

## Investigation of the Antiviral Effect of Vepesid on HSV Type 2

Received: November 07, 2000

**Abstract:** **Objective:** Vepesid is a semisynthetic derivative of podophylotoxin extracted from *Podophyllum peltatum*, which is in the group of plant alkaloids. In this study, the antiviral effect of Vepesid against herpes simplex virus type 2 (HSV-2) in *in vitro* conditions was investigated.

**Materials and Methods:** In this investigation, a HEp-2 continuous cell line that was derived from human larynx cancer cells was used. The experiments were done in culture plates with smooth bases consisting of 96 wells. Cultivation of cells was realized in EMEM medium with 10% fetal bovine serum at an atmosphere of 37°C with 5% CO<sub>2</sub>. The toxicity of investigated agents and sensitivity of HEp-2 cells were evaluated according to the Reed and Muench method. The 50% effective dose (ED<sub>50</sub>) of the antiviral agent was expressed as the concentration that inhibits the cytopathological effect in half of the quadruplicate test cultures. Acyclovir was included as a positive control drug for HSV.

The experiments were done in two stages. In the first stage, the concentration of Vepesid that did not affect proliferation was determined by means of morphological and

biochemical parameters (pH, pCO<sub>2</sub>, pO<sub>2</sub>, Na<sup>+</sup>). In the second stage, the antiviral effect of that dose was examined on 100 TCID<sub>50</sub> of HSV-2.

**Results:** The concentrations of Acyclovir and Vepesid (3.12 µg/ml, 6.28 µg/ml) were toxic and 1.56 µg/ml of both agents did not affect cell proliferation. These amounts of Acyclovir and Vepesid inhibited viral reproduction and had no cytopathological effect on cells. Na<sup>+</sup> content of the culture medium was 138 ± 2.54 mEq/L for the control group, 126 ± 1.65 mEq/L for the virus control, 142 ± 0.3 for the Acyclovir, 142 ± 0.4 for the Acyclovir + virus, 143 ± 1.1 for Vepesid, 141 ± 0.2 for the virus + Vepesid. LD50 of Acyclovir and Vepesid was found to be 71% for a virus titer of 100 TCID<sub>50</sub>.

**Conclusion:** We found that Vepesid prevented the viral replication of herpes simplex virus type 2. We think that the study of Vepesid as the treatment for patients with herpes simplex virus (HSV) is very important in terms of its clinical and epidemiological nature.

**Key Words:** Vepesid, Acyclovir, HSV-2, lethal dose, cell culture.

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### Introduction

The emergence of resistance against antiviral drugs used in the treatment of HSV-1, HSV-2, varicella zoster virus and cytomegalovirus infections, in patients with immune deficiency, especially AIDS patients, poses serious clinical and epidemiological problems (1,2). In recent years, resistance to Acyclovir has been noted in patients receiving cancer chemotherapy due to the treatment. This leads to a more serious clinical cause and spread of the disease. For this reason, new and alternative methods are being sought for species that are resistant to Acyclovir (3).

In view of this, the antiviral effects apart from the anticarcinogenic effects of antineoplastic agents are of clinical and epidemiologic importance. Studies on the antiviral effects of the antineoplastic drugs used in the treatment of cancer are insufficient. The presence of the antiviral characteristics of these agents along with their anticarcinogenic effects could prevent viral infections or the emergence of species resistant to the antiviral drugs (4,5).

In this study, the antiviral effects of Vepesid, the semi-synthetic derivate of the plant alkaloid podophylotoxin, against HSV-2 was examined in an HEp-2 continuous cell culture.

