/5% FBS. The cells were allowed to incubate at standard conditions for 72 hr. Data from the experiment were normalized against the absorbance of the control well, and data were plotted as a percentage of the control (mean ± standard deviation). Each proliferation assay was performed three times in triplicate. F: Farage cell line, P: Pfeiffer cell line, “P < 0.0001 versus the vehicle-treated control (student’s t-test).
RESULTS

Proliferation Study: The anti-proliferative effects of 2ME2 and MTX in Diffuse large B-cell non-Hodgkin’s lymphoma cell lines are shown in Figure 1. The number of viable cells in cultures exposed to 2ME2 or MTX for 72 hr was expressed as a percentage of the number of viable cells in untreated cultures. The concentration of 2ME2 required for 50% inhibition of proliferation (IC_{50}) was 0.4 and ~1 µM for Farage and Pfeiffer, respectively. In contrast, the IC_{50}s for MTX were 0.06 and 0.2 µM for Farage and Pfeiffer, respectively. Thus, the IC_{50}s for MTX were approximately an order of magnitude lower than those of 2ME2. The anti-proliferative effect on the lymphoma cells by 2ME2 began at 0.1 µM whereas that for MTX began at 0.01 µM. The dose-response curves demonstrated that the lowest active concentration of 2ME2 (i.e., the lowest tested concentration at which the maximal effect was observed) was ~ 1 µM whereas for MTX it was ~0.1 µM. MTX exhibited an effect on lymphoma cells at 0.01 ~ 0.1 µM, while 2ME2 exhibited no significant effect at these

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FIGURE 3  Apoptosis study with Farage and Pfeiffer treated with 2ME2 and MTX for 48 hr. Approximately $5 \times 10^5$ cells were seeded in 60-mm tissue culture dish in CM:5% FBS containing 2ME2 or MTX for concentrations of 0 µM (0.1% DMSO vehicle), 0.1 µM, 1 µM, and 10 µM. The cells were incubated in triplicate for an additional 48 hr. Following incubation, approximately $1 \times 10^5$ cells in 100 µL of 1× binding buffer were incubated for 15 min in the dark with 10 µL of Annexin V-FITC solution and 10 µL of 10 µg/mL propidium iodide (PI) solution. A: Farage cell line, B: Pfeiffer cell line, C: Percentage representation of mean apoptotic cell for Farage and Pfeiffer. * $P < 0.0001$ and ** $P < 0.0005$ versus the vehicle-treated control (student’s $t$-test).
concentration ($P < 0.0001$ in $0.1 \mu M$ of 2ME2 and MTX for Farage and Pfeiffer). In the 72-hr dose-response proliferation curve, MTX had more cytotoxic effect on lymphoma cells than did 2ME2 at lower concentrations ($<0.1 \mu M$). However, at high concentrations ($>1 \mu M$), both 2ME2 and MTX had comparable dose-dependent anti-proliferative effects on the lymphoma cells ($P > 0.001$ in 1 and $10 \mu M$ of 2ME2 and MTX for both Farage and Pfeiffer). Farage cells showed more sensitive dose-response to both 2ME2 and MTX compared with Pfeiffer in the anti-proliferation curve. Figure 2 shows a time- and dose-dependant cell proliferation study. For concentrations of $1 \mu M$ and $10 \mu M$, both 2ME2 and MTX showed similar cytotoxic effect on both Farage and Pfeiffer cells at 24 hr, 48 hr, and 72 hr. However, for a concentration of $0.1 \mu M$, MTX had more cytotoxic effect on lymphoma cells than did 2ME2 at each time point. Both 2ME2 and MTX exhibited time and dose dependant anti-proliferative effect on the lymphoma cells.

Apoptosis Study: To investigate whether 2ME2 and MTX were causing lymphoma cells to undergo apoptotic cell death, Farage and Pfeiffer were incubated with varying concentration of 2ME2 or MTX and the percentage of annexin V-reactive cells was assessed as shown in Figures 3 and 4. Figure 3A and B shows representative two-color flow cytometry analysis results for control and $10 \mu M$ drug-treated lymphoma cells stained with PI and annexin V-FITC for Farage and Pfeiffer, respectively. Early apoptotic cells are located in the lower right quadrant, which is positive for annexin V-FITC and negative for PI. Evaluation of the annexin test revealed a clear increase in positively reacting cells in $10 \mu M$ 2ME2 or MTX compared with control. Figure 3C shows the dose-dependant apoptosis study for 48 hr with control, 0.1, 1, and $10 \mu M$ 2ME2 or MTX. In general, both 2ME2 and MTX induced dose-dependant apoptosis. At a low concentration ($0.1 \mu M$), MTX induced more apoptosis than did 2ME2 for both Farage (5.2%) and Pfeiffer (3.8%). In contrast, at $1 \mu M$ concentration, 2ME2 induced more apoptosis than did MTX for both Farage (4.5%) and Pfeiffer (9.5%). At high concentration ($10 \mu M$), 2ME2 induced more apoptosis than did MTX for Farage (11.2%), whereas MTX induced more apoptosis than did 2ME2 for Pfeiffer (4.2%). Figure 4 shows apoptosis vs. time for a fixed concentration ($1 \mu M$) of 2ME2 or MTX with Farage cells. The Farage cells treated with 2ME2 showed more apoptosis than those with MTX from 12 hr to 48 hr. Maximum induction of apoptosis occurred at 24 hr for 2ME2 (25.3%) and at 36 hr for MTX (17.6%).

