Journal of Asian Natural Products Research

Inhibition activities of polysaccharide (RG4-1) from Gentiana rigescens against RSV

Xin Li [a] , Jing-Yu Zhou [a] , Qing-Wei Yang [a] , Yang Chen [a] , Ying-Ai Piao [b] & Hong-Yuan Li [a]

[a] Department of Epidemiology, College of Public Health, Harbin Medical University, Harbin, 150086, China

[b] Department of Pharmacochemistry, Heilongjiang Provincial Institute for Drug Control, Harbin, 150086, China

Available online: 25 May 2011

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis
Taylor & Francis Group

Inhibition activities of polysaccharide (RG4-1) from *Gentiana rigescens* against RSV

Xin Li[a†], Jing-Yu Zhou[a†], Qing-Wei Yang[a], Yang Chen[a], Ying-Ai Piao[b] and Hong-Yuan Li[a]*

[a]Department of Epidemiology, College of Public Health, Harbin Medical University, Harbin 150086, China; [b]Department of Pharmacochemistry, Heilongjiang Provincial Institute for Drug Control, Harbin 150086, China

Respiratory syncytial virus (RSV) is the most important cause of lower respiratory tract infection in infants and young children. With the emergence of drug-resistant strains of RSV, new antiviral agents are needed urgently. *Gentiana rigescens* is a kind of Chinese herb, belonging to Gentianaceae, which has long been used as a folk medicine for curing inflammation, bacterial infection, viral infection, and so on. In this research, polysaccharide designated RG4-1 was isolated from *G. rigescens* by hot water extraction, ethanol precipitation, and macroreticular adsorbing resin column chromatography, and its antiviral activity, cytotoxicity, and possible antiviral mechanisms were assayed by cytopathogenic effect inhibition assay, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and plaque reduction assay. RG4-1 was a fructose-binding lectin. In host cell cultures, RG4-1 was found to be an effective antiviral component against RSV. It showed good inhibitory effect against RSV when it was added 2 h after virus infection with 50% effective concentration of 12.86 μg/ml. RG4-1 also displayed its direct inactivation, attachment inhibition effect, and penetration inhibition effect against RSV. A time-dependent experiment was set up to confirm that RG4-1 blocked RSV infection at early stages of the infection. But RG4-1 seemed to be ineffective against intracellular virus and viral biosynthesis.

Keywords: respiratory syncytial virus (RSV); *Gentiana rigescens*; antiviral agent

1. Introduction

Human respiratory syncytial virus (RSV) belongs to Family Paramyxoviridae, Pneumovirinae subfamily, and is the leading cause of severe lower respiratory tract illness in infants and young children. It is reported that RSV was detected in 77% of children with bronchiolitis [1]. Virtually all children experience RSV infection at least once during the first 2 years of life and part of them develop bronchiolitis and more severe disease requiring hospitalization, usually in the first 6 months of life. Children who

recover from RSV-induced bronchiolitis are at increased risk for the development of recurrent wheeze and asthma in later childhood [2]. In older patients, RSV may exacerbate an underlying condition or pulmonary and cardiac manifestations. It is also reported that RSV produces significant morbidity in those with underlying conditions, especially cardiopulmonary and immunosuppressive diseases. In transplant units, infection of RSV may be occult and is associated with high mortality rates [3]. Recently, only two approved products have been found to be effective for the treatment

*Corresponding author. Email: lihy00@yahoo.com.cn; lihongyuan@ems.hrbmu.edu.cn
†These authors contributed to this paper equally.

or prevention of RSV infection: the nucleoside analog ribavirin for the treatment and the monoclonal antibody palivizumab for the prophylaxis [4]. However, for the significant limitations such as poor safety and high cost of these two agents, new therapeutics with improved efficacy and safety are needed.

A small molecule inhibitor A-60444, which is a highly potent inhibitor of RSV replication, is on its phase II clinical trial. It shows potent sub-micromolar activity in plaque assay against RSV with the 50% inhibitory concentration (IC_{50}) values of $0.7–0.9 \mu M$, approximately 30 times more active than ribavirin. Moreover, natural products from plants have been found to be another potent source for the discovery of antiviral agents now [5]. *Gentiana rigescens*, a Chinese medicinal herb, which is commonly found in China, consisted of *Gentiana manshurica* Kitag, *Gentiana scabra* Bge, *Gentiana triflora* Pall, and *G. rigescens* Franch. Since it was first reported by Hayashi in 1976 [6], many researchers have undertaken numerous studies on *G. rigescens.* It inhibited the release of several intrahepatic enzymes, which was parenchymal injury parameter [7], and caused a 50% increase in benzo(a)pyrene hydroxylase activity in rats [8]. Meanwhile, the aqueous extracts from *G. rigescens* have anti-inflammatory, antimicrobial, and antiviral effects [9].

