:ИНИСТЕРСТВО НА ЗДРАВЕОПАЗВАНЕТО
НАЦИОНАЛЕН ЦЕНТЪР ПО ЗАРАЗНИ И ПАРАЗИТНИ БОЛЕСТИ

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F I N A L R E P O R T

Subject: Performance of a scientific task assignment under a contract between A.V.S.T. TRADING Ltd. and the National Center of Infectious and Parasitic Diseases (NCIPD) on: Topographic study of cells infected with human coronavirus strain 229E (HCoV-229E) and herpes simplex virus type 1 (HSV-1), and treated with Inactivated Pepsin Fragment (IPF) - an active ingredient of the Enzoimmune Active preparation by Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) with varying multiplicity of infection and exposure time.

Scientists:

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I. Relevance of the problem

The increasing number of viruses and microorganisms that are resistant to treatment with approved drugs poses a serious health problem of growing public importance. The emergence of pathogenic strains tolerant to drugs for the treatment of viral diseases and to the latest generation of antibiotics is further exacerbating the need to discover and develop new broad-spectrum pharmaceuticals. Despite the significant success of chemotherapy, it cannot be considered that at the present stage in the treatment of viral diseases any final results have been achieved. The low selectivity, significant toxicity, emergence of resistant mutants, high

number of deaths, and the emergence of new zooanthroponotic viruses are among the main reasons for the search for new antiviral drugs.
There has been an increase in research into the antiviral and immunomodulatory properties of biologically active molecules in recent years. It is extremely important to monitor the changes following the infection and treatment of the cells with certain substances, which allows to draw conclusions about the "target" action of the preparations used, and the changes that occur in the cell membrane and in some organelles. The application of Atomic Force Microscopy (AFM) in the study of neoplastic and infected cells has been developing especially intensively recently, as well as the effect of drugs on cell membranes.

Atomic Force Microscopy (AFM) is a modern and widely used method for studying the surface structure and topography of various biological samples, ensuring high precision and resolution in the nanometric scale. What makes AFM unique, however, is that in addition to imaging the arrangement and structure of various molecular architectures, cell adhesion, and spectroscopic mapping, the microscope is able to "capture" the involvement of vital biomolecules or structures involved in vital processes. As a result, the relief of the sample is reproduced with high resolution, and in principle, depending on the applied scanning method, the quality of the blade, etc., the required resolution in the nanometer scale can be reached with AFM images. The obtained precision AFM images give detailed information about the surface characteristics of the samples, and the sizes of the scanned areas can vary in an extremely wide range – from the nanometric atomic-molecular scale to sizes larger than hundreds of microns. For example, AFM can produce images with an area of the order of $5 \times 5\ nm$, covering about a hundred individual atoms and thus giving information about the crystallographic structure of materials, and at the same time can obtain images of areas larger than $150 \times 150\ \mu m$ which reveal in detail the shape and morphology of individual living cells. AFM also allows scanning of extremely uneven and rough surfaces, as its vertical scope is usually in the range of $10 \div 15\ \mu m$. Compared to the Scanning Electron Microscope (SEM), AFM provides higher contrast and direct topographic relief measurements, thus providing quantitative information on the heights of the sample in the scanning area. In addition, since the test specimens must not be electrically conductive, no dehydration or metal coating is required and the samples are examined in their native state. Compared to the Transmission Electron Microscope (TEM), the images obtained with AFM 3D, which can then be subjected to the so-called profile analysis (cross section), give much more information compared to the 2D images obtained with TEM.

The SEM analysis is a powerful research tool that uses a focused electron beam to create complex, highly magnified images of the surface topography of a sample. The object is scanned (crawled point by point) by an electron beam. The need to have a thin sample is a limitation of TEM – there is no such limitation with the Scanning Electron Microscope. The resolution, although lower than that obtained with TEM, is impressive – from 1 to 10 nm. An important advantage of SEM over optical microscopes, in addition to the higher resolution, is the focus depth. No pre-treatment of the samples is required using ultra-thin sections, coating with precious metal, dehydration, etc.

Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) can be used as precise morpho-functional methods to study physiological and pathological cellular processes. The combined use of the two types of microscopy makes it possible to obtain nanoscale images, to accurately visualize changes in the morphology and elasticity of cell membranes, and others. SEM and AFM studies of infected and treated cells provide valuable information on all pathomorphological processes occurring on the surface (membrane) of the cell, as well as inside it. In the performed experiments a number of modifications were made and the techniques for tracking morphological and torographic changes on the surface of the

infected cells and for assessing the effect of drug administration at different times of exposure with the preparation were adapted.

