Development of a screening method for potential anti-SARS compounds based on Vero-E6 cells

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Abstract: **Object** To develop a Vero-E6 cell line based assay for screening potential anti-SARS compounds. **Methods** Conditions of the Vero-E6 cell line based MTS assay were optimized. Virus titer curve had been generated. To screen anti-SARS virus compounds, Vero-E6 cells were plated in 96-well plates, test compounds were added and immediately followed with BJ-01 SARS virus. Cell morphology was observed by microscope after 48 h of incubation. MTS and PMS mixture was added in 96 h and absorbance values were measured at 490 nm. **Results** More than 4 000 compounds had been screened and ten potential anti-SARS compounds had been found. **Conclusion** The Vero-E6 cell based MTS assay offers a rapid, safe, and convenient method for screening potential anti-SARS compounds.

Key words: severe acute respiratory syndrome (SARS); Vero-E6 cells; 3-(4, 5-dimenthylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS)

基于 Vero E6 细胞的抗 SARS 药物筛选方法的构建

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摘 要: **目的** 构建基于 Vero-E6 细胞的抗 SARS 药物筛选模型。**方法** 运用四唑氮化合物 (MTS) 方法, 对由 SARS 病毒 (BJ-01) 引起的对 Vero-E6 细胞的细胞毒性作用进行检测。首先优化了实验条件(检测了 MTS 不同孵育时间和不同接种细胞数对测试结果的影响, 并绘制了病毒滴度曲线); 在检测化合物的抗病毒作用时, 首先将细胞接种于 96 孔板中, 加入待测药物和 BJ-01 病毒, 48 h 后用显微镜观察细胞形态变化, 96 h 后加入 MTS 和电子耦联剂 (PMS) 的混合溶液, 在 490 nm 检测其吸光度。**结果** 对 4 000 多种化合物的抗 SARS 病毒作用进行了检测。获得 10 种可能有抗 SARS 病毒作用的药物。**结论** 基于 Vero-E6 细胞的 MTS 检测方法提供了一种快速、安全和方便的抗 SARS 药物筛选方法。

关键词: 重急性呼吸综合征; Vero-E6 细胞; 四唑氮化合物

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Introduction

SARS (severe acute respiratory syndrome) is a very contagious disease with high mortality. A new coronavirus (SARS-associated coronavirus, SARS-CoV) was identified as its pathogen[1]. Last year the global outbreak of SARS caused wide-ranging disruption and increased the demand to search for effective therapies for this disease.

Now a rapid, safe, and convenient method—the Vero-E6 cell based 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay for screening potential anti-SARS compounds has been developed. Using this assay, more than 4 000 compounds have been screened, and ten potential anti-SARS compounds have been found.

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1 Materials and methods

1.1 Virus and cells: BJ-01, a strain of SARS-CoV isolated from the lung of a dead SARS patient in Beijing was used. The cell line Vero-E6 derived from the African green monkey kidney was purchased from American Type Culture Collection (ATCC). Cells were plated in 96-well tissue culture plates using Dulbecco's Modified Eagle Media (DMEM, Gibco, USA) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, USA), 200 mmol/L *L*-glutamine (Gibco, USA), 1% Penicillin (Sigma, USA) and 1% Streptomycin (Gibco, USA).

1.2 Other materials: MTS was purchased from Promega (USA). Phenazine methosulfate (PMS) was the product of Sigma (USA). Other reagents and solvents used in the experiment are of analytic grade.

1.3 Compounds preparation: The test compounds were from both natural compounds and chemical systhesis. The natural compounds were extracted and isolated from traditional Chinese herbs including single and complex compounds. All compounds were dissolved in DMSO or water according to their different polarity.

1.4 Time course of MTS assay on Vero-E6 cells: In metabolically active cells, MTS is reduced by dehydrogenase enzymes into an aqueous soluble formazan product. The absorbance can be measured directly at 490 nm from 96-well assay plates and the quantity of formazan product was consi-dered to be directly proportional to the number of viable cells in culture[2, 3].

In order to determine the optimal MTS/PMS incubation time (sufficient color development of formazan dependents on cell lines), the absorbance of formazan at different MTS/PMS incubation time was measured. Vero-E6 cells were plated into a Corning® 96-well tissue culture plate in final volume of 100 μL (4 000 cells/well). After 24 hours of incubation (37 ℃, 5% CO_2), cells were treated with 0.001% and 0.1% triton X-100 (to kill cells). Then 20 μL/well of combined MTS/PMS was added to each well. After 1, 2, 3, 4 hours at 37 ℃ in a humidified, 5% CO_2 atmosphere, 50 μL of 10% sodium dodecyl sulfate (SDS) was added to each well to stop the reaction. The optical density was analyzed on a Multiscan Ascent reader (Lybsystems, Helsinki) at 490 nm. Absorbance values were the mean ± standard deviation (SD) of three replicates for each treatment and Z' factor was calculated.