Cell Cycle Study: After demonstrating the anti-proliferative effect of 2ME2 and MTX on the two types of lymphoma cells, we performed flow cytometry to
FIGURE 5  Cell cycle study with Farage treated with 2ME2 and MTX for 36 hr. Approximately $2 \times 10^6$ cells were seeded in a 60-mm tissue culture dish in CM/5% FBS containing 2ME2 or MTX at $0 \mu M$, $1 \mu M$, and $10 \mu M$. The cells were resuspended in $100 \mu l$ of 0.85% NaCl, fixed in 70% ethanol, and stored at 4°C for at least 2 hr. The cell were then suspended in 0.5 ml PBS containing 50 μg/ml PI and 50 μg/ml RNase A for at least 10 min at room temperature. A: Representative histogram of the data, B: Percentages of the different compartments of the cell cycle. * $P < 0.0001$ versus the vehicle-treated control (student’s t-test).
determine whether the anti-proliferative effect resulted from a cell cycle-specific block. In the cell cycle experiments, 1 and 10 µM concentrations of 2ME2 and MTX were chosen to treat the experimental cells (Farage). Figure 5A shows representative histograms of the data generated by flow cytometry. Figure 5B displays the percentages of the different compartments of the cell cycle for Farage cells treated with 2ME2 or MTX. For Farage cells treated with 2ME2, there was an increase in the G2/M phase and a decrease in the G0/G1 phase when compared with control. However, for Farage cells treated with MTX, there was an increase in the S phase and a decrease in the G2/M phase when compared with control. These results suggest that 2ME2 is a G2/M-phase specific blocker and MTX is an S-phase specific blocker.

**DISCUSSION**

The anti-proliferative, apoptotic, and cell-cycle effects of 2ME2 and MTX in human lymphoma cell lines were compared in this work. 2ME2 is not a proven agent in the treatment of PCNSL and IOL, whereas MTX is an agent known to be active against PCNSL and IOL. From our results, 2ME2 and MTX show comparable dose-time anti-proliferative effect and comparable dose-time apoptotic effect on the lymphoma cells. In cell-cycle results, 2ME2 induces a G2/M phase block and MTX induces an S-phase block.

A dose-dependent G2/M arrest of 2ME2 has been found in a wide range of cancer cells. Reported studies suggest that the antimitic effect of 2ME2 results from its toxicity to microtubules and its effects on the regulators of cell cycle progression. The relationship between cell cycle and MTX activity is not clear, although MTX is considered to prevent DNA synthesis by means of its irreversible binding to the enzyme dihydrofolate reductase during the S-phase.28,29

The IC50 of 2ME2 in 55 different tumor cell lines ranged from 0.08 to 10 µM30 and the IC50 of MTX in 63 different tumor cell lines from 0.1 to 1 µM.31 Our results using lymphoma cell lines were comparable to those obtained in these reported studies. At 1 µM concentration, 2ME2 showed similar anti-proliferative effect on the lymphoma cell lines than did MTX (Fig. 1 and 2 in our results). In previously reported studies, for normal endothelial cells (HUVEC), 1 µM 2ME2 showed no appreciable toxicity,32 while MTX at this same concentration exhibited significant cytotoxicity.33 The previous study34 showed that proliferation and apoptosis of normal lymphocytes was not altered significantly, even at higher doses (3–20 µM) of 2ME2. In contrast, our results have shown that proliferation and apoptosis of lymphoma cells were dose-dependant responses to 2ME2. Taken together, these findings indicate that 2ME2 has selective anti-lymphoma activity. Also, 1 µM 2ME2 showed greater time sensitive anti-proliferative effect than did MTX in tested cell lines. From these results, 2ME2 at a therapeutic target concentration of 1 µM may be an effective and relatively nontoxic drug against PCNSL and IOL. Further studies will be needed to determine whether this therapeutic target concentration of 2ME2 can be achieved by systemic administration, intravitreal injection, or a controlled release implant.

In this study, Farage (EBV positive) and Pfeiffer (EBV negative) cell lines were used to delineate drugs effect on EBV. The origin of IOL and PCNSL is not known. However, it is hypothesized that reactive lymphocytes may be attracted by viral infection into the CNS, where they undergo transformation into neoplastic cells. A defect in the host’s response to EBV has been proposed as a possible cause in immunocompromised patients.35 In human immunodeficiency virus (HIV) positive patients, PCNSL is always EBV positive.36 EBV has also been demonstrated in primary IOL in an AIDS patient.37 Therefore, EBV has been linked etiologically to both PCNSL and IOL. In our proliferation results in Figure 1, both MTX and 2ME2 showed more cytotoxic effects in the EBV positive cells (Farage) than that in the EBV negative cells (Pfeiffer). Our results suggest that the inclusion of 2ME2 into chemotherapy protocols against PCNSL and IOL may be justified.

In conclusion, our study of the effect of 2ME2 and MTX on anti-proliferation, apoptosis, and cell cycling suggests that 2ME2 is a potential agent for treating PCNSL and IOL.

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**REFERENCES**