II. Materials and Methods

Test substance

Inactivated pepsin Fragment (IPF) – an active ingredient of the Enzoimmune Active preparation provided by A.V.S.T. TRADING Ltd.

IPF was dissolved to an initial concentration of 50 mg/ml (background solution). Serial dilutions were prepared *ex tempore* thereof, in a growth medium DMEM (Dulbecco's Modified Eagle Medium, containing 2% fetal bovine serum (FBS, Gibco) and 1% antibiotic solution (100 U/ml penicillin, and 100 µg/ml streptomycin sulphate (Gibco, USA)) (working solutions), which were filtered through sterile antibacterial filters with a pore diameter of 0.45 µm (Sartorius Stedium, Australia).

Cell culture

Two monolayer African green monkey (*Cercopithecus aethiops*) kidney cell lines were used as a model system in the tests (Vero line), and a human embryonic lung (Lep line), courtesy of the Cell Culture Bank at the National Reference Laboratory for Cell Cultures, Rickettsiae and Oncogenic Viruses, NCIPD, Sofia. The cells were cultured in DMEM medium with added thermoinactivated 10% FBS, 1% sodium pyruvate (Sigma-Aldrich, Germany) and antibiotics (penicillin (100 U/ml), streptomycin sulphate (100 µg/ml) (growth medium). The cell cultures were incubated at 37 °C at 5% CO_2 and the air humidity required. The Vero and Lep cells were blended 1:3 at a density of about 2 x 10^5 cells/ml, then resuspended several times. and poured into cell culture mattresses (25 cm^2) (Orange Scientific, Belgium) Before inoculating the cells with virus and/or the corresponding dilutions of IPF, the monolayer was washed three times with Phosphate-Buffered Saline (PBS), pH 7.4, for 1 - 2 minutes, after which the solution was removed. All experiments were performed during the exponential phase of cell growth.

Blending of cell culture

The seeding of the cells from the Vero and Lep lines was performed using a solution containing 0.05% trypsin (Trypsin) and 0.0025% Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Germany), pre-tempered at 37 °C. The cells were treated for about 3-10 minutes with this solution until they began to round and dissociate. Subsequently, the trypsin-versene solution was replaced with a small volume of growth medium, trypan blue test was applied to differentiate living from dead cells, counted using a hemocytometer, diluted to a certain volume with a density of 2 x 10^5 cells/mL, and resuspended in cell culture mattresses. The cells were cultured at 37 °C at 5% CO_2 and at the air humidity required.

Test for staining of dead cells with trypan blue

Aliquots of cell suspension from Vero or Lep cells were mixed with an equal volume of 0.4% solution of trypan blue dye, observed under an inverter light microscope and using a hemocytometer, counting the living (unstained, with a clear cytoplasm) and dead (dark blue) cells.

Freezing and thawing of cells

Freezing and thawing of cells was performed according to generally accepted methods, as described by Pegg D., 2007. Cells in the logarithmic phase of growth were used for freezing. They were washed once with DMEM medium (5 - 10 min, 800 - 1500 rpm/min, 4 °C). The resulting cell depot was resuspended in culture medium and FBS (90%), and the cell

suspension was cooled on ice (2 - 4ºC), then DMSO was added with stirring to a final concentration of 10%. The cells were dispensed into pre-cooled ampoules (0.5 - 1 mL/cell suspension /ampoule) and placed in a styrofoam box in a freezer at -80 °C. After at least 24 hours, the ampoules were transferred to liquid nitrogen (-196 °C). The cells were frozen at a concentration of not less than 1 x 10^6 cells/mL, and most commonly 5 x 10^6 cells/ampoule.

When thawing the cells, the ampoules were quickly placed in a vessel with water heated to 37 - 40 °C and after thawing they were immediately transferred to a medium containing 10% FBS. In order to prevent the toxic effects of DMSO, the culture medium was replaced with a new one immediately after the cells adhered to the medium (no later than 20 hours after seeding).

Determination of cell viability

Three methods were used to assess relative cell survival and proliferation: 1) Microscopic observation of morphological changes in the monolayer of treated cells by inverter light microscope, and through SEM, and 2) Colorimetric MTT analysis.

Microscopic observation of changes in the morphology of the cell monolayer

The cell monolayer was observed every 24 hours under an inverter light microscope for typical cytopathology characterizing the toxic effect in the treated cells. The analysis of the observation of the cell morphology of Vero and Lep cells was synchronized with the MTT analysis of cell survival.

