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Identification of Porcine Reproductive and Respiratory Syndrome Virus Inhibitors Through an Oriented Screening on Natural Products

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Abstract Porcine reproductive and respiratory syndrome(PRRS) caused by porcine reproductive and respiratory syndrome virus(PRRSV) is one of the most infectious diseases in the swine industry worldwide, causing big economic losses. Vaccines are major weapons against PRRSV, however, current available vaccines have several limitations. Developing chemical drugs as alternatives is required. On the basis of traditional medical knowledge, we purposely selected 15 natural products originated from Chinese herbs with anti-infectious effects. Their antiviral activities were evaluated by PRRSV-induced cytopathic effect(CPE) on MARC-145 cells and reverse transcription polymerase chain reaction(RT-PCR) assay. Compounds ethoxysanguinarine(EOSG) and atractylodinol were found to be the hits which could significantly reduce PRRSV-associated CPE with 50% inhibited concentration(IC_{50}) values of 7.9 and 39.4 μmol/L, respectively. Meanwhile, compounds ethoxysanguinarine and atractylodinol significantly decreased mRNA expression of ORF7 gene in a dose-dependent manner. Study results suggest that compounds ethoxysanguinarine and atractylodinol may be useful anti-PRRSV drugs for swine industry or the hits for further lead optimization.

Keywords Porcine reproductive and respiratory syndrome virus(PRRSV); Natural product; Cytopathic effect; Antiviral activity

1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), belonging to the family Arteriviridae, genus *Arterivirus*, has significant impacts on the swine industry worldwide[1—3]. Porcine reproductive and respiratory syndrome (PRRS) caused by PRRSV is characterized by miscarriages, premature farrowing, stillborn pigs, infertility, slow growth, anorexia, fever, cough, dyspnea and so on[4,5]. PRRSV can transmit *via* semen, saliva, urine, and aerosol within and between herds[6—8]. PRRSV was first discovered in the United States in 1987, and swept over the North America and Europe in 1991. To date, even over two decades has gone by since PRRSV emergency. However, it is still a mystery swine disease, and continues to be a serious threat for swine industry, causing great economic losses. Various measures have been taken to control PRRSV. Eradication is an efficient measure, while this method is very expensive and needs early accurate detection in the negative herds. Vaccines have been the major weapon to combat PRRSV. Nevertheless, current commercial vaccines have several limitations. PRRSV-induced immune evasion is becoming serious and none of the vaccines could completely prevent respiratory infection. Up to now, though a great effort has been taken to develop PRRSV vaccines, yet little progress has been made at present[9,10].

In 2002, an emerging infectious disease known as severe acute respiratory syndrome(SARS) broke out in China and soon spread rapidly among 37 countries throughout the world[11]. To fight against SARS, traditional Chinese medicine has played an important role in either improving human constitution or killing SARS virus. Actually, more than one third of marketed antibiotics is directly from natural products or their derivatives[12], which means that natural sources may provide a new hope for anti-PRRSV agents, and prompt us to search for active anti-PRRSV components from traditional medicine.

Based on traditional medical knowledge, 7 herbs(*Papaver*

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somniferum, Atractylodes lancea, Radix aucklandiae, Zingiber officinale, Cortex dictamni, Calculus bovis and *Glycyrrhiza uralensis*) with obvious anti-infectious effect were selected among hundreds of herbs which were considered to be potentially effective on viral diseases, and then 15 representative compounds of these 7 herbs were purchased from a commercial source. The compounds were subjected to an oriented screening by cytopathic effect(CPE) and reverse transcription polymerase chain reaction(RT-PCR) assays, and two hits were fortunately identified. Herein lies the hit identification by oriented screening.

2 Experimental

2.1 Chemical Materials

A total of 15 HPLC(high-performance liquid chromatography) grade chemicals were purchased from National Institutes for Food and Drug Control of China and Jiangxi Bencao Tiangong Science & Technology Co., Ltd.(Nanchang, China). These natural products were ethoxysanguinarine(**1**), atractylodinol(**2**), atractylodin(**3**), atractylenolide I(**4**), asterolide(**5**), atractylenolide III(**6**), costunolide(**7**), dehydrocostuslactone(**8**), isoalantolactone(**9**), alantolactone(**10**), alpinetin(**11**), dictammine(**12**), 2-aminoethanesulfonic acid(**13**), capsaicin(**14**), and glycyrrhizic acid(**15**)(Fig.1). Compound **1** is the active ingredient of *Papaver somniferum*; compounds **2—6** are active ingredients of *Atractylodes lancea*; compounds **7—10** are the active ingredients of *Radix aucklandiae*; and compounds **11—15** are the active ingredients of *Zingiber officinale, Cortex dictamni, Calculus bovis, Capsicum annuum* and *Glycyrrhiza uralensis*, respectively.

