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A novel inhibitor of respiratory syncytial virus isolated from ethnobotanicals

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Abstract

A novel low molecular weight compound, CJ 4-16-4, isolated from ethnobotanicals using bioassay-guided fractionation, was found to be a potent inhibitor of respiratory syncytial virus (RSV) in vitro and in vivo. In vitro, a very low micromolar efficacious dose was obtained against at least four of subtype A (RSV-Long, RSV A2, and RSV A6 57754) and one of subtype B (Washington) RSV strains without seeing any significant cytotoxicity to Hep-2, MDCK or Vero cell lines. The drug inhibits growth of RSV in Hep-2 cells maintained in tissue culture at a very low concentration (~0.07 μM) with cell toxicity >400 μM (TI > 5880). In a cotton rat model of RSV infection, the drug was able to reduce viral titers by ~1 log at dose 12.5 and 25 mg/kg/day, and by >2 log at 100 mg/kg/day. This antiviral activity was specific as influenza A and B and herpes simplex 1 and 2 viruses were not inhibited. The results obtained indicate that CJ 4-16-4 warrants clinical development.

Keywords: Inhibitors; Antivirals; Respiratory syncytial virus; Natural product; Ethnobotanicals

1. Introduction

Respiratory syncytial virus (RSV) belongs to *Pneumovirus* genus of the *Paramyxoviridae* family. RSV is a negative stranded RNA virus comprised of two antigenic subgroups, A and B. The two subgroups are different in their G surface glycoprotein but remain antigenitically similar in their F or fusion surface glycoprotein (Chanock and McIntosh, 1990; Grosfeld et al., 1995).

RSV is the leading cause of severe lower respiratory tract infections in infants and children under 2 years of age (Parrott et al., 1973; Glezen et al., 1982; Chanock and McIntosh, 1990). It has been reported to be responsible for 40–50% of hospitalizations for bronchiolitis in the United States and 25% of pediatric hospitalizations for pneumonia (Dowell et al., 1996). Reinfection with RSV is common, with about 76% of children being reinfected during the second year of life. RSV reinfection also occurs in adults and is a common cause of hospitalization of adult patients with chronic lung conditions such as asthma and chronic obstructive pulmonary disease. It can also impact healthy adults. It was reported that 43% of adults with confirmed RSV missed work for periods up to 2 weeks (Dowell et al., 1996; Falsey and Walsh, 2000; Murry and Dowell, 1997). This virus has also been reported to be an important factor in the development of otitis media infections and hyper-reactive airway disease in later life (La Via et al., 1992).

RSV has also been shown to be significant pathogen in the elderly (Falsey et al., 1992,1995; Mills, 1996; Treanor and Falsey, 1999). In this population, RSV infection often manifests as a flu-like illness that can be misdiagnosed as influenza (Mathur et al., 1980; Osterweil and Norman, 1990). The impact of RSV-related mortality was recently illustrated in an epidemiology study, which estimated that in the United States more than 17,000 deaths per year are caused by RSV infection, with greater than 80% of these deaths occurring in people over 65 years of age (Thompson et al., 2003). Nursing residents are at greater risk, with outbreak rates measured as high as 40% (Mathur et al., 1980; Sorvillo et al., 1984). RSV is also responsible for serious

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lower respiratory tract infection among immunocompromised adults and young adults, as well as elderly who live in nursing homes and the community (Dowell et al., 1996). For example, in bone marrow transplants patients, RSV infection can progress to severe lower respiratory pneumonia, leading to high mortality rates (Englund et al., 1998; Garcia et al., 1997; Martin et al., 1998).

Currently, no vaccines are available for prophylactic use to prevent RSV infections. Only the nucleoside analog, ribavirin (Virazole®) has been approved for the treatment of RSV infection. However, ribavirin is a potential teratogen (Bradley et al., 1990), must be given by continuous small particle aerosol (Committee of Infecious Disease, 1993), and there is some controversy about the positive impact of this compound in mitigating RSV mortality, duration of hospitalization or the need for supportive therapy (Collins et al., 1996). Two antibody preparations are approved for prophylaxis (Prince, 2001): IGIV (RespiGam™), a polyclonal antibody preparation, and palivizumab (Synagis®), a humanized monoclonal antibody. Of these, Synagis® is the prophylaxis treatment of choice. However, its use is restricted to high-risk children up to 2 years of age. It is clear that because of the continued medical impact of RSV infections and the limitations associated with the current therapies, there is an unmet need for the identification of safer and more economical therapies to prevent or ameliorate RSV infections.

Ethnobotanicals may be one source of novel compounds that can be utilized to prevent or treat RSV infections. Traditional medicines utilizing natural products have been shown to have antiviral activity (Ma et al., 2002). Moreover, traditional Chinese herbs have long been used as remedies against infectious diseases in China. Extracts from one such plant, *Lonicera deflexicalyx* (Chin Jinyinhua), have been used in China alone or in medicinal herbal mixtures to treat acute respiratory infections. The main class compounds extracted from this ethnobotanical are derivatives of caffeoylquinic acids. The isolation and utility of this class of compounds have been previously reported (Zhu et al., 1999; Cui et al., 2002; Konczak et al., 2004; Li et al., 2005).

In this report, we independently describe the isolation of 3,5-dicaffeoylquinic acid (CJ 4-16-4) from the ethnobotanical *L. deflexicalyx* and utility of this compound as potential therapy for RSV infection. The property of this compound was elucidated through the use of a stepwise bioassay-guided fractionation of the selected ethnobotanical. CJ 4-16-4 has a promising antiviral activity against RSV with minimal toxicity.

2. Material and methods

2.1. Compounds

3,5-Dicaffeoylquinic acid (CJ 4-16-4; Fig. 2) was isolated from ethnobotanical *Flos lonicerae* or Chin Jinyinhua obtained from Herb King (San Francisco, CA). Ribavirin was obtained from Ribopharm (a division of ICN Pharmaceuticals, Costa Mesa, CA). For in vitro and in vivo experiments, all compounds were dissolved in water.