1) MTT analysis

The MTT [[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye is a water-soluble tetrazolium salt which gives the solution a yellowish color. Placed in the cell culture medium, MTT passes into the cell cytosol, reaches the mitochondria, where mitochondrial dehydrogenases cleave the tetrazolium ring and convert the soluble yellow salt into a water-insoluble blue formazan product. The ability of cells to reduce MTT is an indicator of mitochondrial integrity and activity, which is interpreted as a measure of viability and/or viability of viable cells. Dead cells do not participate in this transformation due to the non-functioning of their mitochondria. The amount of transformed MTT to insoluble formazan crystals is proportional to the number of living cells. To measure the amount of formazan, the cells are treated with a solution that lyses them and at the same time dissolves the blue formazan crystals. The amount of the latter is measured spectrophotometrically at a wavelength $\lambda = 540$ nm.

After cell counting, the latter were resuspended in growth medium supplemented with 2% FBS. The cell suspension was then dispersed at a concentration of 5 x 10^4 cells/well in sterile 96-well plates (Orange Scientific, Belgium) (0.2 mL/well). Due to experimental data that a decrease in volume was observed in the end rows and columns during longer cultivation, no cells were seeded in them. Only medium without FBS was added there. When the cell monolayer reached between 70 - 80% confluence (usually after 24 h), the supernatant was decanted and 0.1 mL of maintenance medium and 0.1 mL of pre-prepared dilutions of IPF in the concentration range 0.0001 - 20 mg/mL were added. IPF was added to a minimum of 3 wells with each dilution. Only nutrient-free nutrient medium (0.2 mL) was added to several wells for cell control. As the volume of substance used (with a given concentration) when diluted in the well was diluted twice, the actual concentration of each dilution added was twice as low as that previously prepared. The plates thus treated were incubated at 37 °C for 72 hours. At the end of the third day, 0.02 mL of MTT working solution (starting concentration 0.05 mg/mL) was added dropwise to each well (excluding end rows and columns), after which the plates were incubated at 37 °C for 3 hours. The culture medium

with the MTT dissolved in it was removed after incubation, then 0.2 mL of the lysol solution containing ethanol: DMSO (v:v) was added. The plate thus treated was read spectrophotometrically at λ = 540 nm using an ELISA reader (Bio-Tek Instruments, Germany). Cell survival was defined as % of living cells in wells treated with different concentrations of test substance compared to control untreated cells.

The following formula was used for this purpose:

% cell survival = $OD_{treated\ cells}$/ $OD_{cell\ control}$ x 100

The values of Maximum Non-toxic Concentration (MNC) and cytotoxic concentration of 50% (CD_{50}) of the test substance relative to Vero and Lep cell lines were calculated based on the plotted curve of "dose (concentration) – cell survival" using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

- Cytotoxic Concentration 50% (CD_{50}) was defined as the concentration of the test substance at which 50% of the cells die as a result of the toxic action of the substance.
- MAXIMUM Non-toxic Concentration (MNC) was defined as the highest concentration of the test substance that does not cause damage or death to the treated cells. The two values in the experiments were expressed in mg/mL.

Determination of infectious viral titer by the Reed-Muench method

Immediately before work, ten-fold logarithmic dilutions of the respective virus strain were prepared in DMEM medium containing 2% FBS, 1% antibiotic solution (100 U/mL penicillin G sodium and 100 µg/mL streptomycin sulphate). When the cell monolayer reaches between 70 - 80% confluence (concentration 5 x 10^3 cells/well, usually after 24 h), the culture medium in the wells was removed. The cell monolayer was infected with the prepared viral dilutions in a volume of 0.1 mL. Four (4) wells were infected with each viral dilution. The virus was adsorbed for 1 hour in a thermostat at 37 °C, after which 0.1 mL of maintenance medium was added to each well. Four wells containing uninfected cells are used for cell control. The plate thus treated was incubated in a thermostat at 37 °C and the air humidity required. When a CytoPathic Effect (CPE) occurs (appearance of round cells, unevenly disseminated in the cell monolayer, with a tendency to foci), wait until the development of 2 - 3 + (50 - 75% of the cell monolayer) or until it stops progressing. Theviral infection titer is expressed as $TCID_{50}$ (50% Tissue Culture Infectious Dose) (reciprocal dilution of the virus in which 50% of the cell culture is infected). The presence or absence of a viral cytopathic effect was determined by the following formula:

T = (dilution log, where % infected is over 50%) + (proportionality factor x log from the dilution factor).

Proportionality factor = [(% infected over 50%) - 50%] / [(% infected over 50%) - (% infected below 50%)].

Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM)

Measurements were taken every four hours to 48 hours of viral infection development, the last hours coinciding with the initial cytopathic cellular changes. For the purpose of the experiment, cells from Vero and Lep lines with a density of 3 x 10on lamellae^3 cells/mL were infected, which were infected with human coronavirus strain 229E (HCoV-229E) and HSV-1 with multiplicity of infection (MOI = 0.1). The lamellae were fixed in 2.5% glutaraldehyde.

Statistical processing of the results

The data obtained were presented with mean values ± standard error of the mean (SEM) obtained from at least two separate experiments, each performed in three to five replicates. Statistically significant differences in terms of survival/proliferation between untreated controls and samples treated at different concentrations were determined by the ANOVA One-Way Analysis of Variance and subsequent Dunnett's test. Statistically significant differences between untreated controls and treated samples were determined by a t-test, with values at $p < 0.05$ being determined as statistically significant.

Results

1. Effect of the Inactivated Pepsin Fragment (IPF) on the survival and proliferative activity of laboratory-cultured cells

The first important step in antiviral experiments involves determining the cytotoxicity of the test substance on laboratory-cultured cells of the Vero and Lep lines. Three methods were concurrently used in the screening analyzes for assessment of the relative cell survival and proliferation: 1) Microscopic observation of the changes in the morphology of the cell monolayer; 2) MTT analysis and 3) Monitoring the kinetics of proliferative activity of treated cells by SPR analysis. Survival was reported at different exposure times after treatment with Inactivated Pepsin Fragment (IPF), as significant cell proliferation could be observed at different time intervals, the direct toxic effect of the test substance leading to cell death could be assessed, and to monitor the morphological changes that occurred in the cells as a result of the treatment.

MTT analysis of cell survival and proliferation was performed over a wide concentration range of the studied pepsin fragment IPF. The limits within which the applied concentrations varied were selected on the basis of preliminary experiments involving larger concentration intervals in order to accurately determine the values of CD_{50} and MNC. The test substance IPF was administered in the concentration range 0.0001 - 20 mg/mL, in the time interval 2 - 96 h. Untreated Vero cells, whose survival was assumed to be 100%, were used as a negative control. Dose-dependent curves on the viability of the cell monolayer of the applied IPF were obtained from the MTT test used. The analysis of the cell morphology of the cells was synchronized with the MTT analysis of cell survival and the kinetics of the proliferative activity of treated cells was assessed by SPR analysis. at different exposure times.

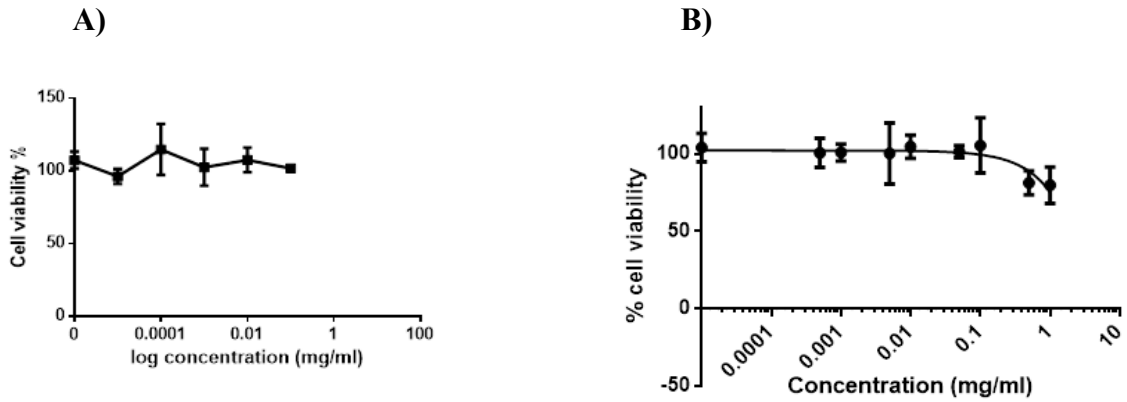
The results obtained show that, in general, the survival of the treated monolayer cells from the Vero and Lep lines did not decrease dramatically under the influence of the tested inactivated pepsin fragment (IPF). The decrease in the number of adherent living cells, as well as the rounding and decrease in the size of the cells follow the trend established in the MTT analysis (Table 1 and Figure 2).