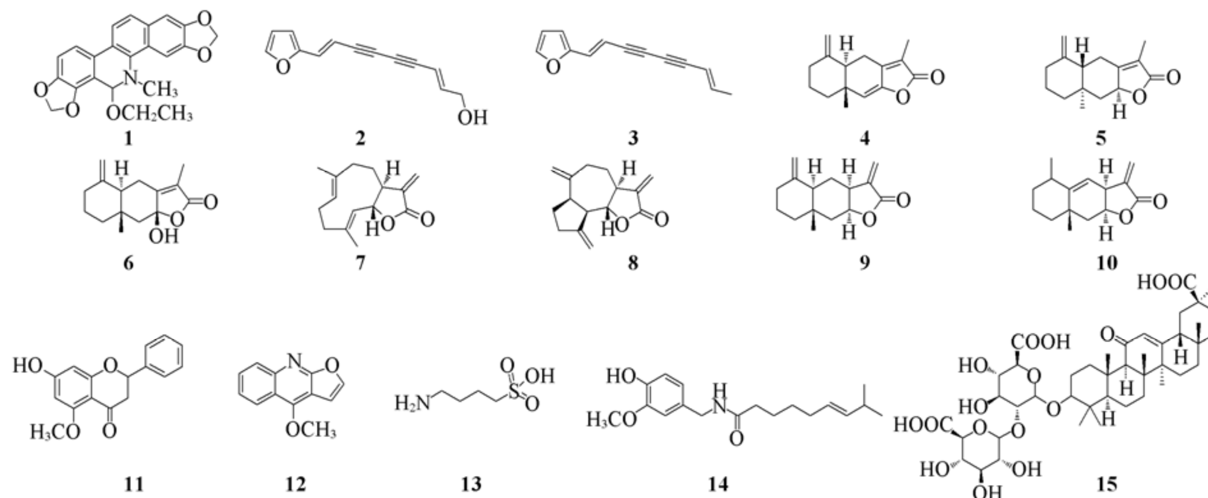

Fig.1 Structures of compounds 1—15

2.2 Cell Line and Cell Culture

Marc-145 cells obtained from Cell Library of Chinese Academy of Sciences were maintained in Dulbecco's modified Eagle's medium(DMEM) supplemented with 10% fetal bovine serum(FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C in an 80%—95% humidity atmosphere containing 5% CO_2 for the time period as noted below. When Marc-145 cells were about 95%—100% confluent, the cells were washed with phosphate buffer solution (PBS), detached with 0.25% trypsin-ethylene diamine tetroacetic acid(EDTA), resuspended, and seeded onto 96- or 6-well plates at an appropriate density according to each experimental until further incubation for 24—48 h. DMEM, FBS and 0.25% trypsin-EDTA were from HyClone Co.(Logau, USA). 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt(WST-8) assay kit was from Beyotime Co.(Shanghai, China). Other chemicals were of the highest quality commercially available.

2.3 PRRSV

YN-1 strain of PRRSV isolated from Yunnan Province of China was used to study antiviral effects of selected natural chemicals[13]. Marc-145 cells were infected with PRRSV in 6-well plates when the cells grow to 80%—90% confluence. After incubation for 72 h, the cells were harvested and centrifuged. The cell pellet was resuspended in 20 mL of the medium, and sonicated. The cell debris was then centrifuged at 10000 r/min for 10 min, and the supernatant was aliquoted(1 mL each tube) and kept at –80 °C. The PRRSV from the cells was titrated in Marc-145 cells *via* the 50% tissue culture infectious dose($TCID_{50}$) assay. The final titer was at $10^{-5.3}TCID_{50}$/0.1 mL.

2.4 Cell Viability Assay

Cell viability was measured with WST-8 assay, and sensitive colorimetric assay was performed for determining the number of viable cells, followed by cell proliferation and cytotoxicity assays, which was based on the conversion of WST-8 to formazan crystals by mitochondrial dehydrogenases[14]. Briefly, Marc-145 cells were seeded onto 96-well plates at a density of 1×10^4 cells/well. Serial two-fold dilutions of the samples were added to confluent cell monolayers and the cells were cultivated at 37 °C for 72 h. Then 10 μL of WST-8 solution was added to each well of the plates after incubation at 37 °C for 1.5 h, and the plates were read on a Bio-Tek ELx 800 enzyme-linked immunosorbent assay(ELISA) reader at 450 nm. The cell viability(percent of control) was calculated by

measured absorbance values.