## Materials and Methods

**Preparation of the Cell Culture:** Experiments were carried out on an HEp-2 continuous cell culture derived from human laryngeal epidermoid carcinoma. The HEp-2 continuous cell culture was obtained from the Virology Department of the Ankara Refik Saydam Central Hifzisi-hha Institute. In preparing the cell culture, EMEM (Eagle's Minimum Essential Medium) (Sigma) containing 10% fetal calf serum (Gibco) was used. Cultivation of cells was realized at an atmosphere of 37°C with 5% CO<sub>2</sub>. The cells were collected and washed with PBS (Phosphate Buffered Saline) and then the surface of the culture cup was completely covered by the cells. Later, versen-trypsin solution (containing 8g NaCl, 0.2 g KCl, 2.37g Na<sub>2</sub>HPO<sub>4</sub> x 12 H<sub>2</sub>O, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 1 g titriplex III, 1.25 g trypsin per liter) was added to cover the surface of the cells. The culture cups were kept at 37°C for 5-10 minutes to allow it to grow and cover the surface of the culture cup. The dispersed cells were centrifugated at 1000 rpm for 10 minutes at + 4°C to remove the versene-trypsin solution. From the pellet of cells precipitated at the bottom of the centrifuge tube a final solution containing 300,000 cells per milliliter was prepared with EMEM containing 10% fetal calf serum.

**Preparation of Drug Dilutions:** Six different concentrations of Vepesid and Acyclovir were prepared by diluting with deionized water.

**Acyclovir:** 1.56 µg/ml, 3.12 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 37.5 µg/ml

**Vepesid:** 1.56 µg/ml, 3.12 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 37.5 µg/ml

**Determination of the Viral Titration:** The HSV-2 species to be determined was removed from liquid nitrogen and immediately dissolved in a 37°C water bath. Then vortexing dilutions of 10<sup>-1</sup>-10<sup>-8</sup> were made in PBS. One hundred microliters of each dilution was then added to 4 wells of the microplate. On to them, 50 µl of the solution containing 300,000 cells/ml, already prepared (with EMEM containing 10% fetal calf serum) was added. Later it was left at 37°C in an atmosphere of 5% CO<sub>2</sub> for incubation. Seventy-two hours after incubation, cells from the wells were examined under a microscope for any cytopathic effects. The 100, 10 and 1 TCID<sub>50</sub> (Tissue Culture Infective Dose 50%) was calculated according to the methods of Reed and Muench (6).

With the aim of determining the concentration of drug not inhibiting the proliferation of cells, 50 µl from each of the six different dilutions of Acyclovir and Vepesid was placed in the 96 well culture plate (Nunc) and 50 µl of the cell suspension (300,000 /ml) was added and incubated for 72 hours in 50 µl EMEM containing 10% fetal calf serum.

### Determination of the Concentration of Drug Inhibiting Viral Proliferation:

#### Viral Inhibition Plate

Drug dilutions (µl/ml)
Diluted virus (100 TCID <sub>50</sub> )
Cell* (50 µl)
Culture Medium (50 µl)

\* HEp-2 Cells: Human laryngeal cancer cells

#### Cell Control Plate

Drug dilutions (µl/ml)
Cell* (50 µl)
Culture Medium (50 µl)

\* HEp-2 Cells: Human laryngeal cancer cells

## Results

Acyclovir and Vepesid at concentrations of 3.12 µg/ml and higher were found to induce clear cytopathic changes such as enlargement of the nucleus, granulation and rounding of the cells. In experiments in which Acyclovir and Vepesid were used at concentrations of 1.56 µg/ml no cytopathological effects were found. At this chosen value, where cellular proliferation was not affected, the optimal concentration, the measured biochemical values (pH, CO<sub>2</sub>, O<sub>2</sub>) after incubation of the cell control groups are shown in Figures 1 and 2.

The EMEM containing 10% fetal calf serum used in the preparation of the HEp-2 cell culture and culture medium taken after incubation of the cells for 72 hours showed the important criteria in the growth and development of the cells as follows: pCO<sub>2</sub>: 5.6 mmHg, 21.2 ± 0.6 mmHg, pO<sub>2</sub>: 189 mmHg, 159 ± 2.9, Na<sup>+</sup>: 147.6 mEq/L, 151 ± 1.5 mEq/L, H<sup>+</sup>: 168 mEq/L, 201 ± 5.8 mEq/L, respectively.