In previous studies, the hot water extract from the roots of *G. rigescens* was found to have good antiviral activity against RSV. It might be a potential anti-RSV substance for treating RSV infection. In this research, the bioassay-directed fractionation was used to isolate a polysaccharide from the roots of *G. rigescens* which displays anti-RSV activity. The isolation, purification, chemical nature, and antiviral activity of the water-soluble polysaccharide and its possible antiviral mechanism are described.

2. Results and discussion

2.1 Extraction and isolation of RG4-1

The Traditional Chinese Drug Laboratory, Provincial Institute for Drug Control of Heilongjiang confirmed that the *G. rigescens* used in this research was genuine by high-pressure liquid chromatography (HPLC)-fingerprint analysis. During the initial screening of herbal extracts for antiviral activity, the hot water extract from the root of *G. rigescens* displayed good activity in inhibiting RSV. As little is known about the anti-RSV activity of *G. rigescens*, experiments for isolating the active anti-RSV principle from this plant were initiated. After ethanol precipitation, the dark precipitate of *G. rigescens* aqueous extract showed anti-RSV activity in cytopathogenic effect (CPE) inhibition assay. The anti-RSV compound present in the precipitate was further fractionated by filtration chromatography on a macroreticular adsorbing resin column. Upon assaying, the active compound was detected in pooled fractions 1, 2, 3, 4, and 5, in which fraction 1 (RG4-1) had the highest activity. The purity of RG4-1 was analyzed by HPLC and spectrophotometry. As shown in Figures 1 and 2, RG4-1 showed a characteristic absorption peak within 260–280 nm in the ultraviolet spectra indicating that RG4-1 contained no protein or nucleic acid. Meanwhile, samples from RG4-1 contained essentially one peak on the ODS2 column indicating that the anti-RSV compound was relatively pure.

2.2 Chemical nature of RG4-1

RG4-1 was soluble in water but insoluble in methanol or ethanol. The Molisch test showed the presence of a purple ring at the junction of two layers, and this indicated the presence of carbohydrates. In the Iodine test, the sample turned violet after the addition of Iodine. The result indicated the presence of polysaccharide. After RG4-1 was hydrolyzed in 2 N trifluoroacetic acid at 120°C for 1 h, the hydrolysate was analyzed by thin-layer chromatography (TLC). One spot that had the same Rf value of fructose standard was identified. The results indicated that
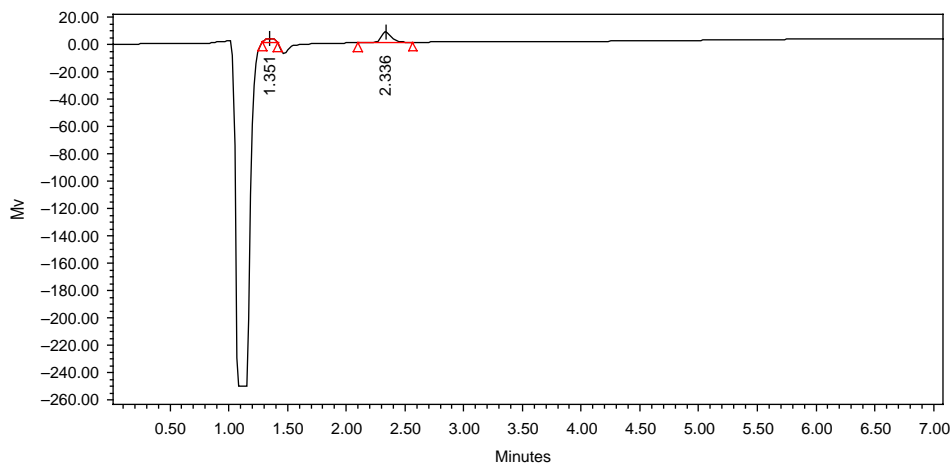
Figure 1. HPLC of RG4-1. RG4-1 was dissolved in deionized water (0.2 mg/ml) and 10 μl was injected into a Spherisorb ODS2 column. Compound was eluted with isotonic sodium chloride at a flow rate of 1.0 ml/min and detected with Differential Refractive Index Detector.

fructose was the constituent monosaccharide of RG4-1.

2.3 Biological characteristics

2.3.1 Cytotoxicity

To delineate whether the suppressive effect of RG4-1 on RSV replication was related to cytotoxicity, the viability of host cells was examined after they were treated with RG4-1 for 3 days by 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Fifty percent cytotoxic concen-

trations (CC_{50}s) of RG4-1 against HeLa cell, Hep-2 cell, and Vero cell were 4.88, 5.01, and 5.22 mg/ml, respectively, and CC_{50}s of ribavirin against HeLa cell, Hep-2 cell, and Vero cell were 1.09, 1.09, and 1.11 mg/ml, respectively.