2.5 Cytopathic Effect Inhibition Assay

The antiviral activity of the compounds against viruses was measured by the CPE inhibition assay[15]. Two-fold dilutions of the compounds were seeded onto cells monolayers cultivated in 96-well culture plates. Wells without the compounds or viruses were used for the negative control. Tilmicosin phosphate was used as a positive drug. An equal volume of virus suspension(100 $TCID_{50}$/mL) was added to the cells monolayers. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 72 h and the CPE was observed. The CPE included the loss of monolayer, rounding, shrinking of the cells observed under an inverted microscope. The concentration reducing CPE by 50% with respect to virus control was estimated from graphic plots and was defined as 50% inhibited concentration(IC_{50}) expressed in μmol/L. The therapeutic index(TI) was calculated from the ratio of TC_{50} to IC_{50}.

2.6 PRRSV mRNA Expression Inhibition Assay

The mRNA expression of PRRSV ORF7 gene was determined by real-time RT-PCR using SYBR Green I[16]. Briefly, primers were selected and designed from conserved regions based on the ORF7 sequences which provided the strain information obtained from the National Center of Biotechnology Information(NCBI) database. A 330 bp fragment of PRRSV was amplified with the following primers: forward primer was 5′-AATGGCCAGCCAGTCAATCA-3′ and reverse primer was 5′-TCATGCTGAGGGTGATGCTG-3′. A 174 bp fragment of *β*-action gene was amplified with the following primers: forward primer was 5′-ATCCAGGCTGTGCTGTCC- 3′ and reverse primer was 5′-GAGGATCTTCATGAGGTAG- TCG-3′. Total RNA was extracted respectively from the wells with or without drugs. Total RNA was isolated *via* RNA isoTMPlus kit(TaKaRa Biotech., Dalian, China) and kept in 0.02% diethyl pyrocarbonate(DEPC)-treated water at –80 °C according to the manufacture instructions. All the samples were quantified and the purity was determined on a spectrophotometer by OD_{260}/OD_{280} ratios. For each sample, 2 μL of total RNA was used for cDNA synthesis *via* SYBR® PrimeScript RT® reagents kit(TaKaRa Biotech., Dalian, China). Reaction mixture containing RNase free dH_2O(4.5 μL), 5×PrimeScript buffer(2 μL), PrimeScript RT enzyme Mix I(0.5 μL), Random 6 mers(100 μmol/L, 0.5 μL), Oligo dT primer(50 μmol/L, 0.5 μL). The conditions were as follows: 37 °C for 15 min, 85 °C for 5 s. The real-time PCR amplification of ORF7 gene used 25 μL of reaction mixture containing 12.5 μL of SYBR®Prime Ex TaqTM(TaKaRa Biotech., Dalian, China), 0.5 μL of PCR forward primer(10 μmol/L), 0.5 μL of PCR reverse primer(10 μmol/L) , 9.5 μL of dH_2O and 2 μL of cDNA. The reactions were carried out on an iQ5 real time PCR(Bio-Rad. Co., Ltd.). The conditions were as follows: one cycle at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative expression ratio of virus control and the tested group ORF7 gene was calculated based on Pfaffl method.

2.7 Statistical Analysis

All the experiments were performed in three replications. Continuous variables, expressed as mean±SD, were compared with one-way analysis of variance(ANOVA). Pairwise comparisons were evaluated by the Student-Newman-Keuls procedure or Dunnett’s T3 procedure when the assumption of equal variances did not hold. The Dunnett procedure was used for comparisons between reference group and each of other groups. Two-tailed p value less than 0.05 was considered statistically significant. Statistical analyses were conducted with SPSS 13.0 software.

3 Results and Discussion

It is well recognized that natural sources play an essential role in drug discovery. Traditional Chinese medicines have been used for millennia for mankind around the world, especially in China. It is estimated that around 10000 plants have been medicinally used, accounting for over one third of terrestrial higher plants[17]. It was reported that 122 plant-derived natural products exhibit therapeutic effects, and over 70% of them is related with ethno-knowledge[18]. As far as epidemic diseases are concerned, hundreds of herbs have been recorded to have anti-infectious effects, such as *Lonicera japonica* Thunb, *Isatis tinctoria* L., *Viola philippica* Car., and so on. Viral diseases are considered to be related to murrain which has been recognized earlier by Chinese ancient doctors. Therefore, there remains a great interest in searching for antiviral agents from natural origin. New methods like the solidification of floating organic drop liquid phase microextraction(SFODLPM) and HPLC are continuously applied to the extract of active compounds[19], and even there is ongoing research of further development cancer therapeutic drugs[20]. However, anti-PRRSV efforts on small molecules from the nature have been neglected. In this study, we selected 15 representative compounds from 7 herbs on the basis of traditional medicinal knowledge and modern scientific research achievements of Chinese medicine. PRRSV-induced CPE assay shows that ethoxysanguinarine(EOSG) and atractylodinol exhibited significant inhibitory effects on PRRSV with IC_{50} values of 7.9 and 39.4 μmol/L, respectively(Table 1). RT-PCR assay demonstrates that EOSG and atractylodinol significantly decreased mRNA expression of ORF7 gene in a dose-dependent manner(Fig.2).