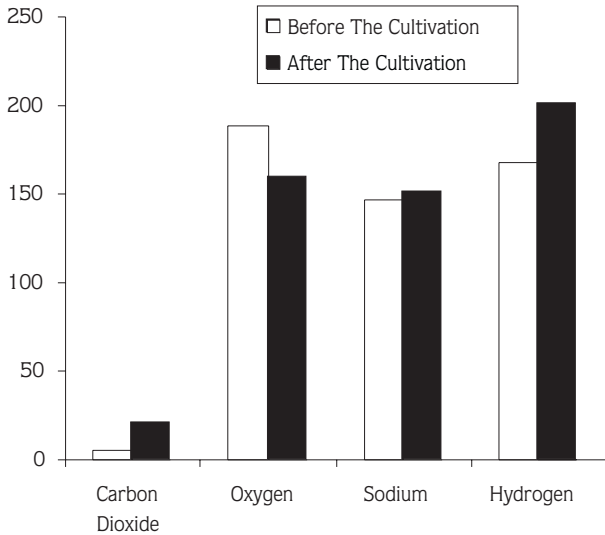


Figure 1. Biochemical values both before and after the cultivation

When the antiviral effects of the drugs of these concentrations against 100 TCID<sub>50</sub> titer of HSV-2 were compared morphologically and biochemically with those of the control group, they were found to inhibit viral reproduction. No cytopathic changes indicative of viral proliferation were found morphologically. The Na<sup>+</sup> content of the culture medium was 138 ± 2.54 mEq/L for the control group, 126 ± 1.65 mEq/L for the virus control, 142 ± 0.3 for the Acyclovir, 142 ± 0.4 for the Acyclovir + virus, 143 ± 1.1 for Vepesid, and 141 ± 0.2 for the virus + Vepesid (Figure 3). The following values were observed for the other biochemical criteria: for the cell control, pCO<sub>2</sub>: 21 ± 1.2 mmHg, pO<sub>2</sub>: 150±3.5 mmHg, H<sup>+</sup>: 202 ±

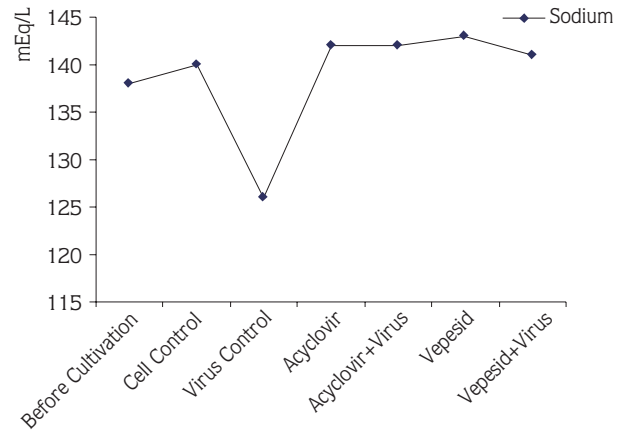


Figure 3. The Effect of Acyclovir and Vepesid against to HSV-2 according to sodium

4.4, for the virus control: pCO<sub>2</sub>: 5 ± 0.2 mmHg, pO<sub>2</sub>: 223 ± 5.2 mmHg, H<sup>+</sup>:176 ± 4.8, for Acyclovir + virus: pCO<sub>2</sub>: 31 ± 0.2 mmHg, pO<sub>2</sub>: 145 ± 2.2 mmHg, H<sup>+</sup>:196 ± 3.8, for the Vepesid + virus pCO<sub>2</sub>: 25 ± 0.4 mmHg, pO<sub>2</sub>: 155 ± 2.7 mmHg, H<sup>+</sup>: 295 ± 3.9.