2.3.2 Anti-RSV activity of RG4-1

RG4-1 showed good antiviral activity against RSV with 50% effective concentration (EC_{50}) of about 15 μg/ml in MTT assay. The activity of RG4-1 against RSV
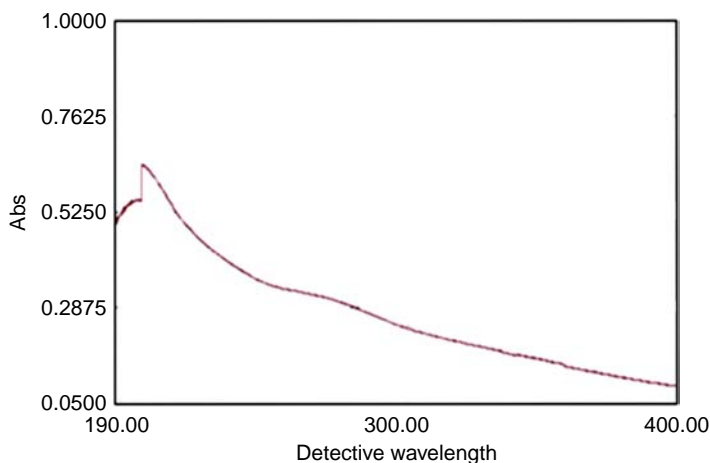

Figure 2. Ultraviolet spectrum of RG4-1. RG4-1 at the concentration of 0.5 mg/ml was scanned by TU-1901 ultraviolet spectrophotometer at the range of 190–400 nm.

Table 1. Anti-RSV effect of RG4-1 and ribavirin *in vitro*.

Culture cell	Tested method	RG4-1 (µg/ml)		Ribavirin (µg/ml)	
		EC_{50}[a]	TI[b]	EC_{50}[a]	TI[b]
HeLa cell	CPE	62.45 ± 8.78	78.14	51.40 ± 5.29	21.23
	MTT	12.64 ± 1.25	386.08	12.86 ± 0.79	87.76
Vero cell	CPE	50.26 ± 3.28	103.86	32.93 ± 3.24	33.40
	MTT	13.67 ± 1.50	381.86	18.66 ± 1.47	58.95
Hep-2 cell	CPE	48.75 ± 5.40	102.97	30.73 ± 4.11	35.47
	MTT	13.48 ± 3.15	372.40	9.20 ± 3.51	118.48
	Plaque reduction assay	40.38 ± 1.49	124.32	22.31 ± 1.40	48.85

Notes: Antiviral drugs were added into cells 2 h after virus inoculation. The antiviral activity of drugs was analyzed by CPE inhibition assay, MTT assay, and plaque reduction assay, respectively, as compared with that of control. Each value of EC_{50} was the mean ± SD of three independent experiments.
[a] EC_{50} was 50% effective concentration in host cells.
[b] TI was the ratio of CC_{50} to EC_{50}.

was further confirmed by plaque reduction assay. RG4-1 showed a dose-dependent inhibition against RSV when RG4-1 was added 2 h after infection. The therapeutic index (TI) was calculated based on their CC_{50}s and EC_{50}s. The narrowest TI of RG4-1 was 78-fold, but the range was as great as 386-fold, which was much higher than ribavirin (Table 1).

2.3.3 Direct killing effects of RG4-1

To investigate the direct inactivating effect of RG4-1 against RSV, serial dilution of RG4-1 was incubated with RSV at 37°C for 2 h before infection. In the virus control group, diluted virus was mixed with equal volume of medium. Pre-incubation of RSV with RG4-1 produced a significant reduction in remaining infectivity of RSV. The data strongly confirmed that RG4-1 acted as a viricidal agent in the research (Table 2).

2.3.4 Attachment inhibition effect of RG4-1

To investigate the attachment inhibition effect of RG4-1 against RSV, serial dilution of RG4-1 was added with RSV simultaneously. In attachment inhibition assays, RG4-1 displayed anti-RSV effect remarkably. The results are given in Table 3. In the experiment, RG4-1

Table 2. Viricidal effect of RG4-1 against RSV *in vitro*.

Culture cell	Tested method	RG4-1 (µg/ml)	
		EC_{50}[a]	TI[b]
HeLa cell	CPE	61.15 ± 7.38	79.80
	MTT	13.59 ± 0.77	359.09
Vero cell	CPE	47.91 ± 1.95	108.95
	MTT	13.62 ± 1.00	383.26
Hep-2 cell	CPE	49.80 ± 1.33	100.80
	MTT	10.45 ± 0.94	480.38
	Plaque reduction assay	26.23 ± 1.08	191.38

Notes: Viral suspension was pre-incubated with different concentrations of RG4-1 at 37°C for 2 h. Then the mixture was used to infect host cells. The direct killing effect of RG4-1 was analyzed by CPE inhibition assay, MTT assay, and plaque reduction assay, respectively, as compared with that of control. Each value of EC_{50} was the mean ± SD of three independent experiments.
[a] EC_{50} was 50% effective concentration in host cells.
[b] TI was the ratio of CC_{50} to EC_{50}.

Table 3. Inhibition effect of RG4-1 on adsorption *in vitro*.

