Curr Microbiol (2014) 69:82–87
DOI 10.1007/s00284-014-0540-9

Antiviral Effect of Diammonium Glycyrrhizinate on Cell Infection by Porcine Parvovirus

Pengchong Li · Hao Zou · Yudong Ren · Dante S. Zarlenga · Xiaofeng Ren

Received: 25 November 2013 / Accepted: 18 December 2013 / Published online: 11 March 2014
© Springer Science+Business Media New York (outside the USA) 2014

Abstract Porcine parvovirus (PPV) can cause reproductive failure in swine, resulting in economic losses to the industry. Antiviral effects of diammonium glycyrrhizinate (DG) have been reported on several animal viruses; however, to date it has yet to be tested on PPV. In this study, the antiviral activity of DG on swine testis (ST) cell infection by PPV was investigated using an empirically determined, non-toxic concentration of DG and three different experimental designs: (1) pre-treatment of virus prior to infection; (2) pre-treatment of cells prior to infection; and (3) direct treatment of virus-infected cells. The results showed that DG possesses potent inhibitory effects on PPV when the virus was treated before incubation with ST cells and that virus infectivity decreased in a dose-dependent manner. Results were confirmed by indirect immunofluorescence assays and real-time quantitative PCR. In addition, deoxycholate was used as a control to exclude the possibility that DG acted as a detergent to inhibit PPV infectivity. The study clearly indicates that DG has a direct anti-PPV effect in vitro.

Pengchong Li and Hao Zou are co-first authors.

P. Li · H. Zou · Y. Ren · X. Ren (✉)
College of Veterinary Medicine, Northeast Agricultural University, 59 Mucai Street, Xiangfang District, Harbin 150030, China
e-mail: renxf@neau.edu.cn; rxfemail@yahoo.com.cn; rxfemail@gmail.com

P. Li · X. Ren
College of Life Science, Inner Mongolia University for Nationalities, Tongliao 028043, China

D. S. Zarlenga
Animal Parasitic Diseases Laboratory, Agricultural Research Service, Beltsville, MD 20705, USA

Introduction

Porcine parvovirus (PPV) belongs to the family *Parvoviridae* and exists as a single-stranded, minus-strand DNA virus ~5,000 bases in length. PPV is among the most common and important causes of infectious infertility in pigs and can result in significant economic losses to the swine industry. It is one of the few viruses that can survive in the environment for extended periods of time and is refractory to most disinfectants. Currently, there are no viable treatments for the disease. Vaccination is effective, but cost and safety issues have been deterrents to routine vaccination in many regions of the world.

Porcine parvovirus was first reported by Mahnel et al. [15] upon culturing a swine fever virus; it was subsequently isolated from diseased pigs in 1967 [3]. Major clinical symptoms of PPV disease include miscarriage, stillbirth, mummified piglets, and newborn piglet death [22]. This disease has been found worldwide since the early 1980s [5, 14].

At present, attenuated and killed viral vaccines are being used to control PPV infection; however, an attenuated vaccine stands the danger of restructuring and reacquiring virulence. Furthermore, evidence has been advanced that vaccination against PPV may protect against the disease, but it does not necessarily prevent viral infection and virus shedding of heterologous strains [10, 26]. As such, conventional use of PPV vaccines on an "as needed" basis provides an opportunity to develop drug therapies for use as supplementary control strategies.

Diammonium glycyrrhizinate (DG), the active ingredient of *Glycyrrhiza* extraction, has significant anti-inflammatory effects and enhances liver cell protection and liver function [7, 16]. This compound is active against human immunodeficiency virus (HIV) [20], hepatitis A virus (HAV) [4], hepatitis B virus (HBV) [21], coronavirus [11],

and herpes virus [23]. In this study, the antiviral effects of DG on PPV in vitro were analyzed. Data presented herein indicate that DG decreases infectivity of PPV significantly if incubated with the virus prior to contact with the cells.