Table 1. Influence of IPF on the survival/proliferative activity of Vero and Lep cells, reported by different methods after 96 h of treatment

Cell culture	Method	MNC (mg/mL)	CD_{50} (mg/mL)
Vero	Microscopic observation of changes in morphology of the cell monolayer	0.1 ± 0.1	15 ± 0.05
	MTT test	0.1 ± 0.12	15 ± 0.22
Lep	Microscopic observation of changes in the	0.1 ± 0.6	10 ± 0.02

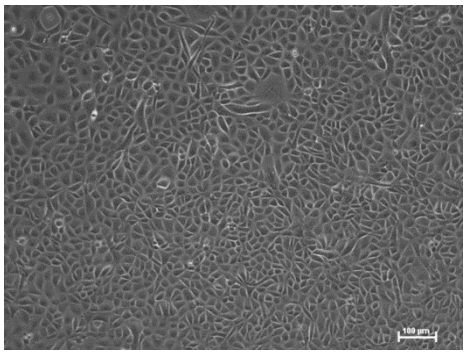
	morphology of the cell monolayer MTT test	 0.1 ± 0.07	 15 ± 0.1

Figure 2. Effect of IPF on the survival/proliferative activity of Vero (A) and Lep (B) cells as reported by MTT analysis after 96 h of treatment. Data is presented with mean ± SEM values.

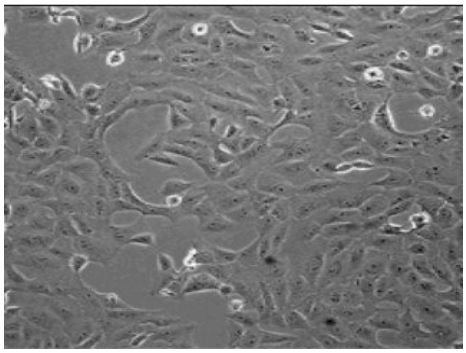


Visualization of changes in the morphology of the cell monolayer after treatment is an accessible and reliable approach in the initial study of the cytotoxicity of various substances. In this regard, concurrently with the MTT analysis, changes in the morphology of the cells exposed to the tested IPF were observed under an inverter light microscope. Untreated control cells and IPF-treated cells in the concentration range 0.0001 - 5 mg/mL retain their morphology and structure (Figures 3 & 4).

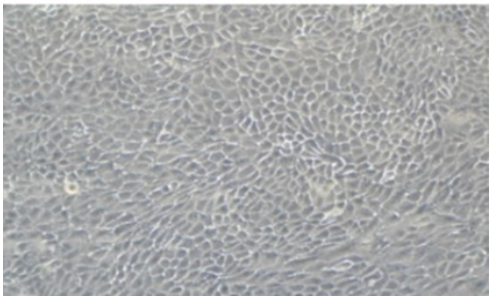
Figure 3. Cytotoxic effect of IPF on Vero and Lep cells after 96 h of treatment monitored by inverted light microscope

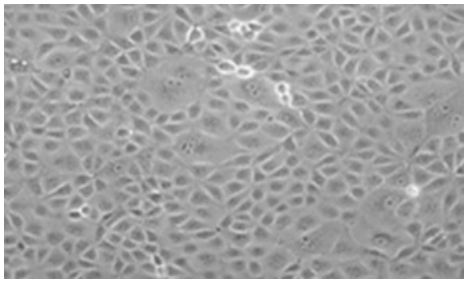


A. Untreated Vero cells



B. IPF-treated (1 mg/mL) Vero cells

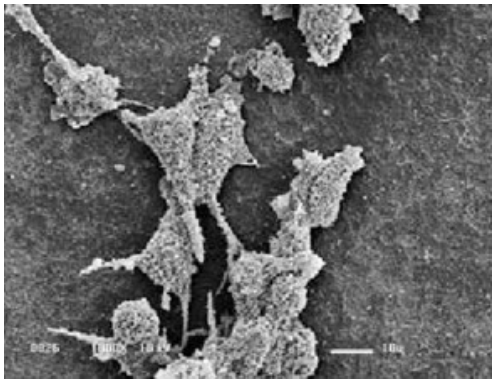




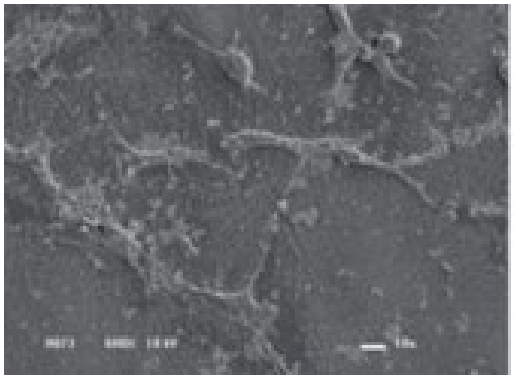
C. Untreated Lep cells

D. IPF-treated (5 mg/mL) Lep cells

Figure 4. Cytotoxic effect of IPF on Vero cells after 96 h of treatment monitored by [i]SEM