Culture cell	Tested method	RG4-1 (μg/ml)	
		EC_{50}[a]	TI[b]
HeLa cell	CPE	127.25 ± 18	38.35
	MTT	34.01 ± 2.23	143.49
Vero cell	CPE	82.42 ± 3.02	63.33
	MTT	47.11 ± 4.90	110.80
Hep-2 cell	CPE	93.71 ± 5.29	53.57
	MTT	27.13 ± 1.50	185.03
	Plaque reduction assay	46.23 ± 2.35	108.59

Notes: Viral suspension together with different concentrations of RG4-1 was added into host cell at 37°C. After adsorbing for 2 h, the mixture was replaced with maintenance medium. The attachment inhibiting effect of RG4-1 was analyzed by CPE inhibition assay, MTT assay, and plaque reduction assay, respectively, as compared with that of control. Each value of EC_{50} was the mean ± SD of three independent experiments.
[a] EC_{50} was 50% effective concentration in host cells.
[b] TI was the ratio of CC_{50} to EC_{50}.

displayed its strong attachment inhibition effect at the smallest EC_{50} of 27.13 μg/ml.

2.3.5 Penetration inhibition effect of RG4-1

To investigate the penetration inhibition effect of RG4-1, RG4-1 diluted in series was added to cells after viral attachment at 4°C. For RSV strain tested, there was significant difference in the RG4-1 treated cells compared with the viral control (Table 4). In the experiment, RG4-1 displayed its strong penetration inhibition effect at the smallest EC_{50} of 26.96 μg/ml.

2.3.6 Effect of RG4-1 on viral growth

The effect of RG4-1 was further investigated in a one-step growth research in which RG4-1 was added at 0, 2, 4, 6, and 8 h post-infection. In the experiment, culture medium instead of RG4-1 was added in virus control group. The extracellular and intracellular viral yields were measured. As expected, the polysaccharide reduced more than 80% of the extracellular viral yield for each of the time points as compared to controls, but the intracellular viral yield was not reduced dramatically for the respective time points. A parallel experiment carried out using ribavirin showed

Table 4. Inhibition effect of RG4-1 on penetration *in vitro*.

Culture cell	Tested method	RG4-1 (μg/ml)	
		EC_{50}[a]	TI[b]
HeLa cell	CPE	94.40 ± 10.81	51.69
	MTT	36.70 ± 6.30	132.97
Vero cell	CPE	70.46 ± 7.35	73.80
	MTT	32.94 ± 1.39	158.47
Hep-2 cell	CPE	68.29 ± 5.84	73.51
	MTT	26.96 ± 3.17	186.20
	Plaque reduction assay	41.98 ± 2.66	119.58

Notes: Viral suspension was infected into host cells followed by 2 h of adsorption at 4°C. After two washes with PBS, different concentrations of RG4-1 were added at 37°C. The penetration inhibiting effect of RG4-1 was analyzed by CPE inhibition assay, MTT assay, and plaque reduction assay, respectively, as compared with that of control. Each value of EC_{50} was the mean ± SD of three independent experiments.
[a] EC_{50} was 50% effective concentration in host cells.
[b] TI was the ratio of CC_{50} to EC_{50}.

Table 5. The virus titer of extracellular virus and intracellular virus at different times after viral infection.

Time course (h)[a]	Drug	Extracellular virus		Intracellular virus	
		Pfu/ml $\times 10^6$	Inhibition (%)[b]	Pfu/ml $\times 10^6$	Inhibition (%)[b]
0	Ribavirin	1.33**	95	1.67**	95
	RG4-1	1.67**	93	22.64	32
	Virus control	24.30	–	33.30	–
2	Ribavirin	2.00**	92	2.70**	92
	RG4-1	2.33**	90	25.61	24
	Virus control	24.30	–	33.70	–
4	Ribavirin	2.33**	91	2.98**	91
	RG4-1	3.00**	88	24.31	27
	Virus control	24.70	–	33.30	–
6	Ribavirin	3.00**	88	7.08**	79
	RG4-1	3.67**	85	26.62	21
	Virus control	24.30	–	33.70	–
8	Ribavirin	3.33**	87	12.24*	64
	RG4-1	4.33**	83	24.14	29
	Virus control	25.30	–	34.00	–

Notes: Hep-2 cells were pre-chilled at 4 °C and infected with RSV for 2 h at 4 °C to synchronize the initial stage of infection and adsorption. Virus inoculum was then removed and cultures were washed three times with pre-chilled PBS. At 0, 2, 4, 6, and 8 h post-infection, RG4-1 at a final concentration of 200 µg/ml was added. In the virus control group, culture medium instead of RG4-1 was added. In the ribavirin control group, ribavirin at a final concentration of 200 µg/ml was added. The culture was further incubated to 24 h post-infection, and then the titer of extracellular and intracellular virus was determined by plaque assay.
[a] The time when compound was added.
[b] Percentage of plaque inhibition is indicated in parentheses = [(Plaque number in the virus control group – Plaque number in the treatment group)/Plaque number in the virus control group] $\times$ 100.
* Compared with virus control group, $P < 0.05$; ** compared with virus control group, $P < 0.01$.

that the extracellular viral yield was also reduced by more than 85%. Moreover, the intracellular viral yield was reduced by 95, 95, 91, 79 and 64% of the controls for the respective time points. These results indicated a clear difference between the effect of RG4-1 and ribavirin on RSV growth. RG4-1 displayed excellent inhibitory effect on extracellular virus (Table 5).

