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Thitima Pengsuparp

Vimolmas Lipipun

Rutt Suttisri

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A Rapid and Simple Colorimetric Assay for Screening of Anti-HSV Agents

Thitima Pengsuparp*, Vimolmas Lipipun, Rutt Suttisri

Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand 10330

* Corresponding author.

ABSTRACT: There are several different approaches to detect the activities of potential antiviral agents *in vitro*. Plaque reduction assay is one of the classical methods for determining antiviral activity that is time-consuming and needs specialized skill in order to evaluate the results. We developed a rapid, simple and high-throughput anti-herpes simplex virus assay in order to be used in bioactivity-guided fractionation of natural product. Our alternative method employed sulforhodamine B (SRB) in measuring cellular protein content as an indicator for cell viability. The results were obtained by measurement of the absorbance on a scanning multiwell spectrophotometer and were comparable with those from classical method. This newly developed assay can be used as rapid screening test to detect both cytotoxicity and antiviral activity of a variety of samples.

Key words: Anti-herpes simplex virus, high-throughput screening, sulforhodamine B, colorimetric assay, plaque reduction

Introduction

Crucial to any investigation of plants with biological activities is the availability of suitable bioassays for monitoring the required effects. In order to cope with the number of extracts and fractions from bioactivity-guided fractionation steps, the capacity for high sample throughput is necessary. Ideally, the test systems should be simple, rapid, reproducible, and relatively inexpensive.

There are several different approaches in detecting potential activities of antiviral agents *in vitro*. Plaque reduction assay is a classical method for determining antiviral activity (1). However, it is time-consuming and needs specialized skill in order to evaluate the results. In order to be used in bioactivity-guided fractionation of natural product, we have developed a rapid, simple and high-throughput anti-herpes simplex virus assay. Sulforhodamine B (SRB) assay methodology, based on cellular protein content measurement, has been developed and used in large scale antitumor drug-screening (2). We have modified this rapid colorimetric assay for use in evaluating the cytopathic effect (CPE) of herpes simplex virus (HSV) in Vero cell cultures.

This assay employs an anionic dye, sulforhodamine B (SRB), in 1% acetic acid to stain cells for the measurement of cell density and virus- or drug-induced cytotoxicity in 96-well microtiter plates. The dye binds electrostatically to macromolecular counter-ions (*e.g.* basic amino acid residues of protein) in cells fixed with trichloroacetic acid (TCA), which allows their binding and solubilization to be controlled by variation in pH (3). At one pH range, the dye binds stoichiometrically to target macromolecular counter-ions, whereas at another they can be quantitatively extracted and measured their optical density on a scanning multiwell spectrophotometer (enzyme-linked immunosorbent assay-ELISA reader).

This assay can be used for several purposes. It allows a rapid and simple screening method for a large number of putative antiviral agents. On the other hand, cytotoxicity

and anti-HSV activity can be monitored simultaneously. The results obtained from this method are comparable with those from classical one.

Materials and Methods

Cultures

Herpes simplex virus tvpe 1 and 2 (standard strain): Virus stocks were prepared as aliquots of culture medium from Vero cells infected at multiplicity of 1 virion per 10 cells and cultured for 3 days. They were stored at -80°C. Working stocks were prepared by titering virus stocks serially diluted by culture medium and assayed in triplicate on Vero monolayers in the wells of microtiter plate.

Vero cell line (African green monkey kidney cell line): Cells were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum, 50 μg/ml of antibiotic-antimycotic (penicillin G + amphotericin B) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2 (water-jacket CO_2 incubator). Vero stocks were maintained at 37°C in culture flasks filled with medium supplemented with 10% fetal calf serum. Subcultures of Vero cells (1×10^4 cells), for virus titration or antiviral screening, were grown in the wells of microtiter plates by suspending Vero cells in medium following trypsin-EDTA treatment, counting the cells in the suspension with a hemocytometer and diluting with medium containing 10% fetal calf serum to 1×105 cells per ml.

Assay of Anti-HSV Activity using Plaque Reduction Method

Cell-free HSV (30 plaque forming unit, PFU) was mixed with the maintenance medium containing various concentrations of acyclovir and incubated at 37°C for 1 hour. After incubation, the mixture were inoculated into monolayer Vero cell culture in 96-well microtiter plates and incubated at 37°C for 2 days in CO_2 incubator. The plaques were counted under an inverted-microscope. The cells were stained with 1% crystal violet in 10% formalin for 1 hour. The cytopathic effect (CPE) of virus was determined as plaque forming unit (PFU).