Materials and Methods

Cytotoxic Assay

Swine testis (ST) cells, a PPV-susceptible cell line, were maintained in our laboratory and grown in Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum at 37 °C and 5 % CO_2. PPV strain PPV2010 was propagated in ST cells in the absence of serum and used for infection analyses [5, 19]. Cytotoxic assays were performed according to published MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) protocols with minor modifications [11, 23]. Cell monolayers plated onto 96-well culture plates were washed three times with phosphate-buffered saline (PBS); then, DG (Zhengda Pharmacy, China) was serially diluted twofold in serum-free DMEM and added onto the wells (5 wells per each dilution). Mock-treated cells containing no DG were used as negative controls. The cells were cultured at 37 °C for 72 h, supplemented with 80 µl per well DMEM and 20 µl per well 0.5 % MTT solution and then incubated for an additional 4 h at 37 °C. After washing with PBS, 150 µl of DMSO was added to each well. The plates were shaken gently for 15 min to dissolve the formazan precipitate, and the OD_{570} was recorded. Cell survival rates were calculated from mean values according to the following equation: (OD_{570} drug)/(OD_{570} control) × 100. Deoxycholate (DOC) (Sigma, China) was serially diluted twofold beginning with 2 mg/ml in serum-free DMEM and added to the ST cell monolayers cultured at 37 °C for 72 h to observe cytopathic effects.

Treatment of Cells Prior to Virus Infection

The ST cells cultured on 96-well plates were treated with an experimentally determined, non-toxic concentration of DG (250 µg/ml) [9, 19] at 37 °C for 1 h and then washed three times with PBS. The cells were subsequently infected with PPV [100 tissue culture infective dose (TCID)50] at 37 °C for 72 h, and MTT assays were performed as described above. Cell survival rates were similarly calculated from mean values.

Treatment of PPV-Infected Cells

To determine the effect of DG on PPV-infected cells, ST cells were infected with PPV (100TCID50) for 1 h at 37 °C, treated with 250 µg/ml DG at 37 °C for 72 h, and then washed three times with PBS. Cell survival rates were calculated using the MTT assays as described above.

Treatment of Virus with Drug

To investigate direct effects of DG on the virus, PPV (100TCID50) was incubated with 250 µg/ml of DG at 37 °C for 1 h. The DG-treated PPV was subsequently used to infect ST cells as previously described [11, 18]. Briefly, confluent ST cells grown on 96-well plates were washed three times with PBS followed by infection with the DG-treated PPV at 37 °C for 72 h. The MTT assays were performed as described above to calculate cell survival rates.

Immunofluorescence and Real-Time PCR

Indirect immunofluorescence assays (IFA) and real-time PCR were performed to confirm and quantify observed inhibitory effects of DG. For IFA, ST cells at 100 % confluency were incubated with drug-treated PPV (100TCID50) at 37 °C for 48 h. In parallel, 31.25 µg/ml of DOC was used to treat PPV as control. After washing with PBS, the cells were fixed with 4 % paraformaldehyde in PBS followed by quenching with 0.1 % glycine in PBS and 1 % Triton-X100 for 10 min. After washing, the cells were incubated with rabbit anti-PPV antibody (1:200) for 1 h followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (1:500) (Zhongshan, China) for 50 min in the dark. All antibodies were diluted in 0.1 % bovine serum albumin.

The effect of DG on PPV infection was quantified by real-time PCR targeting the NS1 gene of PPV. The virus was treated with increasing concentrations of DG at 37 °C for 1 h; DOC-treated PPV was used as control. After washing with PBS, ST cells (100 % confluency in 6-well plates) were incubated with DG-treated PPV (100TCID50) at 37 °C for 48 h. The virus-containing culture was frozen and thawed three times followed by incubation with an equal volume of 20 % polyethylene glycol 8000 (PEG) for 30 min at room temperature. The samples were centrifuged at 15,000 g for 5 min, and the pellets were suspended in DNase-free water.

Total DNA was extracted with a commercial kit (Fastgene, China) according to the manufacturer's instructions. Subsequent PCR was performed using an ABI PRISM 7500 real-time PCR machine (Applied Biosystems, USA). The PCR mixture included 1 µl of DNA template (1 µg), 10 µl of SYBR Taq polymerase, 0.4 µl of ROX reference dye II, 0.4 µl (10 pmol) of sense primer 5′CA ACTGGCTTCAAGATAATGCTC, 0.4 µl (10 pmol) of antisense primer 5′TCTTTGTAGGTCTATCATTTC, and

7.8 µl of sterile water. The PCR parameters were as follows: 95 °C for 10 s; 40 cycles of 95 °C for 5 s; and 60 °C for 34 s. Data analysis was based on the measurement of the cycle threshold (*Ct*) values where expression levels of PPV NS1 were first normalized to those of β-actin. Isolated total DNA from untreated samples was used as a reference. For each treated sample, the ΔCt ($Ct_{\text{sample}} - Ct_{\beta\text{-actin}}$) was used to measure relative amplification using the $2^{-\Delta\Delta Ct}$ method [11, 12, 16, 18].