2.4 Discussion

In this research, RG4-1 was isolated from the roots of *G. rigescens* by hot water extraction, ethanol precipitation, and macro-porous adsorptive resin chromatography. The active principle RG4-1 was a kind of polysaccharide, as indicated by the results of the Molisch test and Iodine test. Polysaccharides, which sometimes play the part of a competitor of receptor to viral glyco-proteins, are known to affect the growth of animal viruses [10]. Polysaccharide such as fucoidan was found to have antiviral activity, which was associated with its effect on adsorption inhibition and preventing viral penetration into the macrophages [11].

The results of our research demonstrated that RG4-1 inhibited RSV *in vitro*. The inhibition was concentration dependent and specific. RG4-1 showed good inhibitory effect against RSV with the minimal EC_{50} of 12.64 µg/ml when it was added 2 h after viral infection. Considering its CC_{50} that was about 5 mg/ml, the therapy index of RG4-1 was up to 386.08, which was much higher than ribavirin. The results showed that RG4-1 might be a potent substance for treating RSV infection.

In the direct killing effects assay, RG4-1 displayed excellent inhibitory effect on extracellular virus with the minimal EC_{50} of $10.45\,\mu g/ml$ and a therapy index of 480. Besides, when RG4-1 was present during adsorption, significant antiviral effect was observed even at an EC_{50} of $27.13\,\mu g/ml$ when comparisons were made to virus control. In penetration inhibition assay, RSV binds to cells but does not penetrate through the cell membrane at $4°C$. The virus starts to penetrate the cell membrane at $37°C$. In the experiment, obvious antiviral effect was observed in the presence of RG4-1 at an EC_{50} of $26.96\,\mu g/ml$.

In the biomacromolecule synthesis inhibition assay, RG4-1 did not show any antiviral effect. As the experiment showed, RG4-1 was added to the infected cells after 2 h of virus infection. Until 8 h later, it was replaced by maintenance media. Finally, RG4-1 did not have any inhibitory effect on the virus biological macromolecule synthesis. An explanation was that the biomacromolecule synthesis processes started at about 4 h after virus infection. Approximately 6–8 hours later, the reproduction processes were done and the filial virus was released. In the first 8 hours, RG4-1 killed parts of extracellular virus. But 8 hours later the filial virus was released and RG4-1 was removed, so that the filial virus continued to infect the other cells. That is to say, the biological macromolecule synthesis processes as well as following assembly release process are not influenced.

Over the time course, the dramatic antiviral effect against RSV was observed at no more than 6 h post-infection. That is to say, RG4-1 impacted the early stage of virus replication. Besides, RG4-1 only had poor inhibitory effect against intracellular virus but had strong efficient inhibitory effect against extracellular virus.

In conclusion, RG4-1 inhibited RSV *in vitro* when present during pre-incubation, adsorption, and penetration. It means that RG4-1 might have interfered with the virus-cell binding and/or virus-cell fusion process. RG4-1 might act with viral G glycoprotein or F glycoprotein directly. One of the mechanisms of the anti-RSV action of RG4-1 has been attributed to an inhibition of virus binding to the cell membrane. This is further supported by other studies, which indicated that polysaccharide inhibited the virus by inhibiting viral adsorption and penetration [12]. Moreover, in the research, RG4-1 also displayed its direct killing effect. Results from this research indicated that one mode of action of RG4-1 against RSV appeared to be dependent on its virucidal activity.

For confidence, three different host cells and three methods were used in the research and the results were highly coincident. In this research, the anti-RSV effect of RG4-1 was proved by these three methods. The result confirmed that RG4-1 might be a potential anti-RSV drug candidate.

A disadvantage of this research was that the EC_{50} of the positive drug ribavirin was much higher than that previously reported, especially in CPE inhibition assay [13]. A possible reason was that the virus infectivity of RSV in the two experiments was not the same, or the therapeutic effect of ribavirin used in the two experiments is not the same.

In conclusion, a polysaccharide from the roots of *G. rigescens* had been isolated and its strong anti-RSV activity was observed. This polysaccharide had strong direct killing activity, adsorption inhibition activity, and penetration inhibition activity against RSV. In further research, many other viruses such as influenza virus, parainfluenza virus, and adenovirus, will be used to test the antiviral activity of RG4-1.

