

Test Summary
of Inactivating Extrasomatic SARS Virus
With NOVARON Inorganic Antibacterial Agent

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Abstract:

Checking effect to inactivate extrasomatic SARS virus in the substrate of VERO E6 with NOVARON inorganic antibacterial agent by adopting virus (CPE) method, the test result manifests that the NOVARON Inorganic antibacterial agent has a certain inactivation effect upon SARS virus after it functions in the room temperature for over 6h.

1. Test Purpose

The test purpose is to detect whether the NOVARON inorganic antibacterial agent possesses inactivation effect upon SARS virus under the condition of ambient temperature and functions on SARS virus for different time duration, and provide test basis for accelerating the progress rate in sifting the stuff for inactivating SARS virus.

2. Test Materials

1) Verification Material:

NOVARON inorganic antibacterial agent: White powder, 10g per bag, provided by Beijing Great Wall Yongyi Science & Technology Development Co., Ltd.

2) Positive Comparison Medicament:

GANCICLOVIR for Injection; Batch No. 020802; Provided by Hubei Keyi Pharmaceutic Co., Ltd.

3) Virus:

SARS-COV-P11 corona-virus separated strain (No.11 specimen of SARS patient blood serum) provided by You'an Hospital; Separated and identified by this room.

SARS-COV-P8 corona-virus separated strain, provided by (Professor Li Dexin) the Hemorrhagic Fever Room of Virosis Prevention Control Institute.

4) Cells:

The generation spreading kidney cells of African green monkey (VERO E6) , provided by this room.

5) The test materials, such as cells culture maintenance media “Eagle’s” etc, were provided by this room.

3. Experimental Method

Preliminary Test

1) Toxicity determination for VERO E6 cells by using NOVARON inorganic antibacterial agent:

Calculate the half toxic concentration (TD₅₀) and maximum non-poisonous concentration (TD₀) through VERO E6 cell culture in substrate, by adopting cellular morphological variation (CPE) method

and Reed-Muenchdrug method.

The pretreatment of the NOVARON inorganic antibacterial agent:

Put 6000µg/ml NOVARON inorganic antibacterial agent into a vessel and have the antibacterial agent diluted in Eagle's cells maintenance media; then lay the vessel on a magnetic stirrer (in ambient temperature); sample from the vessel at separate agitating time, i.e. at 2h, 4h and 6h from the starting time of the agitation. Each specimen sampled at different time should be diluted, i.e. 6000µ g/ml ~ 187.5µ g/ml, and inoculate VERO E6 cells into culture board of 96 orifices.

Cellular morphological variation (CPE) method:

Inoculate the VERO E6 cells with the concentration of 400 thousand unit/ml into the culture board of 96 orifices, and have the object cultured under the condition of 37°C and 5% CO₂ for 24h till cellular mono-layer; Then add the pretreated NOVARON inorganic antibacterial agent, the concentration of which is 6,000µg/ml ~187.5µ g/ml; the multiple proportion of the positive comparison medicament is 6,000µ g/ml ~ 187.5µ g/ml, and that in each kind of the concentrations is to be inoculated into four orifices with 100 µ l/orifice. Meanwhile, normal cellular is to be assumed for control. Lay the normal cells in the environment of 37°C and 5% CO₂ to culture for 5~7 days, and observe and take down the cellular morphological variation (CPE) every 24h under inverted microscope: Denominate the variation under 25% as "+", the variation of 26% ~ 50% as " ++", the variation of 51% ~ 75% as "+++", and the variation of 76% ~ 100% as "++++". The test should be repeated for tree times.