Assay of Anti-HSV Activity using SRB Method

Using the similar procedure as described above, the cytopathic effect of virus was instead determined by staining with SRB. After 3-day incubation of virus and Vero cells with or without test compound, cells were fixed by gently layering 50 μl of cold 50% TCA (4°C) on top of the growth medium in each well to produce a final TCA concentration of 10%. The cultures were incubated at 4°C for 1 hour and then washed 4 times with tap water. Plates were air-dried. TCA-fixed cells were stained for 30 minutes with 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye. The cultures were air-dried until no standing moisture was visible. Bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5) for 5 min on plate shaker. Absorbance was recorded using a scanning multiwell spectrophotometer at 510 nm.

Results and Discussion

SRB is a bright pink aminoxanthene dye with two sulfonic groups (4). Under mildly acidic conditions, SRB binds to basic amino acid residues of protein in TCA-fixed cells to provide an index of cellular protein content that is sensitive and linear over a cell density. The SRB assay was demonstrated as a sensitive method for measuring cytotoxicity in culture as in an NCI's pilot study *in vitro* anticancer-drug discovery project (5).

To determine the feasibility of using SRB assay to screen for anti-HSV agents, Vero cells were pretreated with various concentrations of acyclovir (Sigma) and infected with HSV, as described in Materials and Methods. Cells were cultured for 3 days in 96-well plates and then were fixed with cold 50% TCA (4°C). TCA-fixed cells were stained with 0.4% (w/v) SRB in 1% acetic acid and the colorimetric assay was carried out (Table 1, Figure 1). Cells treated with acyclovir but not infected with HSV were used to determine the toxicity of each compound. Control cells (without virus and without drug) were defined as giving 100% cell viability. No decrease in cell density was observed with concentrations of acyclovir up to 20 μg/ml. Anti-HSV activity of acyclovir was determined at non-toxic concentrations by calculating the treated/untreated infected cell absorbance ratio. By varying the concentration of acyclovir, the dose-response curve of anti-HSV activity could be obtained (Figure 1) and the IC50 could be calculated (Table 2). Alternatively, the IC50 value of acyclovir's anti-HSV activity obtained by the classical plaque reduction method was determined as

described in Materials and Methods. The results were similar to those obtained by SRB method (Table 2).

To evaluate the correlation between this colorimetric and plaque reduction method in measuring anti-HSV activity, 50 plant extracts and 30 pure compounds were tested (data not shown). We found good correlation between these two methods and are working on further bioassay-direct fractionation of the active plant extracts.

In conclusion, this SRB method can be used to monitor the cytopathic effect (CPE) of herpes simplex virus (HSV) in Vero cell cultures. Faster results can be obtained through the use of microplate reader, instead of counting the plaque under microscope. Therefore, the colorimetric method using SRB dye is capable of providing a simple, rapid screening test for putative anti-HSV agents.

Table 1 Toxicity and anti-HSV activity of acyclovir analyzed with SRB colorimetric assay

Acyclovir (μg/ml)	% cell survival		
	Toxicity[a] (no virus)	Activity[b] (with HSV-1 virus)	Activity[b] (with HSV-2 virus)
0	100	0	0
0.05	100	53.7	11.2
0.5	100	100	54.6
1	100	100	100
20	100	100	100

[a] 100% cell survival was determined for untreated Vero cells., [b] Sample activity at non-toxic concentrations was determined by calculating the treated/untreated infected cell absorbance ratio. Each value is the average of triplicates.