3. Materials and methods

3.1 Isolation and purification of polysaccharide from G. rigescens

Dried roots of *G. rigescens* (500 g) from Shiyitang Pharmaceutical Factory (Harbin, China) were authenticated by the Tra-

ditional Chinese Drug Laboratory, Provincial Institute for Drug Control of Heilongjiang. The washed roots were disintegrated with a blender and extracted with 5 L of distilled water at 100°C for 1 h. The aqueous extract was clarified through a cotton cloth and the residue was extracted again as before. Then, the mixed aqueous extract was condensed to 500 ml. The condensate was precipitated by 90% ethanol sequentially at 4°C overnight and the precipitate was collected by centrifugation. The concentrated crude polysaccharide was further fractionated on a macroreticular adsorbing resin column and eluted with deionized water, followed by 30, 60, and 90% ethanol. The collected water-eluted part was further fractionated on a macroporous resin column (50 × 2.5 cm, macroreticular adsorbing resin X-5, purchased from the Chemical Plant of Nankai University, Tianjin, China) and eluted with deionized water. Fractions of 5 ml were collected and the fractions were freeze-dried. The first part of the fractions was an ivory powder named RG4-1.

3.2 HPLC analysis of the extract of *G. rigescens*

To identify the *G. rigescens*, gentiopicrin, a chemical ingredient of *G. manshurica* Kitag, was used as a marker substance for the HPLC-fingerprint analysis. Gentiopicrin (110770–200510) was provided by China Institute for Drug and Biologic Product Standardization. The HPLC system consisted of a Shimadzu LC-10AT Liquid Chromatograph (Tokyo, Japan) equipped with a Shimadzu SPD-10Avp UV–VIS detector, a Shimadzu SCL-10Avp system controller, a Shimadzu CTO-10ACvp column oven, and a Shimadzu DUG-12A Degasser. A Diamonsil C18 (4.6 × 250 mm, 5 μm; DIKMA, St. Louis, MO, USA) and security guard cartridges Gemini C18 (4 × 3.0 mm; Phenomenex, Los Angeles, CA, USA) were used. The sample was eluted with the mobile phase that was composed of

CH_3OH and water (70:30) at a flow rate of 1.0 ml/min. Twenty microliters of the sample were injected into the system and detected with a UV absorbance at the wavelength of 270 nm [14]. The operating temperature was maintained at 25.0°C.

3.3 Purity detection of RG4-1

3.3.1 High-pressure liquid chromatography (HPLC)

For the analysis of the purity of RG4-1, RG4-1 was dissolved in deionized water (0.2 mg/ml) and 10 μl was injected into a Spherisorb ODS2 column (125 × 4.0 mm, 5 μm, Waters, MA, USA). The result is shown in Figure 1. The compound was eluted with isotonic NaCl at a flow rate of 1.0 ml/min and detected with 2414 Differential Refractive Index Detector (Waters, MA, USA).

3.3.2 Ultraviolet spectra scanning

RG4-1 at the concentration of 0.5 mg/ml was scanned by TU-1901 ultraviolet spectrophotometer at the range of 190–400 nm. The result is shown in Figure 2.

3.4 Chemical tests

3.4.1 Test for polysaccharide

3.4.1.1 The Molisch test. For the analysis of the chemical character of RG4-1, two drops of 5% L-napthol solution (in ethanol) was added and mixed to 2 ml of 0.1% RG4-1. Then 2 ml of concentrated sulfuric acid was poured to the side of the tube.

3.4.1.2 The Iodine test. One milliliter of the sample was taken in a test tube and one drop of iodine was added.

3.4.2 Acid hydrolysis of RG4-1

A total of 5 mg of RG4-1 was hydrolyzed in 2 N trifluoroacetic acid at 120°C for 1 h. The hydrolysate was dried using a rotary evaporator under reduced pressure at

40°C and the residues obtained were dissolved in water and analyzed by TLC using the solvent system: *n*-butanol:acetoacetate:water = 5:4.5:0.5. Glucose, galactose, xylose, mannose, rhamnose, levulose, arabinose, and maltose were used as the standards [14]. Sugars were revealed by staining the thin-layer plate with aniline-*o*-phthalic acid at 110°C for 5 min.

3.5 Materials

3.5.1 Virus and cells

RSV (Long strains) and host cells (HeLa cell, Hep-2 cell, and Vero cell) were kindly provided by the Institute of Virology, Chinese Academy of Preventive Medicine. All cells were routinely propagated in Modified Eagle's medium (MEM) and supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 100 IU/ml of penicillin G and 100 μg/ml streptomycin were incubated under 5% CO_2 at 37°C. The concentration of serum was reduced to 2% in antiviral assays.

The 50% tissue culture infective dose ($TCID_{50}$) of RSV was calculated by the Reed–Muench method according to the scores of cytopathogenic effect (CPE) of virus infection.

3.5.2 Positive control drug

Ribavirin used as a positive drug was provided by Hefeng Pharmaceuticals Ltd. (Shanghai, China).