A. Untreated Vero cells (SEM)



B. IPF-treated (1 mg/mL) Vero cells (SEM)

1

A

2. Antiviral activity

A) CPE Inhibition assay

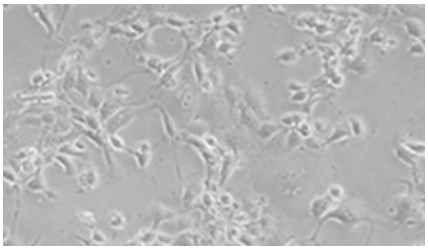
The Inactivated Pepsin Fragment (IPF) was tested for its effect on the viral cytopathic effect against the following reference virus strains:

√ Herpes Simplex virus type 1 (HSV-1) strain Victoria
√ Human coronavirus strain 229E (HCoV-229E)

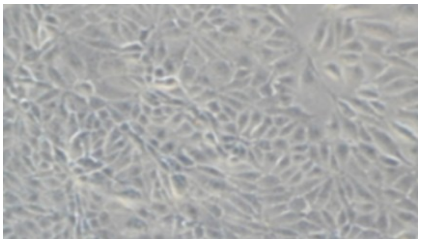
To test the effect of the test substance IPF on the replication of the tested viruses was used in the concentration range - 0.0001 - 1 mg/mL (MNC and close to it). Initially, the antiviral activity of the test substance IPF was assessed by a CPE inhibition test. After infection of the cells with HCoV-229E, CPE developed for about 96 h and resulted in the appearance of round cells, unevenly disseminated in the cell monolayer, with a tendency to foci. Studies have shown that, unlike uninfected Vero cells, HCoV-229E-infected cells have a characteristic round shape, clustered as 'islands', isolated from each other and separated from the medium on which they are cultured. Morphologically, compactinization of chromatin, disintegration of the nucleus, formation of "pseudopods" and vacuolation of the cytoplasm are observed. Cell cultures were observed daily under a light microscope to develop CPE. The effect of the test substance was reported at 96 h (the time for one replicate cycle of coronaviruses) after HCoV-229E infection of Vero E6 cells (*p.i.*). The results of the experiments were evaluated on the basis of comparison with virus-free and no-added cells (cell control), as well as with cells infected with human coronavirus strain 229E, without

addition of substance (viral control) by observation with an inverter light microscope (Figure 5) and by SEM and AFM during different stages of the replication cycle (Figure 6).

Figure 5. Inhibitory effect of IPF evaluated by CPE inhibition assay on HCoV-229E-infected Vero cells after 96 h of treatment by observation under an inverter light microscope.