2.2. Isolation and purification

2.2.1. Preparation of crude CJ 4-16-4

Dried and ground flower bud materials (500 g) from *F. lonicerae* plants were macerated in 1200 ml methanol at room temperature for 3 days. Then the macerate was filtered and the process repeated three times using 1000 ml of methanol for maceration. The combined filtrates were evaporated using 65 °C water bath in vacuo and the residue was freeze-dried in a lyophilizer to yield 117.2 g of a brown powder. The lyophilized methanol extract (40 g) was dissolved in 150 ml methanol. To this solution, 80 g of silica gel 60 (63–200 μm, Sorbent Technologies, Atlanta, GA) were added, and the mixture was dried under vacuum to yield homogeneous extract-loaded silica gel ready for application in a vacuum liquid chromatography (VLC) (Coll and Bowden, 1986). The VLC column was prepared with dried 400 g silica gel 60 (TLC grade, Sorbent Technologies) that was poured into a VLC column 50 mm inner diameter (i.d.) × 240 mm length. The silica gel was allowed to sediment under vacuum. After application of the dried extract-loaded silica, stepwise gradient elution was performed using chloroform/methanol/water (v/v/v = 90/10/1, 2000 ml; 80/20/2, 2000 ml; 70/30/3, 2000 ml; 60/40/4, 2000 ml, and 50/50/5, 2000 ml). Fractions of 400 ml each were collected. The chromatographic process was monitored by UV at λ = 254 nm and sprayed by 20% sulfuric acid, and fractions 5–13 were combined and dried to yield 21.8 g of purified crude extract (ca. 54%). An aliquot of 2.0 g of the purified crude extract was dissolved in 10 ml of 90% methanol/water (v/v = 90/10) and subjected to a Sephadex LH-20 (100 g, 25–100 μ, Sigma, St. Louis, MO) column (19 mm i.d. × 110 mm length). The column was eluted with 90% methanol/water (v/v = 90/10, 300 ml). Fractions of 20 ml each were collected. The chromatographic process was monitored by UV at λ = 254 nm and sprayed by 20% sulfuric acid, and fractions 5–8 were combined and dried to yield 0.33 g of crude CJ 4-16-4 (ca. 9% yield). This process was repeated several times to obtain a larger quantity of crude CJ 4-16-4.

Crude CJ 4-16-4 (500 mg) was dissolved in 4 ml of 90% methanol/water (v/v = 90/10) and subjected again to a Sephadex LH-20 column (19 mm i.d. × 110 mm length). The column was eluted with 90% methanol/water (v/v = 90/10, 300 ml). Fractions of 10 ml each were collected. The chromatographic process was monitored by UV at λ = 254 nm and sprayed by 20% sulfuric acid, and fractions 7–8 were combined and dried to yield 239 mg of 90% pure CJ 4-16-4 (ca. 4% yield). This material was dissolved in 1.0 ml 80% methanol/water (v/v = 80/20) at 65 °C and left at room temperature overnight, then filtered by suction. The precipitate was washed with methanol (1.0 ml) to yield 58 mg 98% pure CJ 4-16-4 (ca. 1% yield; see Fig. 3). The ^{1}H NMR data obtained were essentially identical with previously reported (Basnet et al., 1996). Also, mass spectral data were in accord with the literature values (Miketova et al., 1999).

2.2.2. Thin layer chromatography (TLC)

For monitoring of the isolation process as well as identification of the natural product, TLC on normal phase silica gel (F_{254} precoated aluminum plates, Merck, Darmstadt) was convenient.

TLC plates were developed in chloroform/methanol/water (v/v/v = 60/40/4) as mobile phase with detection at λ = 254 nm and sprayed with 20% sulfuric acid.

2.2.3. High-performance liquid chromatography (HPLC)

This was a modification of the protocol reported by Morishita et al. (1984). The conditions are as follows: column used was Phenomenex Luna 18 Reversed Phase (250 mm × 10 mm), flow rate was 1.0 ml/min, injection was 10.0 μl of a solution prepared by dissolving 5.0 mg product in 1.0 ml methanol/water (v/v = 90/10), detection was at λ = 325 nm, and the mobile phase consisted of solvent A (methanol/HOAc (v/v = 100/2.5)) and solvent B (water/methanol/HOAc (v/v = 95/5/2.5)) at a gradient of 0–10 min, 40–50% A; 10–35 min, 50–80% A; 35–40 min, 80–100% A; 40–50 min, 100% A.

2.3. Viruses and tissue culture

All tissue culture reagents were obtained from GIBCO/BRL (Grand Island, NY). Hep-2 cells and the Long, A2, and B Wash/18537/62 (Bwash) RSV strains were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The Hep-2 cells were propagated at 37 °C in a humidified 5% CO_2 atmosphere in minimum essential medium (MEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), and 5% penicillin G and streptomycin. The different human RSV strains were propagated on 70–85% confluent Hep-2 monolayer in MEM supplemented with 2% FBS. The cells were infected with the virus at a multiplicity of infection of 10–100 tissue culture infectious doses needed to infect 50% of cells (10–100× $TCID_{50}$). Infectivity was monitored by examining the cell monolayers for cytopathic effect (CPE) and the appearance of syncytia. The medium from infected flasks was harvested 4–6 days later when CPE was 90–100% apparent. This material was clarified by low speed centrifugation to remove cell debris. The viral stock was then portioned, labeled, and stored at −135 °C until use.

2.4. In vitro antiviral assay

The antiviral effect of CJ 4-16-4 at different stages of purification was investigated using a CPE assay method as follows: cells (4×10^4 per well) were seeded in 96-well plates and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The overnight medium (MEM containing 10% FBS) was removed, and wells were rinsed twice with 1× phosphate buffer saline (PBS, 200 μl) before adding 10 $TCID_{50}$ of RSV suspension in fresh medium (MEM plus 2% FBS) to each well, except for cell-only control wells. After 2 h of virus adsorption at 37 °C in a humidified 5% CO_2 atmosphere, the medium was removed, and the cells were rinsed once with 100 μl of 1× PBS. Varying concentrations of the compound in culture medium (MEM plus 2% FBS, 200 μl) were added to the appropriate wells, and plates were incubated for 5 days at 37 °C in a humidified 5% CO_2 atmosphere. At this time, the cells in the virus control wells exhibited extensive CPE and destruction. Each concentration of the compound was tested in triplicate wells. CellTiter 96 aqueous non-radioactive cell proliferation (MTS) assay (Promega) was used to monitor the antiviral activity following the manufacturer's instruction. After incubation, the cells were rinsed twice with 200 μl of 1× PBS prior to the addition of 100 μl of fresh medium (MEM plus 10% FBS). Twenty microliters of MTS reagent (Promega) were added to each well, and the plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere until significant color development was observed (1–4 h). The extent of cell viability was measured at 490 nm using an ELISA reader, and the absorbance was used to calculate percent inhibition. Antiviral activity of the compound is expressed as EC_{50}, which represents the concentration of the test substance required to reduce the virus infection (as measured by the reduction in optical density) by 50%.

2.5. Effect of CJ 4-16-4 on influenza virus (FLU) and herpes simplex viruses (HSV)

A CPE assay was undertaken using MDCK or Vero cells infected with FLU A/Shangdong/09/93 (H3N2) or HSV 1 or 2, respectively. The MDCK and Vero cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in a minimum medium and incubated at 37 °C in a 5% CO_2 atmosphere. Serial dilutions of compound were added to cell monolayers; the virus was added within 5 min, and the plates were sealed for incubation. Positive control experiments for FLU or HSV were run in parallel using ribavirin or acyclovir, respectively. Each concentration of the compound was tested in triplicate wells. About 72–168 h after infection, CellTiter 96 aqueous non-radioactive cell proliferation (MTS) assay (Promega) was used to monitor the antiviral activity as describe above.