2) Separate and identify the SARS-COV-P11 in VERO E6 cells culture substrate

SARS-COV-P11 (Separation of No 11 SARS patient blood serum):

Inoculate the VERO E6 cells in the concentration of 400 thousand unit/ml into test tube, and have the cells cultured in the environment of 37°C and 5% CO₂ for 24h; Then discard the culture solution and add the 0.2ml blood serum of SARS patient into each test tube; after the objects are cultured in rotary drum for 5h, add 1ml maintenance media. At the same time, the normal cells control should be assumed; the ambient temperature is 37°C and the culturing duration in the rotary drum is 5 ~ 7 days. After the CPE variation occurs in the cells, detect corona-virus by PCR method. If the specimen No. 11 PCR detection presents "Positive", it can be determined as the corona-virus separate strain. The virus should be purified twice with final dilution method, and then conduct PCR detection. If the S gene sequence of the SARS virus at this time still presents positive, and at the same time if IgM is positive and IgG4 value goes up by times through immuno-fluorescent assay of double SARS patient blood serums, it can be determined as corona-virus. CPE method should be adopted to determine its potency.

Determine the toxicity of SARS-COV-P1be & SARS-COV-P8 toxicant strain in VERO E6 cells culture substrate

Virus CPE Method:

Inoculate the VERO E6 cells in the concentration of 400 thousand unit/ml into culture board of 96 orifices, and have the cells cultured in the environment of 37°C and 5% CO₂ for 24h; Then discard the culture solution and dilute 2 strains of the virus respectively into eight kinds of concentrations from 10⁻¹ to 10⁻⁸. Each kind of the concentration should occupy 4 orifices and each orifice holds 100µL solution. Meanwhile, the control of normal cells should be cultured for 5 ~ 7 days in the environment of 37°C and 5% CO₂. Observe and take down the cellular morphological variation (CPE) under inverted microscope once every 24h: Denominate the variation under 25% as "+", the variation of 26%

~ 50% as " ++", the variation of 51% ~ 75% as "+++", and the variation of 76% ~ 100% as "++++". Calculate the median toxic concentration TCID₅₀ by Reed –Muench Method.

3) **The inactivating effect of NOVARON inorganic antibacterial agent in VERO E6 cells culture substrate upon SARS-COV-P11 and SARS-COV-P8**

Test Purposes:

Virus cells (CPE) method should be adopted to observe the inactivating effect of the NOVARON inorganic antibacterial agents in different concentrations upon the SARS viral in the VERO E6 cell culture substrate, and calculate the median effective concentration (IC₅₀) and the minimum effective concentration (MIC), as well as the therapeutic index (TI) and determine the potency.

Pretreatment of 100TCID₅₀SARS virus & NOVARON inorganic antibacterial agent:

Put two strains of 100TCID₅₀SARS virus diluents and 750μg/ml NOVARON inorganic antibacterial agent into a vessel respectively. After having them agitated in magnetic stirrer for 2h, 4h and 6h, sample object respectively and dilute the specimens into 750μ g/ml ~ 11.7μ g/ml, then inoculate VERO E6 cells into the culture board of 96 orifices.

Virus CPE Method:

Inoculate the VERO E6 cells in the concentration of 400 thousand unit/ml into culture board of 96 orifices, and have the cells cultured in the environment of 37°C and 5% CO₂ for 24h till mono-layer; Then discard the culture solution and add the pretreated solution with SARS virus and NOVARON inorganic antibacterial agent; select two times of the maximum non-poisonous concentration (TD₀) to the cells to be diluted for 7 grade of thickness, i.e. 750μg/ml ~μ g/ml; meanwhile, the pretreated control of NOVARON inorganic antibacterial agent should be two times diluted into 7 grades of thickness, i.e. 750μ g/ml ~ 5.9μ g/ml; take the GANCICLOVIR for Injection as the positive comparison medicament, which would have the maximum non-poisonous concentration (TD₀) to the cells two times diluted into 7 grades of concentrations, i.e.6000μ g/ml ~ 5.9 μ g/ml. Add the diluted medicament into the cell orifices respectively; each grade of the concentration should occupy 4 orifices. At the same time, the normal cells control should be assumed; the ambient temperature is 37 °C and the culturing duration in the rotary drum is 5 ~ 7 days; Observe the virus CPE under inverted microscope every day by comparison. When "+++" ~ "++++" occurs, end off the test. Calculate the median effective concentration (IC₅₀), the minimum effective concentration (MIC) and the therapeutic index (TI) of the medicament to decide the effect by Reed – Muench method. The test should be repeated for tree times.