3.6 Anti-RSV activity assays

3.6.1 Cytotoxicity test by MTT

Host cells were seeded in 96-well plates at an initial density of 5×10^3 cells/well. After the cells had been incubated for 20 h at 37°C, 0.1 ml of doubled dilutions of RG4-1 at the concentration of 25–1.6 mg/ml were added and the incubation was continued for 72 h. Discarding the maintenance medium, 20 μl of MTT at a

concentration of 5 mg/ml was added. Then, all wells were incubated again for 4 h. The supernatants were removed and 150 μl of dimethyl sulfoxide (DMSO) was added to each well. Plates were incubated in the dark for 10 min at room temperature to solubilize the dye and then gently mixed on a 96-well plate-adapted vortexer for 30 s. The absorption was measured at the wavelength of 550 nm. The cytotoxicity was expressed as CC_{50}.

The cytotoxic effect of ribavirin was also observed in the same way.

3.6.2 Inhibition of virus-induced cytopathogenic effect (CPE)

The antiviral activity of RG4-1 against RSV was evaluated by cytopathogenic effect (CPE) inhibition assay [15]. Confluent monolayers of cultured cells were overlaid in 96-well plate with 0.1 ml of virus suspension (63 $TCID_{50}$/ml). Serial two-fold dilutions of RG4-1 were added after the virus was adsorbed for 2 h. Ribavirin was used as positive control group. Virus-induced CPE was scored at the time when complete cell death was observed in the virus control group (usually 3 days). The concentration that reduced CPE by 50% was defined as EC_{50}. The inhibitory activity of that assay was confirmed by MTT [16] assay done on the same plate, and the EC_{50} was determined.

3.6.3 Inhibition effect of RG4-1 against RSV by plaque reduction assay

Plaque reduction assay was carried out to confirm the antiviral activity of RG4-1, after its anti-RSV activity was evaluated by CPE inhibition assay and MTT assay. Monolayers of Hep-2 cells grown on 24-well culture plates were infected with 0.2 ml of virus (63 $TCID_{50}$/ml). After incubation for 2 h with gentle agitation every 15 min, the inoculum was aspirated and the cultures were overlaid with maintenance medium with 1%

methylcellulose containing dilutions of RG4-1. Ribavirin was used as positive control group. After 96 h of incubation, the plates were fixed with 10% formalin, stained with 0.2% crystal violet, and air-dried, and the number of plaques was counted. The concentration of RG4-1 that reduced the number of plaques to 50% of the virus control was determined as EC_{50} [17].

3.6.4 Direct killing effect of RG4-1

The direct killing effect of RG4-1 was determined by the method of Oxford [18]. RSV was diluted to $2 \times 63\, TCID_{50}$/ml in a medium and mixed with an equal volume of medium containing RG4-1 to obtain final concentrations of 200, 100, 50, 25, and 12.5 μg/ml. In virus control group, diluted virus was mixed with equal volume of medium. After the mixtures were incubated at 37°C for 2 h, 0.1 ml of viral sample was removed from each tube to infect confluent host cells at 37°C for 2h. After incubation, viral samples were removed and cells were washed with PBS and then the maintenance medium was added. Using CPE inhibition assay, MTT assay, and plaque reduction assay, the direct killing effect of RG4-1 was investigated.

3.6.5 Attachment inhibition assay of RG4-1

The attachment inhibition effect of RG4-1 was determined by the method described by Zhang [19]. RSV was diluted to $2 \times 63\, TCID_{50}$/ml in a medium and mixed with an equal volume of medium containing RG4-1 to obtain final concentrations of 200, 100, 50, 25, and 12.5 μg/ml. The mixtures in 0.1 ml volume was immediately inoculated onto host cell monolayers. After 2 h of adsorption, inocula were removed from the cultures followed by washing with PBS twice and the addition of maintenance medium, and then the plate was incubated again. Using CPE inhibition assay, MTT

assay, and plaque reduction assay, the attachment inhibition effect of RG4-1 was investigated.

3.6.6 Penetration inhibition assay of RG4-1

The penetration inhibition effect of RG4-1 was quoded vide the paper of Zhang [20]. Host cells were pre-chilled at 4°C and were infected with 0.1 ml of virus suspension ($63\, TCID_{50}$/ml) for 2 h at 4°C. After washing with PBS twice, different concentrations of RG4-1 were added at 37°C. Using CPE inhibition assay, MTT assay, and plaque reduction assay, the penetration inhibition effect of RG4-1 was investigated.