1.5 Cell number course of MTS assay on Vero-E6 cells: To determine the effect of Vero-E6 cell number on the absorbance at 490 nm measured in this assay, different numbers of Vero-E6 cells (from 1 000 to 35 000 cells/well) were plated in a 96-well plate. After 24 hours of incubation, MTS/PMS mixture was added in each well. After incubating 3 hours at 37 ℃ in a humidified, 5% CO_2 atmosphere, absorbance at 490 nm was recorded.

1.6 Virus titer assay: Serial volumes (10^{-5}—10 μL) of BJ-01 SARS viral stocks were added, 24 hours after seeding Vero-E6 cells in 96-well plates. Cell morphology was observed after 48-hour post infected, and 96 hours later, cytopathic effects (CPE) were measured with MTS assay. Cell controls and medium controls were included.

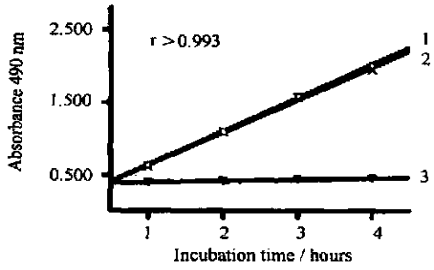
1.7 Visual and MTS inhibition of viral cytopathic effect assay: To screen for anti-SARS virus compounds, Vero-E6 cells were plated in 96-well plates. After 24 hours of incubation, test compounds were added at 10 μmol/L and immediately followed with 2 μL of BJ-01 SARS viral stocks. Cell morphology was observed after 48-hour post-infection. CPE was measured with MTS assay after 96 hours of compound addition. Controls included cells only and cells with viruses.

2 Results

2.1 Time course of MTS assay on Vero-E6 cells: Time course curve after MTS/PMS solution incubation of 1 - 4 hours was determined by absorbance at 490 nm. The absorbance values were the mean ± SD of three replicates for each treatment. As shown in Fig. 1, the absorbance values increased with the incubation time. A linear

ime course of

MTS assay and the correlation coefficient of line was over 0.993. Z′-factor was calculated to assess the assay reliability. In general, assays with Z′-factor values greater than 0.5 are considered good assays. In our assay Z′-factor value was produced greater than 0.6 at 1 hour incubation and more than 0.8 after 2 hours incubation. It indicated this assay worked very well.



1-cell 2-0.001% triton X-100 3-0.1% triton X-100

Fig 1 Time course curve of MTS assay

2.2 Cell number course of MTS assay: Effect of Vero-E6 cell number on absorbance at 490 nm was measured (Fig. 2). Each point represents the mean ± SD of four replicates. The absorbance increased with the cell number. And in the range from 1 000 to 15 000 cells/well (10 000 to 150 000 cells/mL) there was a linear response between cell number and absorbance at 490 nm (r = 0.997). The background absorbance shown at zero cell/well was subtracted from these data.

2.3 Virus titer assay: Effect of dilutions of BJ-01 SARS viral stocks on Vero-E6 cells was measured (Fig. 3). Absorbance values are the mean ± SD of duplicate. The CCID (50% cell culture infectious dose) was generated (0.007 μL).

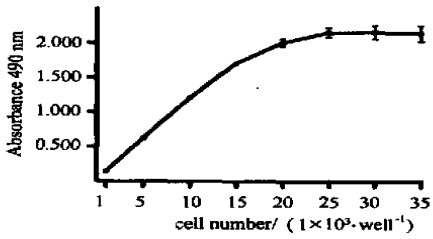


Fig 2 Cell number course of MTS assay

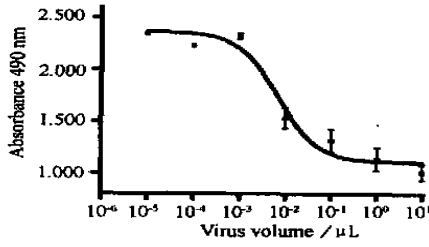


Fig 3 BJ-01 SARS virus titer curve

2.4 Primary screening of compounds by visual and MTS inhibition of viral cytopathic effect assay: The plate layout for primary testing of antiviral compounds was shown in Fig. 4. Cell controls determined the absorbance of uninfected cells and gave values equal to 100% viability. Virus controls determined the absorbance of cells killed by BJ-01 virus treated and equal to 0% viability. Medium controls determined the background absorbance due to MTS/PMS solution in DMEM with 10% FBS and these values were subtracted from corre-sponding efficacy values.

