

## Flow Cytometric Analysis of the Effects of Methotrexate and Vepesid on the HEp-2 Cell Cycle

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**Abstract: Objective:** To determine the mechanism of action of Methotrexate and Vepesid on the HEp-2 cells isolated from human laryngeal cancer cells morphologically and flow cytometrically (G<sub>1</sub>, G<sub>2</sub>, S and P1).

**Materials and Methods:** The HEp-2 continuous cell line was used. Cultivation of the cells was realized in EMEM medium with 10% fetal bovine serum at an atmosphere of 37°C with 5% CO<sub>2</sub>. Six different concentrations of Vepesid and Methotrexate were prepared by diluting with deionized water (5 µg/ml, 50 mg/ml, 500 µg/ml). The morphological and cell cycle parameters of HEp-2 cells were determined by inverted microscope and flow cytometer respectively.

**Results:** In the morphological examination, Vepesid was found to have a more significant cytopathologic effect on the cells than Methotrexate, whereas in the flow cytometric examination, it was found that whilst Methotrexate stopped the cells at the S and G<sub>2</sub> phases, Vepesid did that only at the G<sub>1</sub> phase.

**Conclusion:** Both the flow cytometric and cell morphological analysis showed Vepesid to be more effective than Methotrexate on HEp-2 cells. Results of studies conducted show the mechanism of action of these drugs to be dependent on the origin of the cell and on the drug type.

**Key Words:** Cell cycle, Methotrexate, Vepesid, HEp-2 cell, flow cytometer

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### Introduction

One of the main objectives of modern chemotherapy is to obtain better treatment with minimal toxic effects. The drugs employed in oncology today are mostly synthetic (azacitine, cytarabine, fluorouracil, mercaptopurine, methotrexate, thioguanine) and a minor part is semi-synthetic (vincristine, vinblastine, paclitaxel). The mechanism of action of the drugs in both groups is basically on the intracellular enzymes and substrates, by which they effect the cellular development. Because most of the drugs produced synthetically are extremely toxic to the cell, in recent years investigations have been focused on drugs of plant origin (1).

Among the antimetabolic drugs, one of the most important and widely used drugs is Methotrexate (MTX). MTX is a chemical agent that acts by inhibiting the enzyme dihydrofolic acid reductase, which catalyses the conversion of folic acid to its active form folinic acid, by binding to it (2, 3).

Vepesid is also a drug used in the treatment of cancer.

It interferes with the multiplication of cancer cells and slows or stops their growth and spread in the body. Vepesid (etoposide), which is obtained from the plant *Podophyllum peltatum*, a plant alkaloid, is the semi-synthetic derivative of podophylotoxin. Vepesid, like other drugs in the group, exerts its effects by inhibiting the synthesis of the DNA and RNA of the cells and thereby inhibiting cell division (4).

Information regarding the mechanism of action and pharmacological properties of MTX is abundant because it has been in use various fields of oncology for a long time (2, 5, 6). However vepesid, relative to MTX, is a very new drug and has been introduced in the field of oncology only recently. For this reason, information about its mechanism of action on the different cancer cells is relatively scant and contradictory (4, 7, 8). The number of comparative studies about the therapeutic potency and toxicity of this drug is also limited. In addition, we did not come across any literature on the effects and toxicity of these drugs on HEp-2 cells derived from human laryngeal cancer cells.

For these reasons, we investigated the effects of these two anti-neoplastic drugs on cell growth and development by analyzing the effects on both cell morphology and the cell cycle parameters ( $G_1$ , S,  $G_2$ , and the proliferation index) on HEp-2 cells using the inverted microscope and the flow cytometer.