2.6. In vitro cytotoxicity assay

The cytotoxicity of the compound to host cells was tested in parallel with the antiviral testing and was conducted as follows: uninfected cells were resuspended in medium (MEM supplemented with 10% FBS). The viable cells (determined by trypan blue staining) were dispensed into 96-well (1×10^4 cells per well) microtiter plates. Varying concentrations of the compound or ribavirin (in medium, 20 μl) were added to the appropriate wells, and the plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 7 days. Each concentration of the compound or ribavirin was assayed in triplicate wells. The cytotoxicity of each concentration of the compound or ribavirin was determined using the MTS assay as previously described. The average absorbance for each concentration of the compound or ribavirin was tabulated, and the data obtained were used to calculate CC_{50} (concentration of test article required to inhibit the growth of host cells by 50%).

2.7. Testing of antiviral activity in cotton rats

The cotton rat (CR) host model of RSV infection was used to examine CJ 4-16-4 for in vivo efficacy. CJ 4-16-4 or ribavirin was dissolved in water and administered to the cotton

rats by intraperitoneally (i.p.) at the same time of virus inoculation. Virus (approximately 100 median cotton rat infectious does [$CRID_{50}$] of RSV A2) was given to the animals intranasally (i.n.) after anesthetizing them with Isoflurane (Abbot Laboratories, North Chicago, IL).

Four days after virus infection, the rats were euthanized by CO_2 asphyxiation. The lungs of sacrificed animals were removed, rinsed in sterile phosphate-buffered saline (PBS; pH 7.2), and weighed. The excised lungs were transpleurally lavaged as described elsewhere (Wilson et al., 1980). The resulting lung lavage fluids were placed on ice until assayed for virus in Hep-2 cells. In these tests, each test sample was assayed in quadruplicate in flat-bottom 96-well polystyrene plates (Corning, Acton, MA) using serial 3-fold dilutions in serum-free MEM supplemented with 10 mM HEPES buffer, 2 mM sodium L-glutamine, 10 U penicillin G/ml, 10 μg streptomycin/ml, and 0.025 μg of amphotericin B/ml. An additional 100 μl of supplemented MEM containing 5% (v/v) fetal calf serum was added to each well. After 6 days of incubation, each well was observed for the presence of virus-induced CPE including syncytia formation. The final RSV titers in the lung were calculated as the $\log_{10}$ $TCID_{50}$ per gram of lung. The statistical evaluations were performed using both non-parametric and parametric ANOVA.

3. Results

3.1. Bioassay-guided isolation and purification of the RSV inhibitor compound

The ability to demonstrate the protection of Hep-2 cells from a virus-induced CPE was used as a means to evaluate the anti-RSV activity of hot water extracts of ethnobotanicals, which historically have been used to treat respiratory track infectious illnesses. The ethnobotanical preparations, which inhibit the growth of RSV, are able to protect the host cell from CPE generated by replicating RSV in this 5-day assay. From this evaluation, the crude extract of ethnobotanical *F. lonicerae* (Chin Jinyinhua) was identified to have the best activity as an inhibitor of RSV induced cytopathology. This crude extract yielded protection of Hep-2 cells with an EC_{50} in the assay calculated to 35 μg/ml or 68 μM based on the molecular weight of the active compound (Table 1), at the same time CC_{50} was greater than 1 mg/ml (data not shown).

Using the crude extract of *F. lonicerae* (Chin Jinyinhua) as a reference point, bioassay-guided isolation, and purification was undertaken as outlined in Fig. 1 in an attempt to identify the single chemical entity in this ethnobotanical preparation directly responsible for the anti-RSV activity observed in the crude mixture. Because crude extract is a mixture of many compounds, it was also anticipated that the actual potency of the purified agent would be many-fold greater than the crude extract. In the initial step of the bioassay-guided isolation and purification, 500 g of the flower buds were subjected to exhaustive methanol extraction as described in Section 2, to yield 117.2 g of a brown powder. The brown powder, methanol extract was evaluated for anti-RSV activity in a CPE assay. In a dose titration experiment, the methanol extract protected the Hep-2 cells in cultures from RSV-Long strain-induced CPE with an EC_{50} of 4.7 μg/ml (9.0 μM) (Table 1).

A portion of the methanol extract (40 g) was further purified by two consecutive separations using the silica gel VLC method, and the resulting fractions were evaluated for anti-RSV activity in a CPE assay. In a dose titration experiment, these fractions protected the Hep-2 cells in cultures from RSV-Long strain-induced CPE with varying degrees of activity. The EC_{50} values ranged from 0.359 μg/ml (0.718 μM) (Table 1) to greater than 12.5 μg/ml, the highest concentration tested. At this stage, the fraction that protected Hep-2 cells in culture with the lowest EC_{50} was designated crude CJ 4-16-4 and the yield of the dry material was 0.33g.

Crude CJ 4-16-4 (500 mg) was further purified using a Sephadex column as described in Section 2 to yield 239 mg of 90% pure CJ 4-16-4. The 90% pure CJ 4-16-4 was dissolved in 1.0 ml 80% aqueous methanol at 65 °C and left at room temperature overnight, followed by suction filtration. The precipitated CJ 4-16-4 was washed with 1.0 m of methanol to yield 58 mg 98% pure CJ 4-16-4. In a dose titration experiment, the pure CJ 4-16-4 compound protected the Hep-2 cells in cultures from RSV-Long strain-induced CPE with an EC_{50} of 0.035 μg/ml (0.068 μM) (Table 1), whereas the (CC_{50}) was greater than 400 μM. Ribavirin, the only drug approved for use for the treatment of RSV infection, had an EC_{50} and a CC_{50} of 3.2 and 76 μM, respectively.

The data from the in vitro testing of *F. lonicerae* (Chin Jinyinhua) at different purification stages compiled in Table 1 showed a steady increase of anti-RSV potency of the active compound(s) from crude extract (stage I) to pure compound (stage IV). The ability of the compound CJ 4-16-4 to protect Hep-2 cells from RSV-Long-induced CPE from crude extract to pure compound increased at least 1000-fold.

Fraction CJ 4-16-4 was also examined for activity against RSV in a plaque reduction assay in two separate experiments. The compound was added to cells infected with RSV (~40–50 pfu) and overlaid with SeaPlaque agarose for 5 days

Table 1
Anti-RSV activities of EB preparation at different stages of processing using CPE assay system

Stage	Processing procedure	EC_{50} (μg/ml)[a]	CC_{50} (μg/ml)[a]	TI
I	Crude extract (hot water extract)	35 ± 2.4 (68)	>1000 (ND)	>28.5
II	Methanol extract	4.7 ± 0.7 (9.0)	>200 (>400)	>42.5
III	VLC fraction of methanol extract	0.359 ± 0.02 (0.718)	>200 (>400)	>557
IV	Active compound from fraction CJ 4-16-4	0.035 ± 0.001 (0.068)	>200 (>400)	>5700
	Ribavirin	(3.2)	(76)	23.8

[a] Values in parenthesis are EC_{50} expressed in μM. ND, not determined, TI, therapeutic index.