Sanguinarine(SG) is an alkaloid, which has been found mainly in the plants of Papaveraceae family, such as *Sanguinaria canadesis*, *Eomecon chionantha*, *Corydalis edulis*, *Chelidonium majus* and *Macleya cordata* that have been medicinally used for hundreds of years[21—23]. SG as a representative agent contained in the above plants has been well studied in veterinary and human phytopreparations[24], which shows broad antimicrobial, anti-inflammatory, antioxidant, and antitumor activities. EOSG is a stable sanguinarine derivative prepared in alcohol. Up to now, anti-PRRSV effect is poorly understood. CPE assay indicates that EOSG has potent anti-PRRSV activity, which has been further confirmed by a RT-PCR assay, implying

Table 1 Anti-PRRSV activity of the compounds*

Compound	IC_{50}/(μmol·L^{-1})	TC_{50}/(μmol·L^{-1})	TI(TC_{50}/IC_{50})
1	7.9	53.1	6.7
2	39.4	358.9	9.1
3	>100	—	—
4	>100	—	—
5	>100	—	—
6	99.6	320	3.2
7	>100	—	—
8	>100	—	—
9	>100	—	—
10	>100	—	—
11	>100	—	—
12	89.4	385.4	4.31
13	>100	—	—
14	>100	—	—
15	>100	—	—

* n. d.: Not determined.

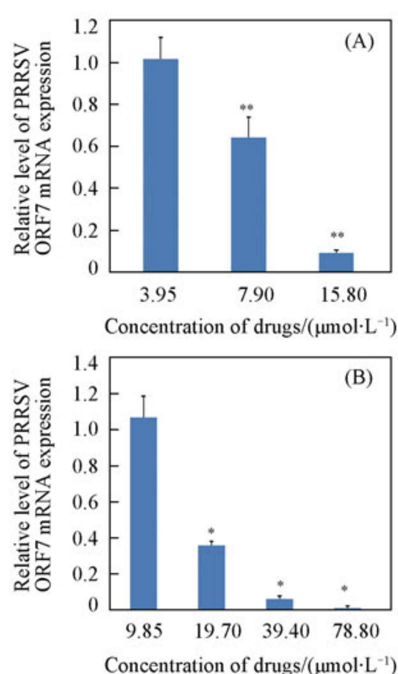

Fig.2 mRNA expression of PRRSV ORF7 inhibited by ethoxysanguinarine(A) and atractylodinol(B)

Data were expressed as mean±SD of three independent experiments (ANOVA). * Difference with virus inoculated group($P<0.05$). ** Significat differences with virus inoculated group($P<0.01$).

that EOSG could inhibit mRNA in a dose-dependant manner.

Atractylodinol is a furan derivative with a polyacetylene side chain. This compound is present in *Atractylodes lancea*, a typical Chinese medicine. It was recorded in ancient book that the smoke of *A. lancea* could prevent murrain transmission. We thus selected some compounds from this herb to test their anti-PRRSV activity. Atractylodinol was found to be against PRRSV in CPE assay and exhibited a good dose-dependant effect in RT-PCR assay, whereas other sesquiterpenoids are inactive. It was noted that atractylodin and atractylodinol are structurally similar compounds, while they showed a distinct anti-PRRSV effect difference, which may imply that the end alcohol group in the structure of atractylodinol is important for keeping the activity.

In summary, we described the screen and identification of PRRSV inhibitor hits(compounds **1** and **2**) *via* a very oriented screening on a small library designed with the help of ethno-pharmacological information, obtaining 15 commercially available natural products. The present study illustrated that oriented screening is a practical and an efficient method for hit identification and will speed up hit-to-lead drug discovery process. SG is naturally abundant, and the stable EOSG could be easily prepared by dissolving SG in ethanol. Atractylodinol has a simple structure which could be chemically synthesized. The anti-PRRSV effects might provide new therapeutic alternatives for PRRSV-induced porcine diseases and will benefit swine industry.

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