3.6.7 Effect of RG4-1 on viral growth

A single-cycle growth of RSV in host cells was performed to determine the inhibitory effect of RG4-1 [21]. Hep-2 cells were pre-chilled at 4°C and infected with RSV for 2 h at 4°C to synchronize the initial stage of infection and adsorption. Virus inoculum was then removed and culture was washed three times with pre-chilled PBS. At 0, 2, 4, 6, and 8 h post-infection, RG4-1 at a final concentration of 200 μg/ml was added. In the virus control group, culture medium instead of RG4-1 was added. The culture was further incubated 24 h post-infection, at which time, the titer of extracellular and intracellular virus was determined. In this research, the culture media were removed for the assay of extracellular virus, and intracellular virus was measured following three frozen–thawed cycles and centrifugation after the culture was gently washed three times with PBS. The virus titer was measured by the plaque assay.

For confidence, in all the experiments described above, monolayers that were neither infected with RSV nor received treatment were used as the normal control, and monolayers infected with RSV but did

not receive treatment were used as the virus control.

3.7   Statistical analysis

TCID$_{50}$ of RSV in HeLa Cells, Hep-2 cells, and Vero cells was calculated by the Reed–Muench method [22]. CC$_{50}$ and EC$_{50}$ were calculated by probit regression analysis [23]. TI was calculated using the formula: TI = CC$_{50}$/EC$_{50}$.

References

[1] H.E. Stempel, E.T. Martin, J. Kuypers, J.A. Englund, and D.M. Zerr, *J. Acta Paediatr.* **98**, 123 (2008).

[2] S.S. Mohapatra and S. Boyapalle, *J. Clin. Microbiol. Rev.* **21**, 495 (2008).

[3] C.B. Hall, *J. Clin. Infect. Dis.* **31**, 590 (2000).

[4] L.R. Krilov, *J. Expet. Opin. Biol. Ther.* **2**, 763 (2002).

[5] S.C. Ma, J. Du, P.P. But, X.L. Deng, Y.W. Zhang, V.E. Ooi, H.X. Xu, S.H. Lee, and S.F. Lee, *J. Ethnopharmacol.* **79**, 205 (2002).

[6] T. Hayashi, *J. Yakushi.* **96**, 356 (1976).

[7] N. Kumazawa, S. Ohta, S.H. Tu, A. Kamogawa, and M. Shinoda, *J. Yakushi.* **111**, 199 (1991).

[8] J.J. Kang, Y.C. Chen, W.C. Kuo, T. Chen, Y.W. Cheng, M.L. Kuo, and T.H. Ueng, *J. Am. J. Chin. Med.* **24**, 19 (1996).

[9] Z.M. Tang, M. Peng, and C.J. Zhan, *China J. Integr. Tradit. Chin. West. Med.* **23**, 447 (2003).

[10] E.B. Damonte, M.C. Matulewicz, and A.S. Cerezo, *J. Curr. Top Med. Chem.* **11**, 2399 (2004).

[11] I.D. Makarenkova, G.G. Kompanets, N.N. Besednova, R.A. Slonova, T.N. Zviagint-seva, and N.M. Shevchenko, *J. Vopr Virusol.* **3**, 12 (2008).

[12] J.F. Liao, Y.M. Jan, S.Y. Huang, H.H. Wang, L.L. Yu, and C.F. Chen, *J. Proc. Natl Sci. Counc. Repub. Chin.* **19**, 151 (1995).

[13] J.F. Hruska, J.M. Bernstein, R.G. Douglas, and C.B. Hall, *J. Antimicrob. Agents Chemother.* **17**, 770 (1980).

[14] B. Classen, K. Witthohn, and W. Blaschek, *J. Carbohydr. Res.* **327**, 497 (2000).

[15] Y.Z. Wang, X.L. Cui, Y.J. Gao, S.S. Guo, X.K. Wang, Y. Huang, Y. Zhao, and W.F. Gong, *China J. Chin. Mater. Med.* **31**, 1176 (2006).

[16] Y. Li, K.T. Leung, F. Yao, L.S. Ooi, and V.E. Ooi, *J. Nat. Prod.* **69**, 833 (2006).

[17] R.O. Suara and J.E. Crowe, *J. Antimicrob. Agents Chemother.* **48**, 783 (2004).

[18] J.S. Oxford, R. Lambkin, I. Gibb, S. Balasingam, C. Chan, and A. Catchpole, *J. Antiviral Chem. Chemother.* **16**, 129 (2005).

[19] M. Zhang, P.C. Cheung, V.E. Ooi, and L. Zhang, *J. Carbohydr. Res.* **339**, 2297 (2004).

[20] Y. Zhang, P.P. But, V.E. Ooi, H.X. Xu, G.D. Delaney, S.H. Lee, and S.F. Lee, *J. Antiviral Res.* **75**, 242 (2007).

[21] K. Sudo, Y. Miyazaki, N. Kojima, M. Kobayashi, H. Suzuki, M. Shintani, and Y. Shimizu, *J. Antiviral Res.* **65**, 125 (2005).

[22] L.J. Reed and H. Muench, *J. Am. J. Trop. Med. Hyg.* **27**, 495 (1938).

[23] W.L. Jiang, X.L. Luo, and S.J. Kuang, *Acad. J. First Med. Coll. PLA* **25**, 454 (2005).