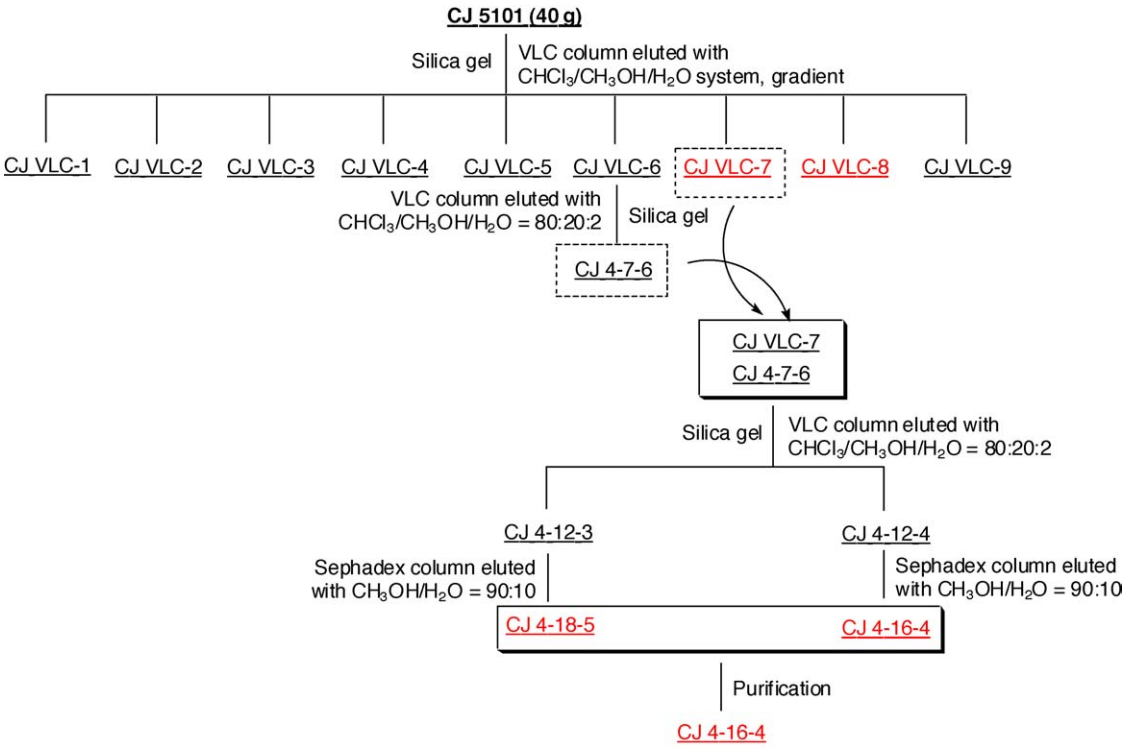


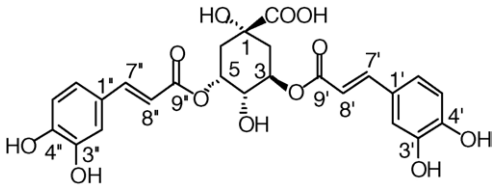
Fig. 1. Isolation and purification of CJ 4-16-4.

before crystal violet staining and analysis. The EC_{50}s of the CJ 4-16-4 for the Long strain RSV in Hep-2 cells were found to range between 0.05 and 0.07 μM (data not shown). These observations suggested that CJ 4-16-4 inhibited RSV replication in culture as measured by cell protection and plaque reduction assays.

3.2. Identification and chemical structure elucidation of the RSV inhibitor

The chemical structure of the most active compound in fraction CJ 4-16-4 is shown in Fig. 2. Its chemical structure was elucidated using chemical and spectral methods, mainly ^{1}H and ^{13}C NMR including DEPT, COSY, and HMQC techniques (Crews et al., 1998). In the mass spectra (MS), the low-resolution electrospray ionization (LR-ESI) method (Crews et al., 1998) was applied.

Thin layer chromatography (TLC): The active compound appeared as a bright yellow zone at $R_f = 0.43$.



CJ 4-16-4

Fig. 2. Structure of 3,5-dicaffeoylquinic acid used in the study.

High-performance liquid chromatography (HPLC): The CJ 4-16-4 peak appeared at approximately 24.2 min (Fig. 3).

Mass spectrometry (MS): LR-ESI mass spectrometry was used in the negative mode. The molecular ion peak of CJ 4-16-4 $[M-H]^{+}$ appeared at m/z 515.2, and the base peak at $m/z = 352.9$ (Fig. 4). This spectrum was in agreement with data previously reported (Miketova et al., 1999).

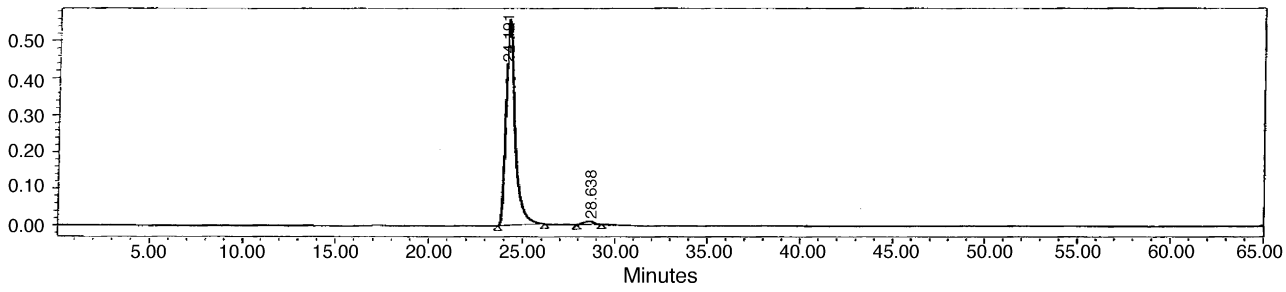


Fig. 3. HPLC profile of 98% pure CJ 4-16-4.

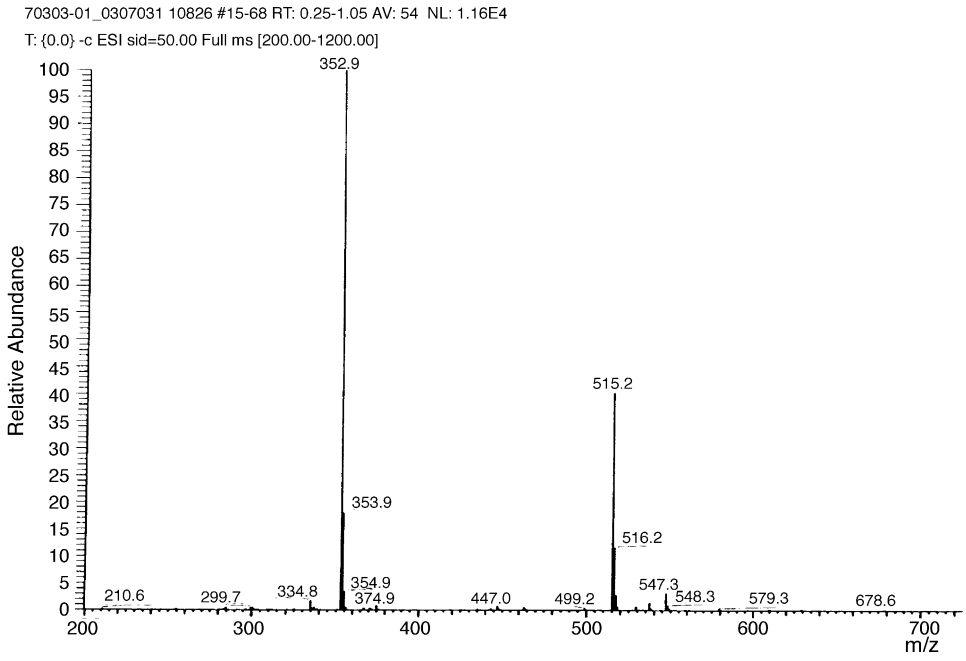


Fig. 4. LR-ESI mass spectrum of CJ 4-16-4.