**Materials and Methods**

Experiments were carried out on HEp-2 continuous cell culture derived from human laryngeal cancer cells. The HEp-2 continuous cell culture was obtained from the Virology Department of the Ankara Refik Saydam Central Hifzisiha Institute. In the preparation of the culture, EMEM (Eagle’s Minimum Essential Medium) (Sigma) was used as the medium and fetal bovine serum (Seromed) was used as the growth factor in the medium at a ratio of 10%. The cells were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. All the culture plates were set to contain a cell count of 100,000/ml.

The effects of MTX and Vepesid on the HEp-2 cells were investigated at concentrations of 5, 50, and 500 mg/ml in an EMEM culture medium containing 10% fetal bovine serum. Also, as the control group, HEp-2 cells were cultivated in media containing no anti-neoplastic drug. After 48 hours of incubation, the cells were examined morphologically with an inverted microscope. Later the samples of cells growing in mono layers in the culture plates were taken and washed with balanced saline solution (PBS) and the cells were then covered with a versen-trypsin solution (containing in 1L: 8 g NaCl, 0.2 g KCl, 2.37 g Na<sub>2</sub>HPO<sub>4</sub>\*12 H<sub>2</sub>O, 0.2 g K<sub>2</sub>HPO<sub>4</sub>, 1 g titriplex III (versen) 1.25 g trypsin (Sigma)). The culture plates were kept at 37°C for 5-10 minutes to allow the cells to come off easily from the plates. Cells that were separated by treatment with versen-trypsin solution were centrifuged at 1000 rpm at +4°C to remove the versen-trypsin solution. Later, the cellular pellets that precipitated at the bottom of the centrifuge tube were prepared with the EMEM culture medium to contain 1,000,000 cells/ml and 12 x 75 ml tubes were each filled with 100 ml portions of the resulting cell suspension for flow cytometric analysis.

**DNA Analysis ( Determination of the Cell Cycle)**

100 ml of the Coulter DNA-prep LPR solution (Coulter Electronics Health, USA), which promotes the opening of the pores on the surface of cells in suspension

was added and within 1-2 seconds 2 ml of the Coulter DNA-prep stain (Coulter Electronics Hialeah, USA) containing small amounts of Pi and RNA was added and after gently stirring for approximately 20 minutes, it was stored at room temperature in the dark. Later the specimens were passed through the flow cytometer equipment (Coulter, Epics XL, USA) for measurement. The results obtained were evaluated using the Mplus AV (version 3.01) computer program (Coulter Electronics, USA). The DNA index was analyzed on the specimens together with the chicken erythrocytes that contained the n number of DNA. The DNA index was calculated by dividing the peak  $G_0/G_1$  of the specimen by the peak  $G_0/G_1$  of the erythrocytes.

Figure 1 shows an example of how a population of cycling cells might appear on a flow cytometer’s data display. The horizontal axis of the graph displays the amount of DNA per cell, identified by relative, arbitrary units. The vertical axis identifies the number of cells at each amount of DNA.

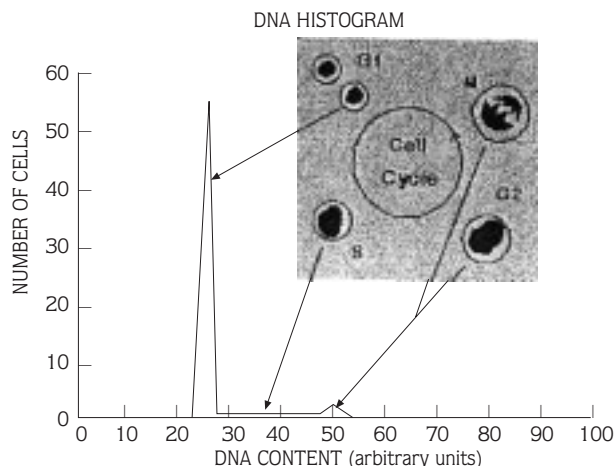


Figure 1. Measuring of the cell cycle by the flow cytometer.

**Results**

**Morphological Analysis:** The morphology of the HEp-2 cells grown on culture media containing various proportions of MTX and Vepesid were examined using the inverted microscope.