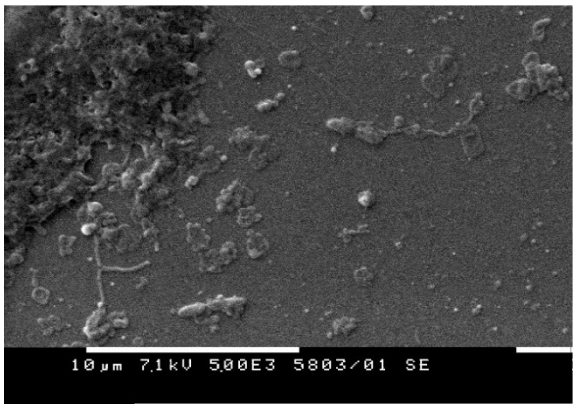
A. Infected Vero cells with HCoV-229E

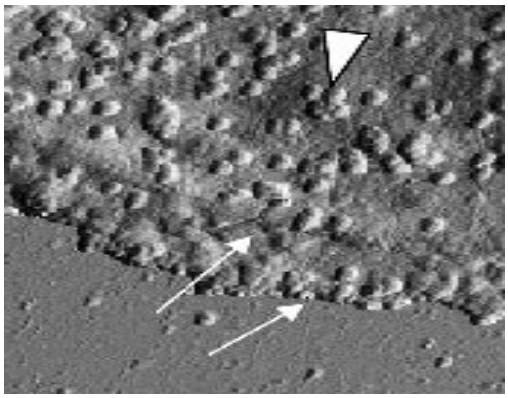


B. Infected Vero cells treated with IPF at MNC (0.1 mg/mL)

C. Uninfected Vero E6 cells

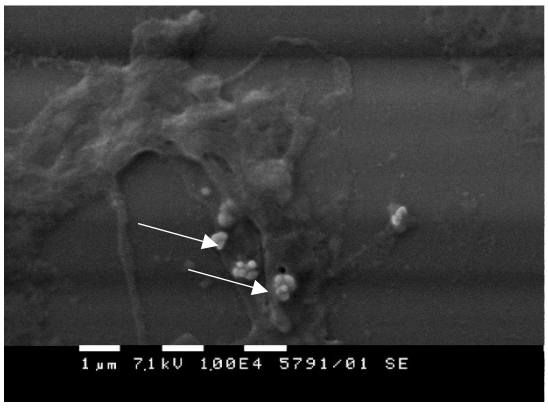
Figure 6. Inhibitory effect of IPF on HCoV-229E-infected Vero cells after 48 h of treatment evaluated by SEM and AFM. The test substance was administered at MNC (mg/mL).

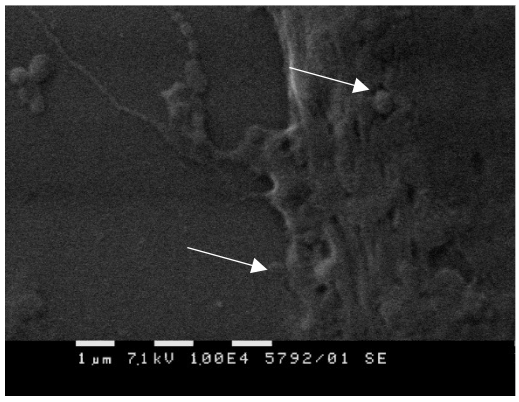




Infected with human coronavirus strain 229E (HCoV-229E) Vero cells (virus control) (SEM) at 48 h

Numerous budding viruses and those that are in the extracellular space 48 hours after infection of the cells.





Infected with human coronavirus strain 229E (HCoV-229E) Vero cells and treated with the IPF test preparation (1 mg/mL) for 48 h (SEM)

Following treatment with cells for IPF for 48 hours, the production of infectious viral progeny is significantly reduced and viruses are rarely detected in the intercellular space, which in turn allows the immune system to cope more easily with the infection.

On microscopic monitoring of the morphology of the monolayers, it can be concluded that the IPF test substance administered at the MNC showed varying degrees of CPE inhibition in HCoV-229E-infected cells after 48 hours of treatment.

The best CPE suppression of HCoV-229E was observed after IPF treatment of infected Vero cells for 48 h.

Similar experiments were performed to evaluate the effect of IPF on HSV-1 infected Lep cells.

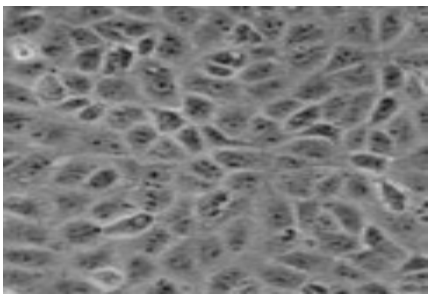
Initially, the antiviral activity of the test substance IPF was assessed by a CPE inhibition test. After infection of the cells with HSV-1, CPE develops in about 72 hours and results in the appearance of round cells, unevenly disseminated in the cell monolayer, with a tendency to foci and the formation of syncytia. Cell cultures were observed daily under a light microscope to develop CPE. The effect of the test substances was reported 72 h (time for one replicate cycle of coronaviruses) after HSV-1 infection of Lep cells (pi.). The results of the experiments were evaluated on the basis of comparison with virus-free and no-added cells (cell control) as well as with HSV-1-infected cells without substance addition (viral control).

It is clear from the studies performed that unlike uninfected Lep cells, HSV-1-infected cells have a characteristic round shape, clustered in the form of "islands", isolated from each other and separated from the substrate on which they are cultured and strongly vacuolated cytoplasm observed by inverter light microscope (Figure 7) and by AFM and SEM (Figure 8).

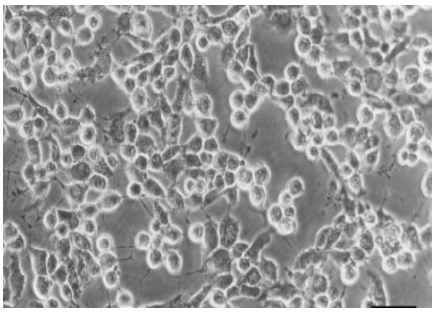
On microscopic monitoring of the morphology of the monolayers, it can be concluded that the substances tested in the MNC showed different degrees of inhibition of CPE of HSV-1/HSV-2 infected cells after 72 h of treatment.

The best CPE suppression of HSV-1/HSV-2 was observed after IPF treatment of infected Vero cells.