Nuclear magnetic resonance (NMR): The proton spectrum at 400 and 600 MHz exhibited 17 protons signals. Aromatic protons at 7.03, 6.94 and 6.76 ppm agree with 1,3,4-trisubstituted benzene group. Mutually coupled proton signals at 7.60 and 6.36 (and 6.27) ppm with *J*-values 16.0 Hz suggest *trans*-orientated protons on a double bond (Fig. 5A). The proton decoupled carbon spectrum at 100 MHz exhibited 23 signals, and its DEPT-135 spectrum, indi-

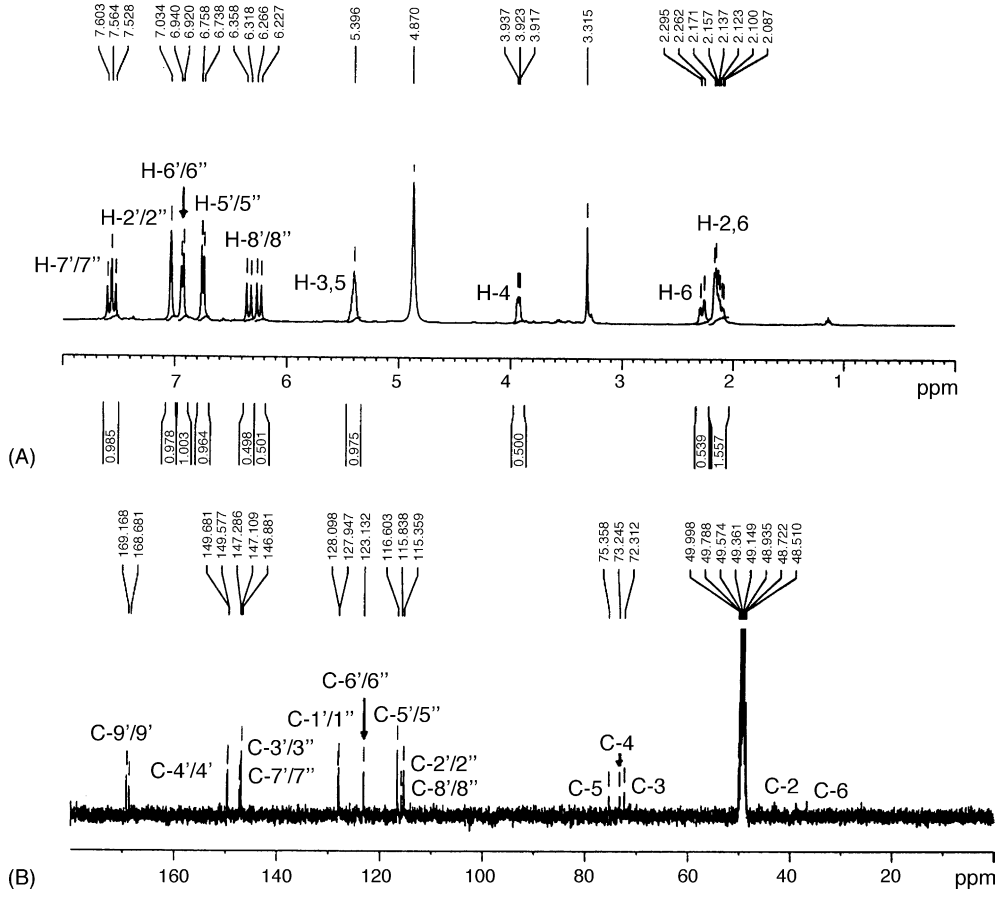


Fig. 5. Nuclear magnetic resonance (NMR): (A) ^{1}H NMR spectrum of CJ 4-16-4; (B) ^{13}C NMR spectrum of CJ 4-16-4.

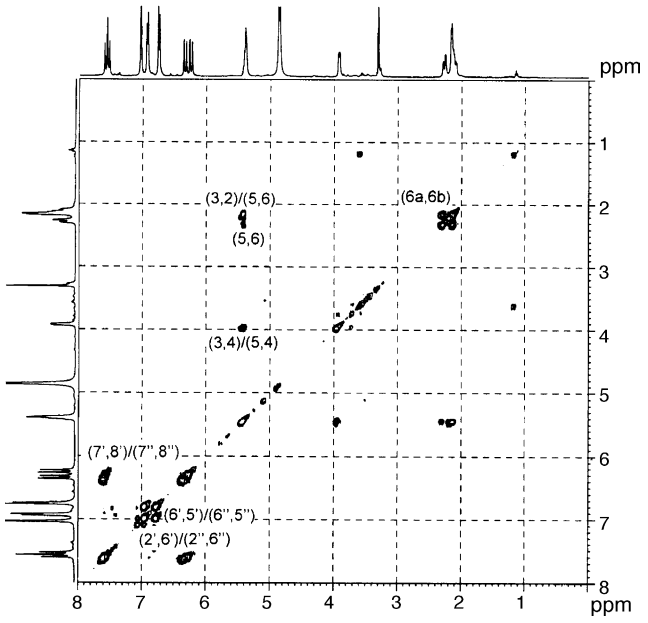


Fig. 6. ^{1}H-^{1}H COSY spectrum of CJ 4-16-4.

cated 2 methylene, 13 methines, and 8 quaternary carbons (Fig. 5B).

In COSY spectrum, the correlation peaks between proton-2 and proton-3, proton-3 and proton-4, proton-4 and proton-5, and proton-5 and proton-6 suggest that there is a quinic acid group in this compound. The cross peaks between proton-2′ (or 2″) and proton-6′ (or 6″), proton-6′ (or 6″) and proton-5′ (or 5″), and proton-7′ (or 7″) and proton-8′ (or 8″) gives the evidence of existence of two caffeoyl group in this compound (Fig. 6).

In HMQC spectrum (Fig. 7), correlation peaks between carbon-2 and proton-2, carbon-3 and proton-3, and carbon-6 and proton-6 gives the evidence of existence of quinic acid group. Signals between carbon-2′ (or 2″) and proton-2′ (or 2″), carbon-5′ (or 5″) and proton-5′ (or 5″), carbon-6′ (or 6″) and proton-6′ (or 6″), carbon-7′ (or 7″) and proton-7′ (or 7″), and carbon-8′ (or 8″) and proton-8′ (or 8″) proves there are two caffeoyl groups in CJ 4-16-4.