At concentrations of 1.56 µg/ml, the LD<sub>50</sub> for Vepesid against HSV-2 at three different titrations (1 TCID<sub>50</sub>, 10 TCID<sub>50</sub>, 100 TCID<sub>50</sub>) are seen in Figure 4. The LD<sub>50</sub> for the 1 and 10 TCID<sub>50</sub> was found to be 100% and that at the 100 TCID<sub>50</sub> to be 71%.

### Discussion

Herpes simplex virus infections form an important group among viral diseases. Of these, especially those due

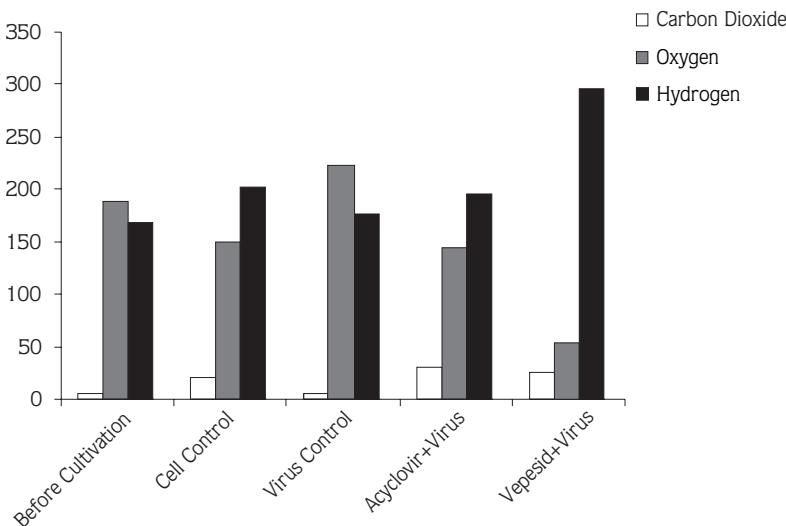


Figure 2. The effects of Acyclovir and Vepesid against to HSV-2 according to biochemical criteria

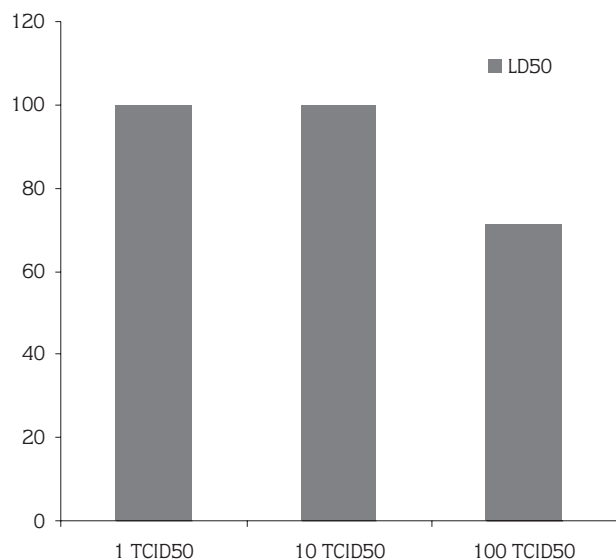


Figure 4. The LD<sub>50</sub> for Vepesid against to HSV-2 at three titrations

to HSV-2 are of most importance to human health. They cause serious infections like neonatal infections, meningitis and cervical cancer (1,7).

Today, in the treatment of herpes viral infections, drugs like 5-iodo-2-deoxyuridine, cytarabine, vidarabine and trifluorothymidine are used. The mechanism of action of these drugs rests basically on their ability to inhibit the virus specific thymidine kinase and the DNA polymerase enzymes. However, because of the cytotoxic effects of these drugs, their use is not widespread. Acyclovir is the most frequently chosen and most potent of the drugs in herpes virus infections. As is known, Acyclovir is the acyclic nucleoside analogues of guanosine. It has a powerful antiviral activity against most herpes viruses. Nonetheless, Acyclovir resistant mutants have been documented in recent years (2,8,9).