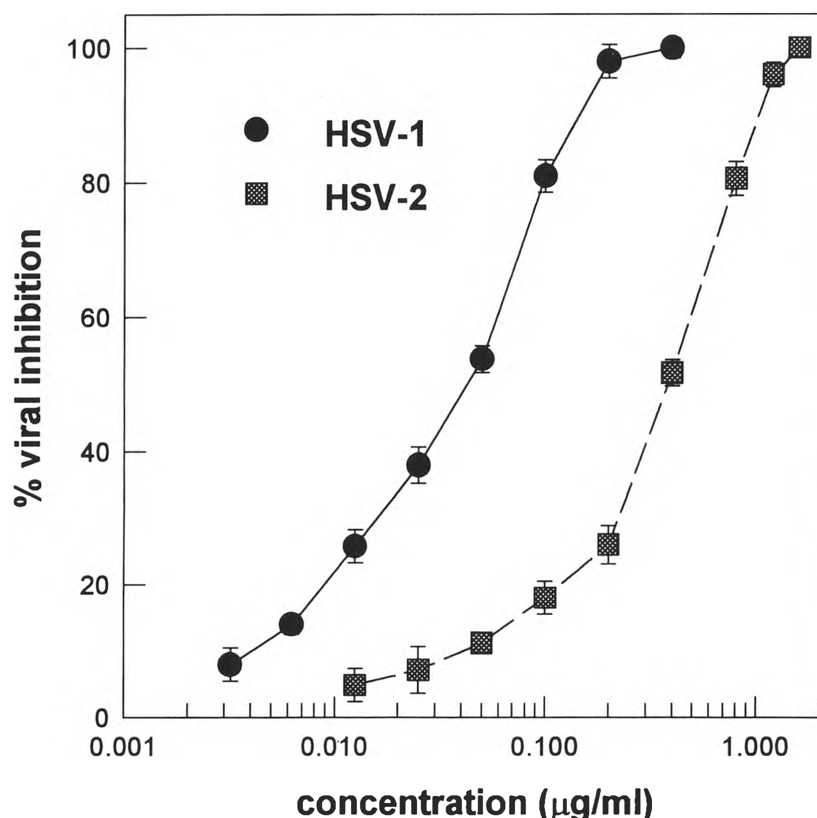

Figure 1 Dose-response curve of anti-HSV activity of acyclovir (using SRB colorimetric method)

Table 2 Anti-HSV activity of acyclovir

IC_{50} (μg/ml)			
Plaque Reduction Assay		**SRB Colorimetric Method**	
HSV-1	HSV-2	HSV-1	HSV-2
0.06 ± 0.01	0.5 ± 0.1	0.04 ± 0.02	0.4 ± 0.1

IC50 value is the concentration of sample at which 50% inhibition was obtained. (n = 3)

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วิธีการทดสอบฤทธิ์ต้านไวรัสก่อโรคเริมที่ง่ายและรวดเร็วโดยอาศัยหลักการวัดการดูดกลืนแสงของสี

ธิติมา เพ็งสุภาพ* วิมลมาศ ลิปิพันธ์ รุทธ์ สุทธิศรี

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพมหานคร 10330

* ผู้เขียนที่สามารถติดต่อได้

บทคัดย่อ : แนวทางการพัฒนาการตรวจสอบฤทธิ์ของสารที่มีศักยภาพในการต้านไวรัสในหลอดทดลองนั้นมีได้หลายแบบต่าง ๆ กันไป วิธีการตรวจสอบการลดปริมาณ plaque เป็นหนึ่งในวิธีดั้งเดิมที่ใช้ในการทดสอบฤทธิ์ต้านไวรัสซึ่งสิ้นเปลืองเวลาและจำเป็นต้องใช้ความชำนาญเฉพาะทางในการประเมินผลการทดลอง คณะผู้วิจัยได้พัฒนาวิธีการตรวจสอบฤทธิ์ต้านไวรัสเฮอร์ปีส์ซิมเพล็กซ์ซึ่งรวดเร็ว ง่าย และสามารถทดสอบสารตัวอย่างได้ทีละจำนวนมาก ๆ ขึ้นมา เพื่อใช้ในการสกัดแยกผลิตภัณฑ์ธรรมชาติโดยมีการติดตามฤทธิ์ทางชีวภาพ วิธีการที่ผู้วิจัยได้พัฒนาขึ้นนี้อาศัย sulforhodamine B (SRB) ในการวัดปริมาณโปรตีนในเซลล์เพื่อเป็นตัวบ่งชี้ถึงความอยู่รอดของเซลล์เหล่านั้น ปริมาณโปรตีนดังกล่าวสามารถวัดผลจากการดูดกลืนแสงโดยใช้เครื่อง scanning multiwell spectrophotometer ซึ่งผลการทดลองที่ได้นี้มีค่าใกล้เคียงกับเมื่อทดสอบโดยใช้วิธีดั้งเดิม วิธีการตรวจสอบที่ได้พัฒนาขึ้นใหม่นี้สามารถใช้สำหรับทดสอบเบื้องต้นอย่างรวดเร็วเพื่อตรวจวัดทั้งความเป็นพิษต่อเซลล์และฤทธิ์ต้านไวรัสของตัวอย่างที่ได้มากมายหลายชนิด

กุญแจคำ : ฤทธิ์ต้านไวรัสเฮอร์ปีส์ซิมเพล็กซ์, การทดสอบที่สามารถทดสอบสารตัวอย่างได้ทีละจำนวนมาก ๆ (high-throughput screening), sulforhodamine B, การทดสอบที่ใช้การวัดการดูดกลืนแสงของสี (colorimetric assay), การลดปริมาณ plaque