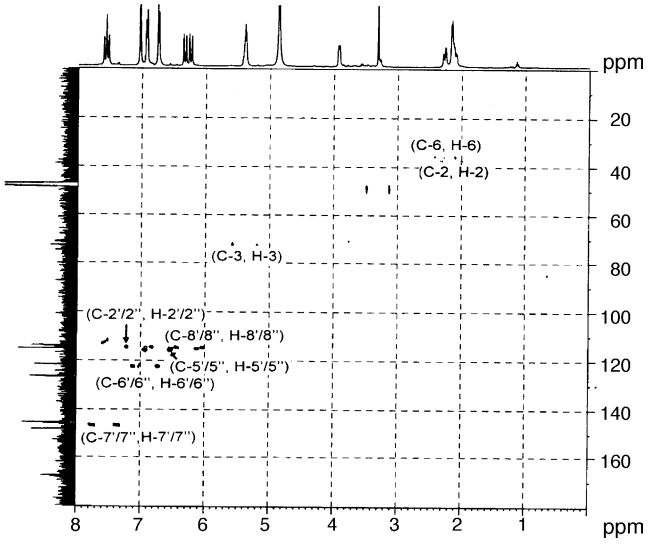


Fig. 7. HMQC spectrum of CJ 4-16-4.

The identity and purity of the compound was shown by NMR (400, 600 MHz, methanol-d$_4$, δ in ppm), and solvent signal used as reference signal ($\delta = 4.87$); ^{1}H and ^{13}C NMR data are essentially identical with data reported in the literature (Basnet et al., 1996).

Based on the information obtained using both chemical and spectral methods, the chemical structure of the single chemical entity (CJ 4-16-4) present in the most active fraction was deduced to be 3,5-dicaffeoylquinic acid.

3.3. Activities of CJ 4-16-4 against multiple laboratory isolates in Hep-2 cells

All the experiments described above were performed using the Long strain (subtype A). In order to determine the spectrum activity of CJ 4-16-4 against multiple RSV isolates, representatives of both the A and the B subtypes were obtained and used in the cell protection assay. In each experiment, the compound was evaluated in a series of eight concentrations in triplicate. The end-points of antiviral effect were monitored by a CPE assay method. CJ 4-16-4 was efficacious against laboratory strains A2, A6, and B Washington, with EC$_{50}$s of 0.16, 0.16, and 0.17 μM, respectively (Table 2). The data presented here and recent independently reported data by Li et al. (2005) suggest that CJ 4-16-4 compound is effective against all strains of RSV tested to date in the sub-micromolar range and may be capable of treating infections caused by all major strains of RSV.

3.4. Selectivity of CJ 4-16-4 against several viruses

In order to determine the specificity of CJ 4-16-4, efficacy studies were conducted with a number of related and unrelated viruses. The three viruses selected for these experiments included influenza type A/Shangdong/09/93 (H3N2), herpes simplex type 1 (MacItyre), and herpes simplex type 2 (MS). When the antiviral activity of CJ 4-16-4 was examined in cell protection assay, CJ 4-16-4 do not exhibit any inhibitory activity at the highest concentrations tested (Table 3). The highest concentration tested was greater than 1470-fold above the EC$_{50}$ of CJ 4-16-4 for the Long strain RSV. The results demonstrate that compound CJ 4-16-4 is a specific inhibitor of RSV. In addition, the cytotoxic effect of CJ 4-16-4 was also determined in Vero or MDCK cells in culture, which are the host cells for

Table 2
Effect of CJ 4-16-4 on multiple RSV strains

RSV-strains	EC$_{50}$ (μM)	TI
RSV-Long	0.068 ± 0.002	>5800
RSV-Long (cotton rat adapted strain)	2.7 ± 0.160	>148
RSV-B (Washington)	0.167 ± 0.007	>2395
RSV-A2	0.163 ± 0.008	>2453
RSV-A6 57754	0.16 ± 0.021	>2500

TI, therapeutic index.

Table 3
Specificity of CJ 4-16-4

Viruses	Cell lines	EC_{50} (μM)	CC_{50} (μM)
Influenza A/Shangdong/09/93 (H3N2)	MDCK	>100	>400
Herpes simplex virus type 1 (MacIntyre strain)	Vero	>100	>400
Herpes simplex virus type 2 (MS-strain)	Vero	>100	>400

HSV and influenza virus, respectively. In each experiment, the compound was evaluated in a series of eight concentrations in triplicate. The end-points of antiviral effect were monitored by a CPE assay method. The CC_{50}s values of CJ 4-16-4 in all these three cells were greater than 400 μM, the highest concentration tested.

The data on specificity presented here are consistent with recently reported data by Li et al. (2005). In the study, it was independently shown that 3,5-di-*O*-caffeoylquinic acid was inactive against Coxsackie B3 virus in addition to the other viruses described above. Furthermore, it has also been independently reported (Li et al., 2005; Cui et al., 2002; Zhu et al., 1999) that dicaffeoylquinic acids are relatively non-toxic in cell culture with CC_{50} greater than 1000 μM.

3.5. Antiviral efficacy in cotton rats

The cotton rat host model of RSV infection was used to examine CJ 4-16-4 for its anti-RSV activity in vivo. In these studies, ribavirin was used as the positive control, and water was used for the placebo group. All groups consisted of randomly selected rats and cohorts of four animals were used. CJ 4-16-4 was administered at 100, 25, and 12.5 mg/kg/day. Ribavirin was administered at 90 mg/kg/day. Each drug was administered i.p. with twice daily dosing with the first dosing being given at the same time of virus infection. Infected animals were sacrificed 4 days later. At that time the lung lavage fluids were tested to determine the $TCID_{50}$ titers of the virus present in each.

As shown in Table 4, the infected control animals that were treated only with vehicle had titers ranging from 4.3 to 4.8 $TCID_{50}$s (log_{10}/g lung), with a mean $TCID_{50}$ of 4.4 ± 0.3. The mean viral titers were reduced by 2.9, 1.2, and 1.1 log_{10}/g lung in the cohorts treated with 100, 25, and 12.5 mg/kg/day, respectively. No virus could be detected in two animals in the high dose of CJ 4-16-4–treated group. In the same experiments, the mean viral titer was reduced by 1.3 log_{10} in the cohorts treated with 90 mg/kg/day of ribavirin. The mean viral titer reductions by 100 and 25 mg/kg/day of CJ 4-16-4 and 90 mg/kg/day of ribavirin were statistically significant.

In addition to the efficacy studies discussed above, a minimal toxicity study was performed in the cotton rats. In these studies, the weights of test animals were determined at the beginning and conclusion of each experiment. Each animal was also observed daily for morbidity, mortality, diarrhea or other untoward responses. No evidence of toxicity was observed on these animals treated with up to 250 mg/kg/day for 4 days (data not shown).