4. Test Result

Preliminary Test Result:

The toxic action of NOVARON inorganic antibacterial agent upon VERO E6:

Calculate the maximum nontoxic concentration (TD₀) and median toxic concentration (TD₅₀) of the NOVARON inorganic antibacterial agent and positive comparison medicament i.e. the GANCICLOVIR for injection in VERO E6 cells culture substrate by adopting Cellular morphological variation (CPE) method provided by Beijing Great Wall Yongyi Science & Technology Development Co., Ltd. And the following test data is the average values of thrice test results.

1) The test result for the toxicity of NOVARON inorganic antibacterial agent to the VERO E6

cells

Verification Material:

NOVARON inorganic antibacterial agent: The maximum nontoxic concentration (TD0) is $750 \pm 0 \mu$ g/ml and the medium toxic concentration (TD50) is $1500 \pm 0 \mu$ g/ml.

Positive comparison medicament:

GANCICLOVIR for injection: The maximum nontoxic concentration (TD0) is $>6000 \pm 0 \mu$ g/ml, and the medium toxic concentration (TD50) is $>6000 \pm 0 \mu$ g/ml.

2) The test result for the toxicity in VERO E6 cells culture substrate to SARS-COV-P11 & SARS-COV-P8

SARS-COV-P11: Median infective dose (TCID₅₀) is 10^{-7}

SARS-COV-P8: Median infective dose (TCID₅₀) is 10^{-7}

Official Test Result:

1) After the NOVARON inorganic antibacterial agent takes action at the SARS-COV-P11 for 2h, 4h and 6h, the inactivation effect could be checked in the VERO E6 cells culture substrate, by taking IC50, MIC, TI and the percentage of inactivation as the indices (The following test data are the average values of thrice test results).

Detect the inactivation effect (taking IC50, MIC and TI as the indices).

Verification Material:

NOVARON inorganic antibacterial agent:

After the medicament action for 2h under ambient temperature:

With virus CPE method, median effective concentration (IC₅₀) is $188 \pm 0 \mu$ g/ml, and the minimum effective concentration (MIC) is $94 \pm 0 \mu$ g/ml; the therapeutic index (TI) is 8.

After the medicament action for 4h under ambient temperature:

With virus CPE method, median effective concentration (IC₅₀) is $188 \pm 0 \mu$ g/ml, and the minimum effective concentration (MIC) is $94 \pm 0 \mu$ g/ml; the therapeutic index (TI) is 8.

After the medicament action for 6h under ambient temperature:

With virus CPE method, median effective concentration (IC₅₀) is $94 \pm 0 \mu$ g/ml, and the minimum effective concentration (MIC) is $46.8 \pm 0 \mu$ g/ml; the therapeutic index (TI) is 16.

Detection of inactivation effect (Taking inactivation percentages as the indices):

After the medicament action for 2h under ambient temperature:

With virus CPE method, when the diluted concentration of the NOVARON inorganic antibacterial agent $>375 \mu$ g/ml, the 100% SARS virus can be inactivated; the diluted concentration is 188μ g/ml, 50% SARS virus can be inactivated; when the diluted concentration is 94μ g/ml, 25% SARS virus can be inactivated.

After the medicament action for 4h under ambient temperature:

With virus CPE method, when the diluted concentration of the NOVARON inorganic antibacterial agent $>375\mu\text{g/ml}$, the 100% SARS virus can be inactivated; the diluted concentration is $188\mu\text{ g/ml}$, 50% SARS virus can be inactivated; when the diluted concentration is $94\mu\text{ g/ml}$, 25% SARS virus can be inactivated.