Statistical Analysis

All experiments were performed in triplicate. Statistical significance was evaluated using the *t* test by SPSS software. Values where $P < 0.01$ ('*') were considered highly significant.

Results and Discussion

The antiviral activities of glycyrrhizin have been well documented [1, 2, 4, 6, 8, 13, 23, 24]; however, they vary among the different viruses. As example, dissimilar effects on viral replication were observed on cultured HIV isolates depending on whether or not the isolate was a syncytium-inducing variant. This difference was linked to the ability of glycyrrhizin to induce certain ß-chemokines in the cultured cells used to propagate the virus [20]. Glycyrrhizin inhibited early stage replication of HAV and the penetration of HAV into the plasma membrane in PLC/PRF/5 cells, an Alexander hepatoma cell line, but exhibited no virucidal or inhibitory effects on the adsorption of HAV [4]. In a guinea pigs model, one group found that intravenously administered glycyrrhizin bound to hepatocytes and modified the expression of HBV-related antigens on the hepatocytes and suppressed sialylation of HBsAg. This was proposed as one mechanism by which intravenously injected glycyrrhizin improves liver function resulting in occasional complete recovery from hepatitis [21].

In a previous report, both in vitro and in vivo assays indicated that DG had antiviral activity on cell infection by avian infectious bronchitis virus (IBV) and decreased apoptosis of infected cells [11]. Also, pharmacological activities of DG on pseudorabies virus (PrV) infection were evaluated using virus plaque-reduction assays, PCR, and RT-PCR [23]. All data points demonstrated inhibition of PrV infection and PrV-induced cell apoptosis; however, these results were observed only if the PrV was pre-treated with DG, indicating that cellular factors for PrV might be involved.

As noted above, the activities of DG on virus infection and replication are various; however, currently there are no reports on the effects of DG on PPV infection. Our studies showed that the maximum, non-toxic concentration of DG on ST cell viability and proliferation was experimentally determined to be 250 µg/ml as indicated in MTT assay and well below that observed in previous studies [9, 19]. At this concentration, there were no differences between DG-treated cells and mock-treated cells relative to cell morphology (data now shown). DOC was used at a non-toxic concentration of 31.25 µg/ml which was also determined experimentally.