The cells in the control growth media at the end of the 48 hour incubation period were observed to have grown with adhesion in a mono layer covering the entire surface

of the culture plate. Microscopic examination of the cells did not reveal cytopathologic changes such as granulation, fusion, nuclear enlargement or cell rounding.

In all culture media that contained MTX, cellular adhesion and pathologic changes were observed in the cells. Microscopic examination of the cells from the culture media containing 5 µg/ml of MTX revealed, in addition to the disruption of the cell membrane integrity, findings such as granulation, nuclear enlargement and rounding of cells. These morphological changes were observed to be more pronounced with increasing concentration (50 µg/ml and 500 µg/ml) of MTX.

In culture media that contained 5 µg/ml and 50 µg/ml of Vepesid, morphologic changes similar to those of MTX were found. These effects on the cells were observed to be more striking for Vepesid than for MTX at the same concentrations. In culture media that contained 500 µg/ml of Vepesid, however, disturbance in the adhesion pattern, failure to grow in a mono layer, and microscopically, cytopathologic changes like cell rounding and significant nuclear enlargement were observed.

**Flow Cytometric Analysis:** These findings were evaluated in two groups under cell cycle and proliferation index.

Figure 2 shows the results of the comparison between the effects of Vepesid and MTX on the cell cycle of the HEp-2 cells and the effects on the control group. In the figure, almost all the cells in the control group are seen to exist in the G<sub>2</sub> phase of the cell cycle. In media containing MTX, most of the cells are in the S and a small fraction in the G<sub>2</sub> phase, whilst almost all those in media containing Vepesid were in the G<sub>1</sub> phase.

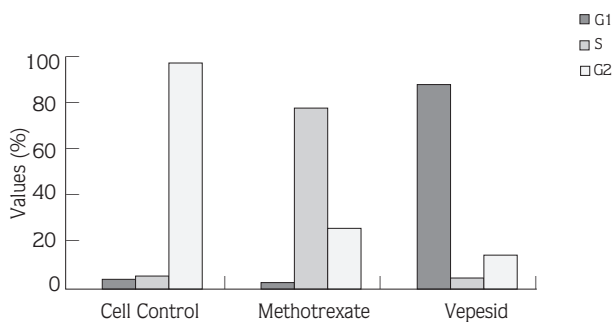


Figure 2. The effects of Vepesid and Methotrexate on the cell cycle of the HEp-2 cells.

In Figure 3, the proliferative indices of the culture media containing MTX and Vepesid are shown together with those of the controls. The proliferative index of Vepesid is seen to be relatively lower than that of MTX.

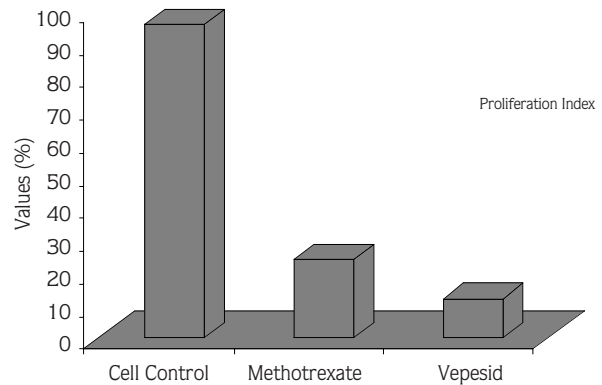


Figure 3. Proliferation indices of HEp-2 cells in the media containing Methotrexate and Vepesid.

### Discussion

Cancer is a disease where regulation of the cell cycle goes awry and normal cell behavior is lost. Cancer begins when a single cell is transformed or converted from a normal cell to a cancer cell. Often this is because of a change in function of one of several genes that normally function to control growth. Once these crucial cell cycle genes start behaving abnormally, cancer cells start to proliferate wildly by repeated, uncontrolled mitosis (9, 10).