Although in the years of its discovery it was known as a revolutionary step, the development of resistance against Acyclovir in the treatment of HSV-1 and HSV-2 infections has led to the search for new antiviral drugs. HSV infections especially present important problems in patients in cancer chemotherapy and those immunocompromised. For this reason, any antiviral effects of the drugs employed in cancer chemotherapy could help to a large extent in preventing these secondary infections as well as the emergence of resistant mutants against the antiviral drugs (1,10-12).

Studies on the presence of antiviral characteristics of the antineoplastic drugs used in the therapy of the cancer today, however, are limited. In this study the antiviral effects of Vepesid, an antineoplastic drug, against HSV-2 were examined in comparison with those of Acyclovir.

Although literature on the antiviral effects of Vepesid is available, there is insufficient knowledge about its use clinically against HSV (13).

Vepesid is a semisynthetic derivative of podophylotoxin obtained from the plant alkaloid *Podophylum peltatum*. It is a highly lipophilic compound. It is one of the effective drugs against small cell cancers. It also has effects against non-hodgkin lymphomas, choriosarcomas, acute myelocytic leukemias and neuroblastomas (14).

Determination of the toxicity and antiviral effects of various drugs and compounds in cell cultures under *in vitro* conditions generally depends on morphological evaluations (calculation of the LD<sub>50</sub> due to cytopathological effect, LD: lethal dose) (8).

In this study, however, in addition to the morphological evaluations, we also examined the cell metabolic values (Na<sup>+</sup>, K<sup>+</sup>, pH, pCO<sub>2</sub>, pO<sub>2</sub>) in the culture media. Of the determinants of the cellular function, the Na<sup>+</sup> ion regulates the membrane potential of the cells. Oxygen is recovered in the provision of energy through the aerobic glycolysis as well as the use of some of the products of the kreb's cycle in the synthesis of the cellular components. Carbon dioxide controls the pH of the cell culture media. The hydrogen ion concentration in the culture is an important criterion necessary for growth development of the cells (15-17).

In the first group of experiments, with the aim of determining the concentration of Vepesid and Acyclovir that did not affect the proliferation of cells, the effects of these two drugs at various concentration on HEp-2 cells were investigated. Except for the 1.56 µg/ml concentration, Vepesid and Acyclovir were found to induce cytopathological changes like nuclear enlargement, granulation and rounding of the cells at all concentrations linearly. The nontoxic concentration of Acyclovir and Vepesid was determined morphologically to be 1.56 µg/ml. At this concentration the drugs were not found to produce any cytopathologic changes in the cells.

Whether Acyclovir and Vepesid at this concentration had any effect on HEp-2 cells or not was investigated by examining parameters related directly to the cell metabo-

lism like the cells and the Na<sup>+</sup> ions, pH, pCO<sub>2</sub> and pO<sub>2</sub> of the culture media containing the drugs. In the experiments, no significant difference was found between the Na<sup>+</sup> ion content of the control group and those of the Vepesid and Acyclovir groups of nontoxic concentration. The Na<sup>+</sup> ion content of the culture media was found to be 138 ± 2.54 mEq/L for the control cells, 126 ± 1.65 mEq/L for the virus control, 142 ± 0.3 for the Acyclovir, 142 ± 0.4 for the Acyclovir + virus, 143 ± 1.1 for Vepesid, and 141 ± 0.2 for the virus + Vepesid (Figure 3).

These results show that the addition of Acyclovir and Vepesid to the HEp-2 cell growth media did not affect cellular proliferation and that the Na<sup>+</sup> pump, a determinant of cellular metabolism, functions normally.