Some results were shown in Fig. 5. The absorbance values of compounds without potential anti-SARS function were similar to the virus controls and percents of CPE reduction were about 0%. Some compounds showed weak inhibition of

	1	2	3	4	5	6	7	8	9	10	11	12
A	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	virus controls
B	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
C	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
D	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	cell controls
E	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
F	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	medium
G	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	controls
H												

Fig 4 Plate layout for primary screening of anti-SARS compounds

BJ-01 SARS-CoV with 20%- 50% CPE reduction. In the test, dilutions of viral stocks with 200 CCID were added to the cells. A few compounds with over 60% of CPE reduction (i.e. A3, B4, G3 in the sheet below, Fig. 5) were considered as strong inhibitors of SARS-CoV and as hits. Examples of morphology observation were shown in Fig. 6.

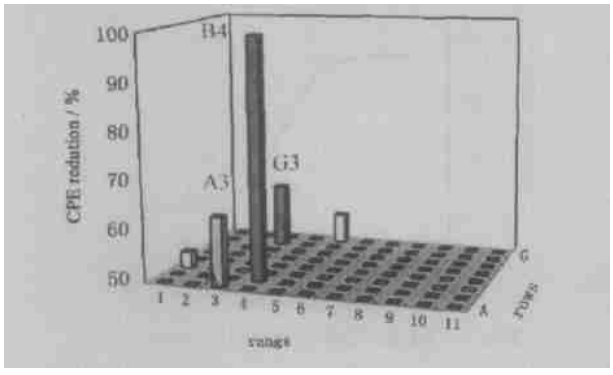
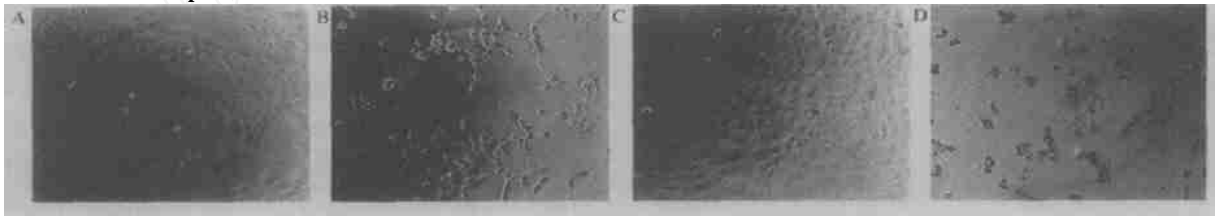


Fig. 5 Result of one typical 96-well plate shown as values of percent of CPE reduction

3 Discussion and conclusion

During replication, many viruses (i.e. HIV-1, SARS-CoV) destroy not only the host cells that they infect but also neighboring uninfected cells by CPE. So the CPE inhibitory assays are widely used in the field of identifying potential antiviral agents by evaluating the inhibition of this virus induced cell death[4]. Traditional CPE assays provide visual cell morphology and credible evidence of antivirus of compounds. However, they could not offer quantitative results and high efficiency when great number of samples need to be tested.

Neural red (NR) vital stain and MTT assay used in many laboratories including the Centers for Disease Control and Prevention (CDC) in USA. and China offer more quantitative results[5]. But during these tests free neutral red dyes must be washed or volatile organic solvent is required to solubilize the formazan product. It increases not only the steps of the screening but also the danger of the test because of SARS-Cov in the supernatants of wells. Besides that these assays are not suitable for every cell line[4].



A-compound TY-AD-97 (position B4 in Fig. 4 and 5) with 99.2% of CPE reduction. B-compound TY-AD-90 (position G3 in Fig. 4 and 5) with 62.9% of CPE reduction. C-uninfected Vero-E6 cells (cell control). D-infected Vero-E6 cells without treatment (virus control).