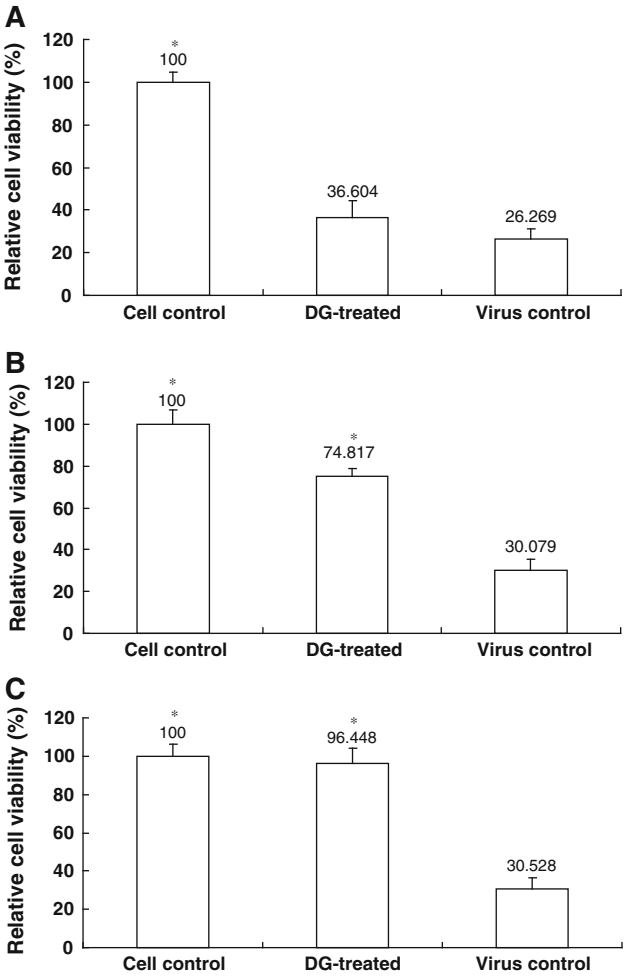

Fig. 1 Effect of DG on viral infection and cell viability. Experiments were conducted to assess the effects of changing the order of DG treatment on virus infectivity and, therefore, cell viability. Each of the 3 separate experiments (**a**, **b**, and **c**) consisted of non-DG-treated cells (cell control), cells or virus treated with DG (DG-treated), and non-DG-treated virus (virus control) where the order of DG treatment was varied. All DG treatments were performed with 250 µg/ml DG and porcine parvovirus (PPV) [100 TCID50]. Cell viability was measured using MTT assays. Data were graphed as relative cell viabilities [$(OD_{570}$ drug)/$(OD_{570}$ virus control)] for each treatment group. *t* tests were performed by SPSS software; *asterisk* means $P < 0.01$ relative to non-drug-treated virus control. All experiments were performed in triplicate. **a** ST cells treated with DG prior to incubation with PPV; **b** ST cells treated with DG following incubation with PPV; **c** ST cells infected with PPV pre-treated with DG