4. Discussion

Novel anti-RSV compounds with better therapeutic efficacy and safety than ribavirin is a target for research. Natural products traditionally have been a rich source of therapeutic agents (Baker et al., 1995). A few familiar examples include aspirin, which was discovered from the bark of the willow tree. Modifications to these salicylate compounds have given us many of the non-steroidal anti-inflammatory therapeutics. Narcotic analgesics such as codeine, morphine, and papaverine were alkaloids isolated from the juice of the opium poppy. Newer drugs bear chemical modifications of these alkaloids to increase efficacy. Taxol was discovered from the bark of the Pacific Yew tree and is currently approved for use in treating ovarian and breast cancer. Vincristine and vinblastine were identified from the extracts of the periwinkle plant and have been important in the treatment of leukemias and lymphomas. Digitoxin and other digitalis glycosides were discovered from the leaves of the foxglove plant and are used in the treatment of congestive heart failure.

A variation in this search for active natural products that significantly narrows the field of investigation is to limit evaluation to natural products from ethnobotanical (EB) sources historically reported to have medicinal value. We have exploited this approach to identify EB sources that had historically been used for respiratory viruses treatment, and from this approach, we isolated and chemically characterized a low molecular weight compound, CJ 4-16-4, that exhibited significant anti-RSV activity both in our in vitro and in vivo tests. The ability of CJ 4-16-4

Table 4
Activity of CJ 4-16-4 in vivo

Treatment (dose)	RSV pulmonary titer (log_{10}/g lung) in CR				Mean ± S.D.	Reduction (log_{10})
	A	B	C	D		
Placebo	4.3	4.8	4.3	4.3	4.4 ± 0.3	0
CJ4-16-4 (100 mg/kg/day)[a]	0	2.8	0	3.3	1.5 ± 1.8	2.9[$]
CJ4-16-4 (25 mg/kg/day)	2.8	3.3	3.3	3.3	3.2 ± 0.3	1.2[$]
CJ4-16-4 (12.5 mg/kg/day)	3.3	3.3	3.3	3.3	3.3 ± 0.0	1.1
Ribavirin (90 mg/kg/day)	2.8	3.3	3.3	2.8	3.1 ± 1.8	1.3[$]

[a] This study was performed on a separate experiment on a different day from the other. S.D.: standard deviation and CR: cotton rats.
[$] $P < 0.05$.

to protect the Hep-2 cell culture from RSV-induced cytopathology was enhanced in every step of subsequent purification. The EC_{50} values increased over 1000-fold from crude extract to pure compound. This improvement of antiviral activity corresponded nicely with the enrichment of the active compound in the crude mixture from this EB source, which has historically been used alone or in combination with other therapies as a treatment regiment for respiratory-related illnesses.

The results presented above taken together with recently reported data (Cui et al., 2002; Li et al., 2005) and previous report on the derivatives of dicaffeoylquinic acids (Zhu et al., 1999) indicate that CJ 4-16-4 is a highly selective and potent inhibitor of RSV replication in tissue culture with activity against a broad-spectrum of virus isolates belonging to both the A and B subgroups of RSV. In addition, CJ 4-16-4 demonstrated excellent selectivity, showing no activity against other viruses when concentrations greater than 1000 times the EC_{50} for RSV were used. The mechanism of action for CJ 4-16-4 is still in progress, however, the recent report by Li et al. (2005), indicate that CJ 4-16-4 may be a fusion inhibitor.

The compound was also active against RSV in vivo in cotton rats. The value of animal models of RSV infection as a predictor of human RSV infection remains questionable (Cianci et al., 2004); however, the cotton rat model has been utilized to predict the usefulness of Respigam™ and Synagis® (Prince, 2001). Moreover, for a new chemical agent with inhibitory activity against RSV in vitro, the cotton rat model of RSV infection can impart proof principle for efficacy in vivo, which can validate the need for further clinical development. In the cotton rat model, CJ 4-16-4 administered i.p. at 100, 25 and 12.5 mg/kg/day at the same time of infection with twice daily dosing, reduced the viral titer in the lungs of RSV-infected cotton rats on average by 1–3 logs as compared to the placebo. Indeed, the lowest dose of CJ 4-16-4 tested (12.5 mg/kg/day) inhibited RSV infection by over 1 log, which was comparable to the effect observed after dosing with 90 mg/kg/day ribavirin. Thus, in both in vitro and in vivo experiments, CJ 4-16-4 antiviral activity against RSV was better than ribavirin.

To ensure that the in vivo effect of CJ 4-16-4 was not due to low drug levels in homogenized organs (drug carry over effect), uninfected cotton rats were given the compound at a dose of 100 mg/kg/day or placebo (two animals in each group) in the morning and afternoon and the next morning the animals were sacrificed and their lungs lavaged. The resulting lavage fluid from treated animals was tested in vitro by serial dilution into a 96-well plates and then adding approximately $10\times$ $TCID_{50}$ of virus. The control wells contained lavage fluids obtained from animals not given the test material. The cells were incubated until CPE was observed. Comparable CPE was observed in both CJ 4-16-4 and control wells (data not shown) suggesting that there was minimum or no drug carry over effect.

The work presented here was initiated independent of any knowledge of chemical compounds potentially contained in these ethnobotanicals. The identification of active fractions with in vitro activity against RSV and the subsequent identification of the active chemical compound being 3,5-dicaffeoylquinic acid was fortuitous. However, the data are consisted with previous independent reports in terms of anti-RSV activity (Cui et al., 2002; Li et al., 2005), specificity (Li et al., 2005), and toxicity profile (Li et al., 2005; Cui et al., 2002; Zhu et al., 1999) of derivatives of dicaffeoylquinic acids. Based on the current unmet need for the treatment of RSV infection and the results presented above, further studies on chemical synthesis and structure–activity relationship testing of CJ 4-16-4 based derivatives is merited.

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References

Baker, R., Borris, B., Carte, B., Cragg, G., Gupta, M., Iwu, M., Madulid, D., Tyler, V., 1995. Natural product drug discovery and development: new perspective on international collaboration. J. Nat. Prod. 58, 1325–1357.

Basnet, P., Matsushige, K., Hase, K., Kadota, S., Namba, T., 1996. Four di-*O*-caffeoyl quinic acid derivatives from propolis. Potent hepatoprotective activity in experimental liver injury models. Biol. Pharm. Bull. 19, 1479–1484.

Bradley, J.S., Conner, J.D., Campogiannis, L.M., 1990. Exposure of health care workers to ribavirin during therapy for respiratory syncytial virus infection. Antimicrob. Agents Chemother. 34, 68–70.

Chanock, R.M., McIntosh, K., 1990. Respiratory syncytial virus. In: Fields, B., Knipe, D.M., Chanock, R.M., Hirsch, M.S., Melnick, J.L., Monath, T.P., Roizman, B. (Eds.), Fields Virology, second ed. Raven Press, New York, pp. 1045–1074.