If cell division is understood completely, it might be possible to control many forms of cancer. Basically, the cell cycle is the "program" for cell growth and cell division (proliferation). There are 4 broad phases of the cell cycle: G<sub>1</sub> (and G<sub>0</sub>), S, G<sub>2</sub>, and M. The G<sub>1</sub> (Gap 1) phase is characterized by gene expression and protein synthesis. This is really the only part of the cell cycle regulated primarily by extracellular stimuli (like mitogens and adhesion). Anyway, this phase enables the cell to grow and to produce all the necessary proteins for DNA synthesis. It primes the cell to enter the next phase: The S (Synthesis) phase. During this phase, DNA is synthesized. DNA is synthesized only during the S phase between G<sub>1</sub> and G<sub>2</sub>. During the G<sub>2</sub> phase, the cell again undergoes growth and protein synthesis; it needs enough proteins for 2 cells, priming it to be able to divide. Once

this is complete (by the way, there are many "checkpoints" along the way), the cell finally enters the fourth and final phase of the cell cycle: the M (Mitosis) phase. During the M phase, the cell splits apart (called cytokinesis) into two daughter cells. Following mitosis, the daughter cells may re-enter the G<sub>1</sub> phase, or proceed to a 5<sup>th</sup> phase called "G<sub>0</sub>", where growth and replication stops. Cells in G<sub>0</sub> are said to be "quiescent". G<sub>0</sub> cells may eventually re-enter G<sub>1</sub> or perhaps die (11). (Figure 4).

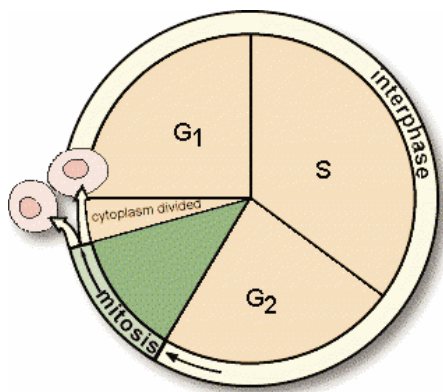


Figure 4. Phases of the cell cycle.

Cancer cells escape from the controls on cell division, and the action mechanism of anti-neoplastic drugs is based on the stopping of uncontrolled cells at any one stage of the cell cycle (9). It is very important that the action mechanism of the anti-neoplastic drugs is known for the treatment of cancer cells.

We therefore set out to investigate the effects of MTX, a drug that has been in use clinically in the antimetabolic group of drugs, and Vepesid, a semi-synthetic derivative of podophylotoxin obtained from the plant *Podophyllum peltatum*, on the cells of HEP-2 cell culture isolated from human laryngeal cancer, in the light of the above. With this objective, we investigated the effects of varying concentrations of these two agents on both the morphology and cell cycle parameters (G<sub>1</sub>, S, G<sub>2</sub>, and PI) of the HEP-2 cells.

In recent years, new drugs in clinical practice continue to emerge in the field of chemotherapy every passing day. Most of the drugs, although known to have toxic effects, have succeeded in gaining clinical use. MTX and Vepesid are the most important drugs among these antineoplastic

agents. MTX, which has been in use for a long time in cancer chemotherapy as an anti-neoplastic agent, continues to be employed widely in the various fields of oncology (2, 12, 13). Vepesid is an antineoplastic drug that is produced as the semi-synthetic derivative of podophylotoxin. Although its biochemical mechanism is not well understood, it has effects similar to those of etoposide and tenoposide. Etoposide and tenoposide both block the cell cycle in two specific places: they block the phase between the last division and the start of DNA replication (the G<sub>1</sub> phase) and they block the replication of DNA (the S phase). However, researchers do not fully understand of how the compounds do this. Vepesid at low concentrations blocks growing cells at the S and G<sub>2</sub> phases of the cell cycle, and at higher concentrations blocks cells at the G<sub>2</sub> phase (1, 7, 8, 14, 15, 16).