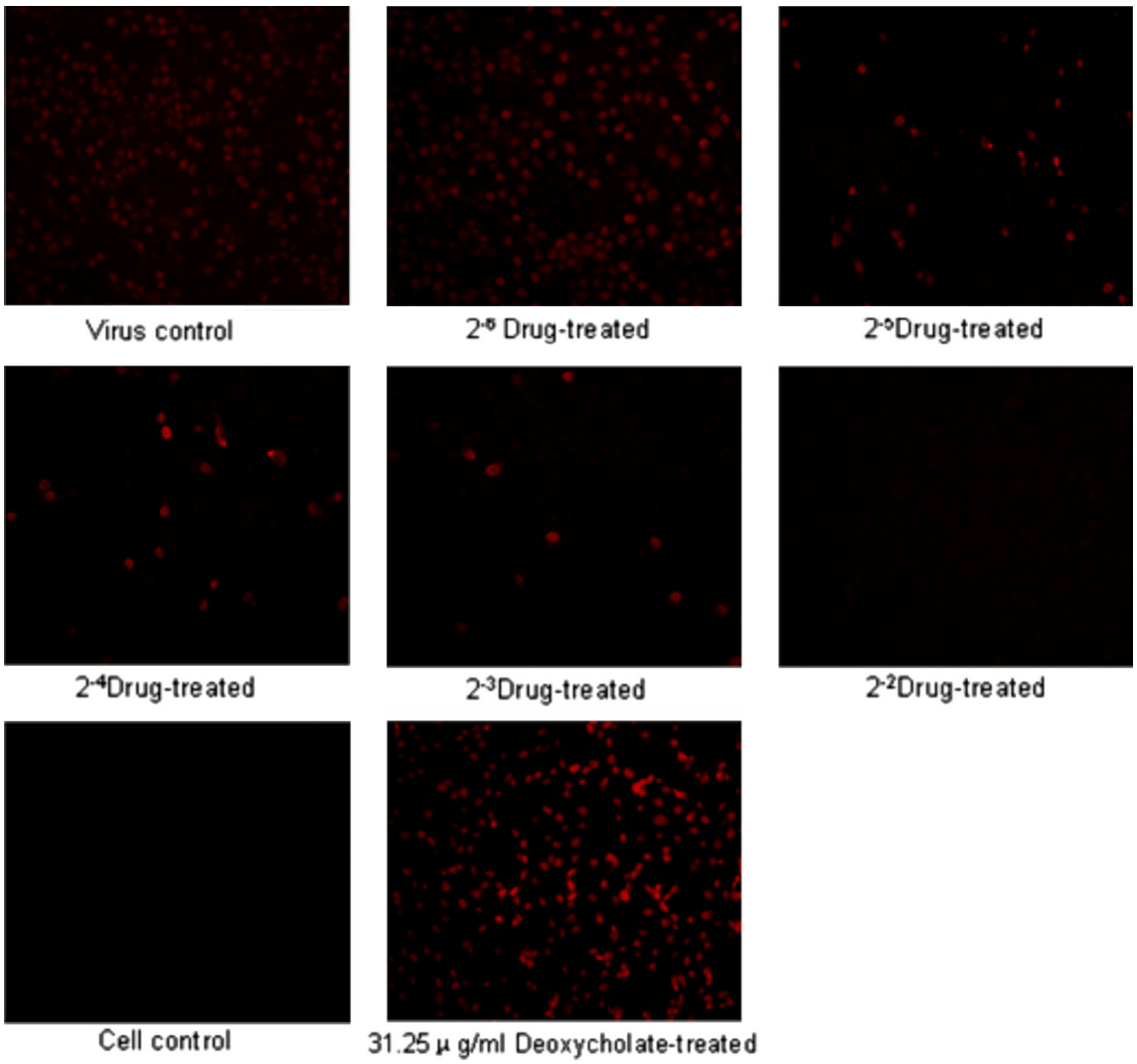

Fig. 2 Inhibitory effects of DG on PPV by immunofluorescence assays (IFA). Porcine parvovirus (PPV) [100 (TCID)50] was pre-treated with serially diluted DG starting at a concentration of 250 μg/ml at 37 °C for 1 h followed by incubation with ST cells and IFA analysis. The 31.25 μg/ml of DOC-treated PPV was used as control. Fluorescence intensity (40×) is proportional to viral infectivity and inversely related to DG concentration

When cells were treated with DG prior to PPV infection, minimal anti-viral effects were observed; the cell survival rate was low at 36.6 % and slightly better than virus controls (26.3 %) but not significantly ($P > 0.05$) (Fig. 1a). Significant differences were, however, observed between the cell and virus control groups ($P < 0.01$). The effect of DG on PPV-infected cells indicated that DG also significantly reduced the infectivity of PPV where the cell survival rate was 74.8 % (Fig. 1b). When PPV pre-treated with DG was used to infect ST cells, a significant decrease in infectivity was observed where the cell survival rate was 96.4 % ($P < 0.01$) relative to controls (Fig. 1c). The results of this treatment regimen were confirmed by IFA. As shown in Fig. 2, PPV-infected ST cells generated strong fluorescence signals by 48 h post-infection. In contrast, the fluorescence from ST cells infected with DG-treated PPV decreased in a dose-dependent manner. Although the inhibitory effects of DG on PPV-infected cells were lower than those observed on the DG-pretreated virus, high cell survival rates are still important because under most circumstances the antiviral therapy will be used on animals which have been previously diagnosed as positive. Given the difference between the cells pretreated with DG and washed, versus the cells treated with DG post-infection with virus, it is possible that the DG internalized by the cells is functioning intracellularly to bind to and inactivate viral particles within the cells. Indeed, current research is being targeted at finding ways to bind DG to nanoparticles to help better internalize the drug and target specific cell types [17, 25] As such, the presence of DG may have a meaningful effect on the virus even after it has entered the cell.

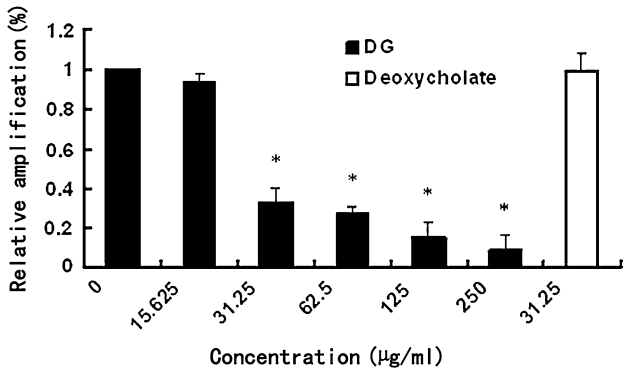

Fig. 3 Quantification of DG on PPV by real-time PCR. PPV [100 (TCID)50] was pre-treated with different concentrations of DG as indicated followed by incubation with ST cells and real-time PCR analysis. The 31.25 μg/ml of DOC-treated PPV was used as a control. *t* tests were performed using SPSS software. *Asterisk* means $P < 0.01$ relative to the 0 μg/ml DG group. The experiment was performed in triplicate