Examination of the biochemical criteria shows that while CO<sub>2</sub> increased from 10 mmHg to 19 mmHg, O<sub>2</sub> fell from 200 mmHg to 160 mmHg in the cell control group. Similarly, in culture media that contained Acyclovir and Vepesid, CO<sub>2</sub> content increased, while O<sub>2</sub> content decreased. When both the morphology and biochemical criteria were considered, Acyclovir and Vepesid at concentrations of 1.56 µg/ml were observed not to induce any toxic effects on the cells.

In the second group of experiments, the antiviral effects of Vepesid and Acyclovir at this nontoxic concentration on HSV-2 were examined in comparison with the cell and viral control groups morphologically and biochemically (Na<sup>+</sup> ions, pH, pCO<sub>2</sub> and pO<sub>2</sub>). In the morphologic examination, no cytopathologic changes were observed in the cell control, whereas in the viral control, as expected, cytopathologic changes specific to the herpes viruses like rounding and granulation of cell and nuclear enlargement were found.

In the culture media containing Acyclovir and Vepesid of concentrations of 1.56 µg/ml, the Na<sup>+</sup> ions were found to be 138 ± 2.54 mEq/L for the cell control, and 126 ± 1.65 mEq/L for the viral control, whereas for the Acyclovir and Vepesid they were 141 ± 2.11 mEq/L and 142 ± 1.86 mEq/L respectively. Here it can be understood that in culture media containing Acyclovir and Vepesid, viral proliferation was inhibited while the Na<sup>+</sup> ions content approached that of the cell control.

It can be seen clearly that apart from the Na<sup>+</sup> ions the pCO<sub>2</sub>, pO<sub>2</sub> and H<sup>+</sup> ion concentration of the culture medium also affects the inhibition of the growth of the HSV-

2 by Acyclovir and Vepesid. This can be inferred from the observation that in the culture media containing the drugs, just as in the control group while the CO<sub>2</sub> and H<sup>+</sup> ions concentrations increased, a decrease in the O<sub>2</sub> pressure was found. This shows that the metabolic activity of the cells continued with the O<sub>2</sub> pressure falling because it is used for cellular activity, whereas as metabolic bio-products the CO<sub>2</sub> and H<sup>+</sup> ions concentration increased.

In addition, the LD<sub>50</sub> of Acyclovir and Vepesid at this concentration against 1, 10, and 100 TCID<sub>50</sub> titers of HSV-2 was calculated. For 1 and 10 TCID<sub>50</sub>, the LD<sub>50</sub> was found to be 100%, whereas for the 100 TCID<sub>50</sub> it was 71%.

Thus, experiments in which various concentrations of Acyclovir and Vepesid were used the antiviral effects of these drugs against HSV-2 at 1.56 µg/ml concentrations were determined by examination of both morphological and biochemical criteria.

In conformity with one study, Fahad et al. found a decrease in the Na<sup>+</sup> ions content of the cell culture medium infected by HSV (18).

Vepesid, which is employed today as an antineoplastic agent, has been reported in two separate studies to possess antiviral activity against HSV-1. In addition, Lewis et al. reported that Vepesid at a concentration of 3 µg/ml incubated the proliferation of HSV type 1 in vero (African green monkey kidney) cells (13).

In a study by Nishiyama et al., the antiviral activity of the semisynthetic antineoplastic drug Epipodophylotoxin VP-16-23 against HSV-2 was observed and found to inhibit the replication of HSV-2. The mechanism of the antiviral action of this compound was shown to depend upon its inhibition of both the topoisomerase II enzyme of the cells and the viral DNA replication (19). Considering the fact that Vepesid is also from the semisynthetic group of drugs it can be assumed to have the same mechanism of action.

The exhibition of antiviral effects against HSV-2 under in vitro conditions by Vepesid can be extremely important clinically. As is known, in patients receiving cancer chemotherapy, and in bone marrow transplant patients with HSV infections, resistance to Acyclovir develops and the disease follows a more severe course. The fact that Vepesid is an anticarcinogenic drug and is used clinically suggests that it can prevent the development of resistance to Acyclovir in herpes virus infections.

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