Whilst the flow cytometric analysis of the control, which was observed not to exhibit any pathologic morphological changes, revealed most of the cells to be in the G<sub>2</sub> phase in the media containing MTX, a small proportion of the cells were observed to have passed into the G<sub>2</sub> phase where synthesis of cellular components necessary for mitotic cell division takes place, with the majority of them being blocked at the S phase where replication of the DNA genome was observed to take place. In the cultures that contained Vepesid, most of the cells, unlike those of the MTX medium, were found to be in the G<sub>1</sub> phase where the cellular components necessary for the synthesis of the DNA genome occur, and only a small proportion of the cells in the S and G<sub>2</sub> phases. With cells in culture under normal conditions, as in the control group, most of the cells are expected to be in the G<sub>2</sub> phase. In contrast, however, for cells from the media with MTX, the number of cells that were able to pass into the G<sub>2</sub> phase was found to be exactly 4 times lower than in the control group, whereas those of the Vepesid group were exactly 8 times lower than those of the control group.

In this study, where similar concentrations of each antineoplastic agent were used, Vepesid was clearly seen to be more potent than MTX on the laryngeal cancer cells. These effects are also seen clearly in Figure 2 in which the proliferative indices of the two drugs are shown. Whilst the cells from the control group were found to have a PI close to 96%, that of cells from the culture media containing MTX was found to be lower (approximately 24%) whereas that of the cells from the Vepesid media was the lowest (12%).

In studies with MTX, which is widely used nowadays in the treatment of leukemia and several other tumors, Huang et al. (17) reported that MTX lead to a reduction in the level of ribonucleotide reductase, which plays an important role in the synthesis and repair of DNA and is also an important regulator of cell cycle activity. Huschtcha et al. also showed MTX to induce morphological changes in cancer cells and also bring about changes that lead to inhibition of DNA synthesis within 48 hours (18).

In our study, however, we found that, like in the two above-mentioned studies, MTX induced significant pathological changes, and stopped the cells from proliferating by inhibiting the synthesis of DNA at the S (76%) and G<sub>2</sub> (24%) phases.

Concerning the effect of Vepesid on cells under in vitro conditions, Chatterjee et al. (19) reported that it stops proliferation of prostate cancer cells by inhibiting their growth at the G<sub>1</sub> phase of the cell cycle. Bonelli et al. (7) investigated the effect of Vepesid on the L 929 cell series and found Vepesid to reduce DNA synthesis, thereby resulting in an imbalance in the cell protein/DNA ratio of the cells. They also showed that it blocks cells at the S and G<sub>2</sub> phases of the cell cycle. Pellicciari et al. (16)

reported that the phase in which Vepesid will inhibit cell division depended on the cell type and concentration of the drug employed. In our study, Vepesid was seen to more effective than MTX at the same concentrations on the HEp-2 cells within 48 hours and also brought about significant cytopathological changes. In the flow cytometric analysis, a larger proportion of the cell division was found to have been inhibited at the G<sub>1</sub> (86%) and S (12%) phases. In one of the studies involving Vepesid, quite unlike our results, Bonelli et al. found Vepesid to inhibit the cell cycle at both the S and G<sub>2</sub> phases on an L 929 cell series. Chatterjee et al., in their study with prostate cancer cells, found the cells to be inhibited at the G<sub>1</sub> phase, similar to our results.

In the present study, whilst MTX was seen to block cell division of the HEp-2 cells derived from human laryngeal cancer at the S and G<sub>2</sub> phases, Vepesid was found to do so at the G<sub>1</sub> phase. In comparison of the cytopathological changes induced by these two different drugs of different grouping, Vepesid was found to be more effective than MTX. From these results, the mechanism of action of MTX and Vepesid can be seen to vary with the cell type as well as the concentration of the drug.

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