Our results clearly showed that DG deactivated PPV. The use of DOC excluded the possibility that the detergent characteristics of DG were the cause of non-specific, antiviral activity. Indeed, it has been reported recently that the oral bioavailability of glycyrrhizin could be improved by formulation of this drug in association with sodium DOC/phospholipid-mixed nanomicelles (SDC/PL-MMs) [9]. To further support the specific nature of the activity, when the PPV group was treated with DOC the fluorescence signal was not diminished; this observation was quantified by RT-PCR. The results showed that relative amplification of the PPV-derived NS1 gene decreased in a dose-dependent manner to a maximum of 92.3 % in the presence of 250 μg/ml DG and this coincided with a decrease in PPV infectivity (Fig. 3). Real-time PCR was performed at 24, 36, and 48 h post-PPV infection, and the results showed at all time points that DG treatment inhibited cell infection (data not shown) with maximum inhibition occurring at 48 h.

As noted, pre-treatment of ST cells with DG did not result in significant increases in cell survival; however, treatment of PPV prior to incubation with ST cells positively influenced cell survival. This suggests that the activity of DG is more direct than indirect where contact with the virus is essential to prevent infectivity. This contrasts with hypotheses suggesting that DG interaction with cell-related factors such as viral receptors is critical for preventing infection. This also suggests that maintaining sufficiently high levels of DG will be necessary to prevent any progeny virus from infecting new cells and propagating the infection after a short latent period. This is important and beneficial as a therapeutic drug for PPV. This can be seen in Fig 1b where DG was used to directly treat virus-infected cells for an extended period of time (72 h) during which multiple rounds of virus replication would have occurred. Based on these observations, it becomes important to identify the mechanism of binding of DG to PPV as well as other viruses in order to advance development of newer, more effective therapies against viruses known to be affected by DG treatment. In conclusion, DG has negligible adverse effects on ST cells. Further, this study reveals that DG exhibits direct inhibition on PPV in vitro and may be considered an effective anti-PPV agent.

Acknowledgments This work was supported by the Program for New Century Excellent Talents at the Heilongjiang Provincial University (1155–NCET–005), National Natural Science Foundation of China (31200122; 31270187), the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (SKLVEB2012KFKT007), Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2013BAD12B04), and Research Fund for the Doctoral Program of Higher Education of China (Ph.D. Student Supervisor Type) of Ministry of Education of P.R. China, No. 20122325110019, and sponsored by Chang Jiang Scholar Candidates Program for Provincial Universities in Heilongjiang (20123CJHB002).

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Arase Y, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, Suzuki Y, Saitoh S, Kobayashi M, Kumada H (1997) The long term efficacy of glycyrrhizin in chronic hepatitis C patients. Cancer 79:1494–1500
2. Baba M, Shigeta S (1987) Antiviral activity of glycyrrhizin against varicella-zoster virus in vitro. Antiviral Res 7:99–107
3. Cartwright SF, Lucas M, Huck RA (1969) A small haemagglutinating porcine DNA virus: I Isolation and properties. J Comp Pathol 79:371–377
4. Crance JM, Leveque F, Biziagos E, van Cuyck-Gandre H, Jouan A, Deloince R (1994) Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication in vitro. Antiviral Res 23:63–76
5. Cui J, Wang X, Ren Y, Cui S, Li G, Ren X (2012) Genome sequence of Chinese porcine parvovirus strain PPV2010. J Virol 86:2379
6. Hoever G, Baltina L, Michaelis M, Kondratenko R, Baltina L, Tolstikov GA, Doerr HW, Cinatl J Jr (2005) Antiviral activity of glycyrrhizic acid derivatives against SARS-coronavirus. J Med Chem 48:1256–1259
7. Hou SZ, Li Y, Zhu XL, Wang ZY, Wang X, Xu Y (2012) Ameliorative effects of diammonium glycyrrhizinate on inflammation in focal cerebral ischemic-reperfusion injury. Brain Res 1447:20–27
8. Ito M, Nakashima H, Baba M, Pauwels R, De Clercq E, Shigeta S, Yamamoto N (1987) Inhibitory effect of glycyrrhizin on the in vitro infectivity and cytopathic activity of the human immunodeficiency virus [HIV(HTLV-III/LAV)]. Antiviral Res 7:127–137
9. Jin S, Fu S, Han J, Jin S, Lv Q, Lu Y, Qi J, Wu W, Yuan H (2012) Improvement of oral bioavailability of glycyrrhizin by sodium deoxycholate/phospholipid-mixed nanomicelles. J Drug Target 20(7):615–622