Cianci, C., Yu, K.-L., Combrink, K., Sin, N., Pearce, B., Wang, A., Civiello, R., Voss, S., Luo, G., Kadow, K., Genovesi, E.V., Venables, B., Gulgeze, H., Trehan, A., James, J., Lamb, L., Medina, I., Roach, J., Yang, Z., Zadjura, L., Colonno, R., Clark, J., Meanwell, N., Krystal, M., 2004. Orally active fusion inhibitor of respiratory syncytial virus. Antimicrob. Agents Chemother. 48, 413–422.

Coll, J.C., Bowden, B.F., 1986. J. Nat. Prod. 49, 934–936.

Collins, P.L., McIntosh, K., Chanock, R.M., 1996. Respiratory syncytial virus. In: Fields, B., Knipe, D.M., Chanock, R.M., Howley, P.M., et al. (Eds.), Fields Virology, third ed. Lippincott-Raven Press Publishers, Philadelphia, pp. 1313–1351.

Committee of Infecious Disease, 1993. Use of ribavirin in the treatment of respiratory syncytial virus infection. Pediatrics 92, 501–504.

Crews, P., Rodriguez, J., Jaspars, M., 1998. Organic Structure Analysis. Oxford University Press, New York.

Cui, X., Xu, Q., Lin, L.-Z., Zhao, L., Zhang, S., Wu, L., Lu, Y., 2002. Antiviral compounds and methods of treating viral infection. US Patent Application #20020111382.

Dowell, S.F., Anderson, L.J., Gary Jr., H.E., Erdman, D.D., Plouffe, F., File Jr., T.M., Marston, B.J., Breiman, R.F., 1996. Respiratory syncytial virus is an important cause of community acquired lower respiratory infection among hospitalized adults. J. Infect. Dis. 174, 456–462.

Englund, J.A., Sullivan, C.J., Jordan, M.C., Dehner, L.P., Vercellotti, G.M., Balfour, H.H., 1998. Respiratory syncytial virus infection in immunocompromised adults. Ann. Intern. Med. 109, 203–208.

Falsey, A.R., Cunningham, C.K., Barker, W.H., Kouides, R.W., Yuen, J.B., Menegus, M., Weiner, L.B., Bonville, C.A., Betts, R.F., 1995. Respiratory syncytial virus and influenza A infections in the hospitalized elderly. J. Infect. Dis. 172, 389–394.

Falsey, A.R., Treanor, J.J., Betts, R.F., Walsh, E.E., 1992. Viral respiratory infections in the institutionalized elderly: clinical and epidemiologic findings. J. Am. Geriatr. Soc. 40, 115–119.

Falsey, A.R., Walsh, E.E., 2000. Respiratory syncytial virus infection in adults. Clin. Microbial. Rev. 13, 371–384.

Garcia, R., Raad, I., Abi-Said, D., Bodey, G., Champlin, R., Tarrand, J., Hill, L.A., Umphrey, J., Neumann, J., Englund, J., Whimbey, E., 1997. Nosocomial respiratory syncytial virus infections: prevention and control in bone marrow transplant patients. Infect. Control Hosp. Epidemiol. 18, 412–416.

Glezen, W.P., Loda, F.A., Denny, F.W., 1982. The parainfluenza viruses. In: Evans, A.S. (Ed.), Viral Infections of Humans: Epidemiology and Control. Plenum Press, New York, pp. 337–349.

Grosfeld, H., Hill, M.G., Collins, P.L., 1995. RNA respiratory syncytial virus (RSV) is directed by the N, P, and L protein; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. J. Virol. 121, 503–510.

Konczak, I., Okuno, S., Yoshimoto, M., Yamakawa, O., 2004. Caffeoylquinic acids generated in vitro in a high-anthocyanin-accumulating sweet potato cell line. J. Biomed. Biotechnol. 5, 287–292.

La Via, W.V., Marks, M.I., Stutman, H.R., 1992. Respiratory syncytial virus puzzle: clinical features, pathophysiology, treatment and prevention. J. Pediatr. 69, 5677–5686.

Li, Y., But, P.P.H., Ooi, V.E.C., 2005. Antiviral activity and mode of action of caffeoylquinic acids from *Schefflera heptaphylla* (L) Frodin. Antiviral Res. 68, 1–9.

Ma, S.-C., Du, J., But, P.P.-H., Deng, X.-L., Zhang, Y.-W., Ooi, V.E.-C., Xu, H.-X., Lee, S.H.-S., Lee, S.F., 2002. Antiviral Chinese medicinal herbs against respiratory syncytial virus. J. Ethnopharm. 79, 205–211.

Martin, M.A., Bock, M.J., Pfaller, M.A., Wenzel, R.P., 1998. Respiratory syncytial virus infections in adult bone marrow transplant recipients. Lancet 1, 1396–1397.

Mathur, U., Bentley, D.W., Hall, C.B., 1980. Concurrent respiratory syncytial virus and influenza A infections in the institutionalized elderly and chronically ill. Ann. Intern. Med. 93, 49–52.

Miketova, P., Schram, K.H., Whitney, J., Kearns, E.H., Timmermann, B.N., 1999. Mass spectrometry of 3,5- and 4,5-dicaffeoylquinic acids and selected derivatives. J. Mass Spectrosc. 34, 1240–1252.

Mills, J., 1996. Management of Respiratory Syncytial Virus Infections. Plenum Press, New York, NY.

Morishita, H., Iwahashi, H., Osaka, N., Kido, R., 1984. Chromatographic separation and identification of naturally occurring chlorogenic acids by ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry. J. Chromatogr. 315, 253–260.

Murry, A.R., Dowell, S.F., 1997. Respiratory syncytial virus: not just for kids. Hosp. Pract. 15, 87–88.

Osterweil, D., Norman, D., 1990. An outbreak of an influenza-like illness in a nursing home. J. Am. Geriatr. Soc. 38, 659–662.

Parrott, R.H., Kim, H.W., Arrobio, J.O., Hodes, D.S., Murphy, B.R., Brandt, C.D., Camargo, E., Chanock, R.M., 1973. Epidemiology of respiratory syncytial virus infection in Washington, DC. Am. J. Epidemiol. 98, 289–300.

Prince, G.A., 2001. An update on respiratory syncytial virus antiviral agents. Expert Opin. Investig. Drugs 10, 297–308.

Sorvillo, F.F., Huie, S.F., Strassburg, M.A., Butsumyo, A., Shandera, W.X., Fannin, S.L., 1984. An outbreak of respiratory syncytial virus pneumonia in a nursing home for the elderly. J. Infect. 9, 252–256.

Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Cox, N., Anderson, L.J., Fukuda, K., 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 289, 179–186.

Treanor, J., Falsey, A., 1999. Respiratory viral infections in the elderly. Antiviral Res. 44, 79–102.

Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S., Couch, R.B., 1980. Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. 17, 642–648.

Zhu, K., Cordeiro, M.L., Atienza, J., Robinson Jr., W.E., Chow, S.A., 1999. Irreversible inhibition of human immunodeficiency virus type 1 integrase by dicaffeoylquinic acids. J. Virol. 73, 3309–3316.