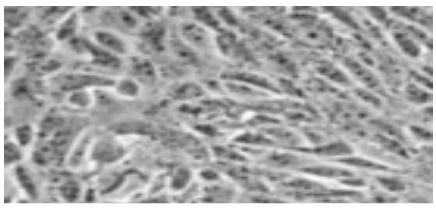
Figure 7. Inhibitory effect of IPF evaluated by CPE inhibition test on HSV-1-infected Lep cells after 24 h of inverter light microscope treatment.



A. Uninfected Lep cells (cell control)

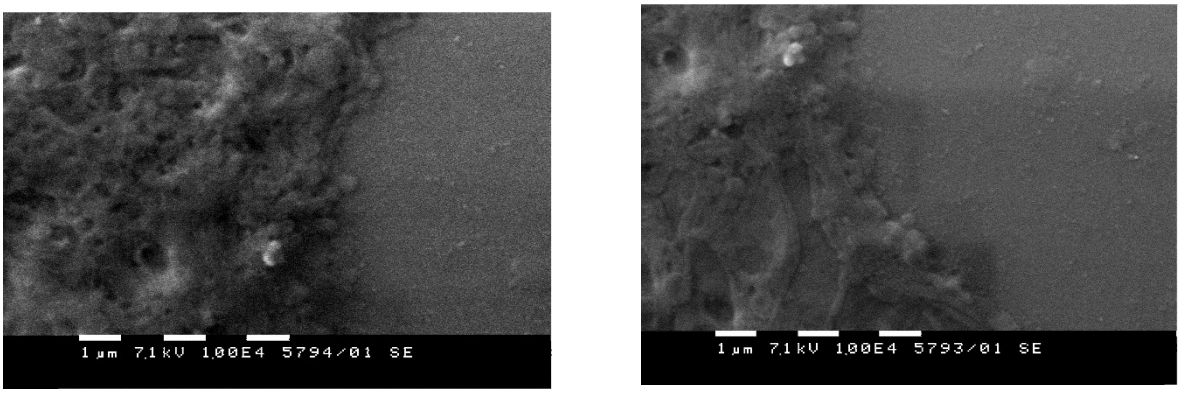


B. HSV-1-infected Lep cells (virus control)

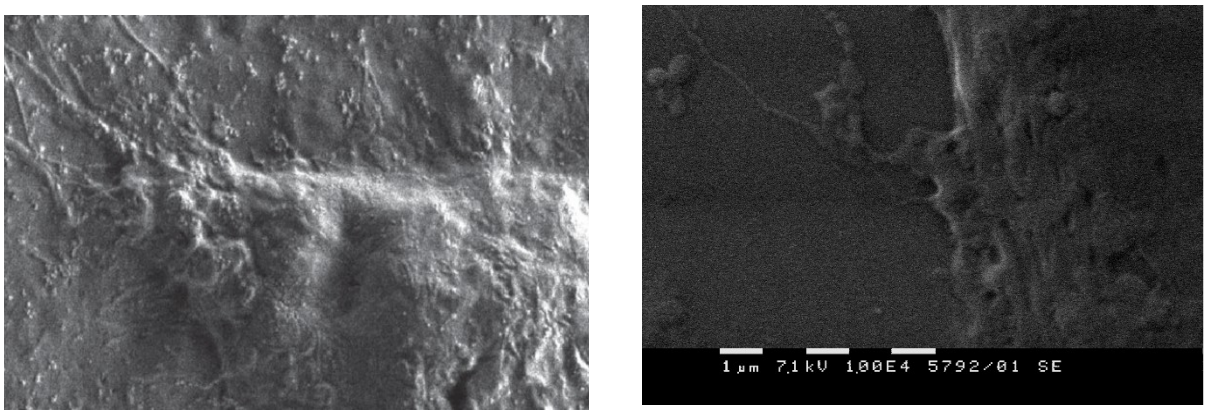


C. HSV-1-infected Lep cells treated with IPF
at MNC (0.1 mg/mL)

Figure 8. Inhibitory effect of IPF on HSV-1-infected Lep cells after 24 h of treatment evaluated by SEM and AFM



HSV-1-infected cells from the Lep line (virus control) (SEM) at 24 h



HSV-1-infected cells from the Lep line treated with the IPF test preparation
(5 mg/mL) at 48 h (SEM) and (AFM)

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I, the undersigned Ivan Petrov Tortorochev, hereby declare that this is, to the best of my knowledge and belief, a correct and complete translation from Bulgarian into English of the document enclosed. The translation comprises thirteen (13) pages.

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