10. Jozwik A, Manteufel J, Selbitz HJ, Truyen U (2009) Vaccination against porcine parvovirus protects against disease but does not prevent infection and virus shedding after challenge infection with a heterologous virus strain. J Gen Virol 90:2437–2441
11. Li J, Yin J, Sui X, Li G, Ren X (2009) Comparative analysis of the effect of glycyrrhizin diammonium and lithium chloride on infectious bronchitis virus infection in vitro. Avian Pathol 38:215–221
12. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(t)) method. Methods 25:402–408
13. Lin JC (2003) Mechanism of action of glycyrrhizic acid in inhibition of Epstein–Barr virus replication in vitro. Antiviral Res 59:41–47
14. Lutz W (2006) Serological investigation to demonstrate the presence of antibodies to the viruses causing porcine reproductive and respiratory syndrome, Aueszkys disease, hog cholera and porcine parvovirus among wild boar (Sus scrofa, L.1758) in Norh rhine-wesdelia. Zeischrif Fur Jagdwissensch Aft 42:123–133
15. Mahnel H, Mayr A, Bibrack B (1966) Multiplication of swine fever virus with cytopathogenic effect in piglet testis culture. Zentralbl Veterinarmed B 3:250–259
16. Mukhtar M, Arshad M, Ahmad M, Pomerantz RJ, Wigdahlnad B, Parveen Z (2008) Antiviral potentials of medicinal plants. Virus Res 131:111–120
17. Patel MP, Patel RR, Patel JK (2010) Chitosan mediated targeted drug delivery system: a review. J Pharm Pharm Sci 13:536–557
18. Ren X, Meng F, Yin J, Li G, Li X, Wang C, Herrler G (2011) Action mechanisms of lithium chloride on cell infection by transmissible gastroenteritis coronavirus. PLoS One 6:e18669
19. Ren X, Tao Y, Cui J, Suo S, Cong Y, Tijssen P (2013) Phylogeny and evolution of porcine parvovirus. Virus Res 178:392–397
20. Sasaki H, Takei M, Kobayashi M, Pollard RB, Suzuki F (2002) Effect of glycyrrhizin, an active component of licorice roots, on HIV replication in cultures of peripheral blood mononuclear cells from HIV-seropositive patients. Pathobiology 70:229–236
21. Sato H, Goto W, Yamamura J, Kurokawa M, Kageyama S, Takahara T, Watanabe A, Shiraki K (1996) Therapeutic basis of glycyrrhizin on chronic hepatitis B. Antiviral Res 30:171–177
22. Shangjin C, Cortey M, Segales J (2009) Phylogeny and evolution of the NS1 and VP1/VP2 gene sequences from porcine parvovirus. Virus Res 140:209–215
23. Sui X, Yin J, Ren X (2010) Antiviral effect of diammonium glycyrrhizinate and lithium chloride on cell infection by pseudorabies herpesvirus. Antiviral Res 85:346–353
24. Utsunomiya T, Kobayashi M, Pollard RB, Suzuki F (1997) Glycyrrhizin, an active component of licorice roots, reduces morbidity and mortality of mice infected with lethal doses of influenza virus. Antimicrob Agents Chemother 41:551–556
25. Yang KW, Li XR, Yang ZL, Li PZ, Wang F, Liu Y (2009) Novel polyion complex micelles for liver-targeted delivery of diammonium glycyrrhizinate: in vitro and in vivo characterization. J Biomed Mater Res A 88:140–148
26. Zeeuw EJL, Leinecker N, Herwig V, Selbitz HJ, Truyen U (2007) Study of the virulence and cross-neutralization capability of recent porcine parvovirus field isolates and vaccine viruses in experimentally infected pregnant gilts. J Gen Virol 88:420–427