Fig. 6 Effects of two positive compounds on morphology of BJ-01 infected Vero-E6 cells

In the test a Vero-E6 cell based MTS CPE assay was developed for screening potential anti-SARS compounds. It provides a faster, safer, and more convenient screening method as compared to the traditional visual CPE assays, NR vital stain and MTT assay. Unlike MTT formazan, the MTS formazan product is soluble in tissue culture medium. So the step of decanting supernatants and adding volatile organic solvent was not needed. The absorbance spectrum generated by culture medium containing MTS is 382 nm and the bioreduction is 490 nm. So there was no washing or cell harvesting required in the test. Besides that, SDS was added to each well after MTS/PMS solution incubation. The step was not just to stop reaction, but more importantly to kill the virus in supernatants in order to reduce the spreading of viruses.

In the whole, a Vero-E6 cell based MTS CPE assay for screening potential anti-SARS compounds was developed. This aqueous soluble formazan assay eliminates the need to remove culture media or perform other sample manipulations. It results in high efficiency and throughput as well as decreased well-to-well variation. So this assay can be considered as a

ARS efficacy

of test compounds in a high-throughput format. Using this assay, more than 4 000 compounds have been tested in the primary screening and ten compounds showed potential anti-SARS inhibition.

References:

[1] Ksiazed T G, Erdman D, Goldsmith C S, *et al*. A novel coronavirus associated with severe acute respiratory syndrome [J]. *N Engl J Med*, 2003, 348(20): 1953-1966.

[2] Sutherland M W, Learmonth B A. The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase [J]. *Fre Rad Res*, 1997, 27(3): 283-289.

[3] Goodwin C J, Holt S J, Downes S, *et al*. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS [J]. *J Immunol Methods*, 1995, 179(1): 95-103.

[4] Morrey J D, Smee D F, Sidwell R W, *et al*. Identification of active antiviral compounds against a New York isolate of West Nil virus [J]. *Antiviral Res*, 2002, 55: 107-116.

[5] Gieni R S, Li Y, HayGlass K T. Comparison of [^{3}H] thymidine incorporation with MTT- and MTS-base bioassays for human and murine IL-2 and IL-4 analysis. Tetrazolium assays provide markedly enhanced sensitivity [J]. *J Immunol Methods*, 1995, 187(1): 85-93.

山茱萸鞣质活性部位对佐剂性关节炎大鼠免疫功能的影响

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摘 要: 目的 研究山茱萸免疫抑制活性部位(鞣质活性部位, F-1C) 对佐剂性关节炎 (AA) 大鼠免疫功能的影响。**方法** 运用淋巴细胞增殖反应、流式细胞技术和酶联免疫吸附试验 (ELISA) 观察 F-1C 体内和体外对 AA 大鼠免疫功能的影响, 并与环孢素 A (CsA) 和雷公藤多苷片 (TW) 进行比较。**结果** ig 给予 F-1C (30 mg/kg) 对 AA 大鼠原发性足肿胀具有明显的治疗作用, 该作用较 TW 强, 较 CsA 弱; F-1C 对 AA 大鼠低下的脾细胞增殖反应具有改善作用; 对亢进的胸腺细胞增殖反应具有抑制作用。F-1C 体外对 AA 大鼠低下的脾细胞产生 IgG 水平具有明显的促进作用, 并能抑制亢进的胸腺细胞增殖反应。**结论** F-1C 能够治疗 AA 大鼠原发足肿胀, 该作用可能与纠正 AA 大鼠的异常的免疫反应有关。

关键词: 山茱萸; 免疫抑制; 佐剂性关节炎; F-1C

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Effect of tannin from Cornus officinalis on immune function of adjuvant arthritis rats

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Abstract: Object To study the therapeutic effect and the possible mechanism of F-1C, a tannin fraction with immunosuppressive effect isolated from *Cornus officinalis* Sieb. et Zucc., in the treatment of adjuvant arthritis (AA) rats. **Methods** AA rat model was reproduced to observe the effect on immune function both *in vivo* and *in vitro* and compare with Cyclosporine (CsA) and *Tripterygium wilfordii* (TW). The effect of F-1C on joint swelling and the immune respones of AA rats were evaluated by lymphocytotic reaction, flow cytometry, and ELISA. **Results** F-1C by ig administration to AA rats decreased the primary joint swelling of AA rats, stronger than TW and weaker than CsA, promoted the decreased splenocyte proliferation induced by Con A, and inhibited the augmented thymocyte proliferation induced by Con A in AA rats. The evaluation *in vitro* showed the F-1C inhibited the augmented thymocyte proliferation from AA rats and promoted the deficient IgG produced by splenocyte from AA rats. **Conclusion** F-1C has therapeutic effect on AA rats and the modulating immune function is one possible underlying mechanism.

Key words: *Cornus officinalis* Sieb. et Zucc.; immunosuppressive; adjuvant arthritis (AA); F-1C

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