After the medicament action for 6h under ambient temperature:

With virus CPE method, when the diluted concentration of the NOVARON inorganic antibacterial agent $>188\mu\text{g/ml}$, the 100% SARS virus can be inactivated; when the diluted concentration is $94\mu\text{ g/ml}$, 50% SARS virus can be inactivated; When the diluted concentration is $46.8\mu\text{ g/ml}$, 25% SARS virus can be inactivated.

Positive comparison medicament:

GANCICLOVIR for injection: With virus CPE method, median effective concentration (IC_{50}) is $11.7\pm 0\mu\text{ g/ml}$, and the minimum effective concentration (MIC) is $23.44\pm 0\mu\text{ g/ml}$; the therapeutic index (TI) is 256.

- 2) After the NOVARON inorganic antibacterial agent takes action at the SARS-COV-P8 for 2h, 4h and 6h, the inactivation effect could be checked in the VERO E6 cells culture substrate, by taking IC_{50} , MIC, TI and the percentage of inactivation as the indices (The following test data are the average values of thrice test results).

Detect the inactivation effect (taking IC_{50} , MIC and TI as the indices).

Verification Material:

NOVARON inorganic antibacterial agent:

After the medicament action for 2h under ambient temperature:

With virus CPE method, median effective concentration (IC_{50}) is $188\pm 0\mu\text{ g/ml}$, and the minimum effective concentration (MIC) is $94\pm 0\mu\text{ g/ml}$; the therapeutic index (TI) is 8.

After the medicament action for 4h under ambient temperature:

With virus CPE method, median effective concentration (IC_{50}) is $188\pm 0\mu\text{ g/ml}$, and the minimum effective concentration (MIC) is $94\pm 0\mu\text{ g/ml}$; the therapeutic index (TI) is 8.

After the medicament action for 6h under ambient temperature:

With virus CPE method, median effective concentration (IC_{50}) is $94\pm 0\mu\text{ g/ml}$, and the minimum effective concentration (MIC) is $46.8\pm 0\mu\text{ g/ml}$; the therapeutic index (TI) is 16.

Detection of inactivation effect (Taking inactivation percentages as the indices):

After the medicament action for 2h under ambient temperature:

With virus CPE method, when the diluted concentration of the NOVARON inorganic antibacterial agent $>375\mu\text{g/ml}$, the 100% SARS virus can be inactivated; the diluted concentration is $188\mu\text{ g/ml}$, 50% SARS virus can be inactivated; when the diluted concentration is $94\mu\text{ g/ml}$, 25% SARS virus can be inactivated.

After the medicament action for 4h under ambient temperature:

With virus CPE method, when the diluted concentration of the NOVARON inorganic antibacterial agent $>375\mu\text{g/ml}$, the 100% SARS virus can be inactivated; the diluted concentration is $188\mu\text{ g/ml}$,

50% SARS virus can be inactivated; when the diluted concentration is 94 μ g/ml, 25% SARS virus can be inactivated.

After the medicament action for 6h under ambient temperature:

With virus CPE method, when the diluted concentration of the NOVARON inorganic antibacterial agent >188 μ g/ml, the 100% SARS virus can be inactivated; when the diluted concentration is 94 μ g/ml, 50% SARS virus can be inactivated; When the diluted concentration is 46.8 μ g/ml, 25% SARS virus can be inactivated.

Positive comparison medicament:

GANCICLOVIR for injection: With virus CPE method, median effective concentration (IC_{50}) is 11.7 \pm 0 μ g/ml, and the minimum effective concentration (MIC) is 23.44 \pm 0 μ g/ml; the therapeutic index (TI) is 256.

Summary

With the ZEOMIC - AJ10N inorganic antibacterial agent and the positive comparison medicament i.e. GANCICLOVIR for injection provided by Beijing Great Wall Yongyi Science & Technology Co. Ltd. in VERO E6 cell culture substrate, while adopting virus CPE method, the result, verified by the tests with two SARS virus isolated strains, indicates that the NOVARON inorganic antibacterial agent has definite inactivation effect upon the SARS virus if it acts with the SARS virus for over 6h in ambient temperature.

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