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Antiviral effect of diammonium glycyrrhizinate and lithium chloride on cell infection by pseudorabies herpesvirus

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ABSTRACT

Diammonium glycyrrhizin (DG), a salt from glycyrrhizinate (GL) that is a major active component of licorice root extract with various pharmacological activities was investigated for its inhibitory effect on pseudorabies virus (PrV) infection. In parallel, lithium chloride (LiCl), a chemical reagent with potential antiviral activity was compared with DG for their inhibitory ability against PrV infection in vitro. Virus plaque-reduction assays, PCR and RT-PCR analysis indicated that both drugs inhibited cell infection by PrV. Moreover, addition of the drugs resulted in fewer apoptotic cells during PrV infection.

1. Introduction

Glycyrrhizin (GL) is the most important bioactive compound of licorice root (*Glycyrrhiza radix*), and it has been used as a traditional Chinese medicinal herb for treating hepatitis due to its anti-inflammatory property (Yuan et al., 2006). GL is active against herpes simplex type 1 (HSV-1), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), human cytomegalovirus, hepatitis A, B, and C viruses, influenza virus, human immunodeficiency virus (HIV), and SARS coronavirus (Arase et al., 1997; Baba and Shigeta, 1987; Crance et al., 1994; Hoever et al., 2005; Ito et al., 1987, 1988; Lin, 2003; Numazaki et al., 1994; Pompei et al., 1979; Sato et al., 1996; Utsunomiya et al., 1997). Diammonium glycyrrhizinate (DG) is derived from the glycyrrhizin modified in the two carboxyl groups at the 6′ and 6″ position (Fig. 1). DG is more stable, soluble and has more significant bioactivities than GL. Lithium salts have been used as therapeutic substances for the treatment of 'gout and rheumatic gout', 'Bright's disease', epilepsy, syphilis, acute mania and depressive episodes (Skinner et al., 1980). The effect of lithium salts on the replication of herpes simplex virus and vaccinia virus was documented (Skinner et al., 1980; Ziaie and Kefalides, 1989). Recently, it was reported that lithium chloride inhibits infectious bronchitis virus, an RNA coronavirus in cell culture and such inhibition was due to a cellular rather than virucidal effect (Harrison et al., 2007).

Pseudorabies virus (PrV) is a swine neurotropic herpesvirus that has common genome arrangements with those of HSV-1. PrV is a useful model organism for exploring herpesvirus biology. In swine and cattle, PrV infection can cause Aujeszky's disease (AD), which is one of the List B diseases of the Office international des épizooties (OIE). Although pig is the natural reservoir for the virus, PrV has a broad host range and can infect most mammals and some avian species (Klupp et al., 2004). In susceptible species, PrV infection is often lethal and infected animals may die from central nervous system disorders (Tirabassi and Enquist, 2000). PrV infection poses a severe threat to animal husbandry and the development of effective antiviral agents is one important strategy to decrease the occurrence of PrV infection in addition to vaccination.

In this study, we demonstrated the inhibitory effect of DG and LiCl on PrV infection. Moreover, the effect of the drugs on the cell apoptosis induced by PrV was investigated. The data is helpful for better understanding the antiviral mechanism of both compounds.

2. Materials and methods

2.1. Cell and virus

Vero cells (African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Shanghai ExCell Biology, Inc.) at 37 °C in an incubator containing 5% CO_2. PrV (strain Bartha K-61) was propagated in Vero cells.

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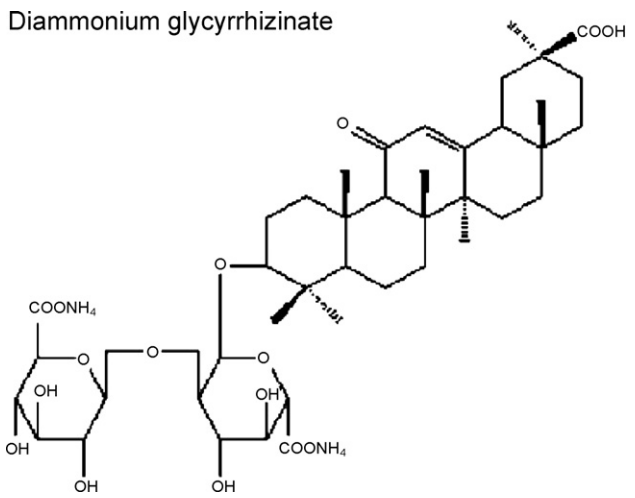

Fig. 1. Chemical structure of diammonium glycyrrhizinate (DG).

2.2. Cytotoxic assay

2.2.1. Cell morphology analysis

Vero cell monolayers in 96-well culture plates at a density of 10^4 cells/well were washed three times with phosphate-buffered saline (PBS), then DG (Zhengda Pharmacy, China) and LiCl (Sigma, St. Louis, MO) diluted serially in serum-free DMEM were inoculated into the wells (6 wells/each drug). Mock-treated cells were served as control. All the cells were cultured at 37 °C for 48 h, and the cell morphology was analyzed by light microscope.

2.2.2. MTT assay

Cell monolayers in 96-well plates were incubated with drugs as above. After washing with PBS, the cells were incubated with 80 μl/well DMEM culture and 20 μl/well 0.5% MTT solution at 37 °C for 4 h. After washing with PBS, 150 μl DMSO were added to each well. The tray was shaken gently for 10 min to dissolve the precipitate of formazan and the optical density (OD) value of the wells was determined using a plate reader at a test wavelength of 570 nm. Cell survival rate was calculated as drug average OD value/control average OD value and 50% above cell survival rate was regarded as a non-toxic concentration of the drug.

2.2.3. Trypan blue staining

Trypan blue staining was used to confirm the maximum non-toxic concentration of drug. Briefly, 1 ml of the Trypan Blue Mix (0.5% (w/v) in PBS) was added into each dish of cells, and incubated for 5 min at room temperature. The percentage of viable cells was determined by dividing the number of unstained cells by the total number of cells and multiplying by 100. The equation is as follows: (number of unstained cells/total number of cells) × 100 = percent viable cells.

2.3. Virus titration and infection assay

Virus titration or subsequent infectivity analysis was performed by plaque-reduction assays (Burleson et al., 1992). Briefly, Cell monolayers in 24-well plates were inoculated with serially diluted viruses. The inoculums were replaced with 1% methylcellulose in DMEM, after 1 h absorption of the virus. The cell monolayers were incubated at 37 °C for 48 h and the overlay medium was removed. Then the cells were gently washed three times with PBS and fixed with 3% formalin in PBS for 30 min at room temperature. Then, 1% crystal violet (v/v) diluted in 5% EtOH in PBS was used to stain the cells for 20 min at room temperature. The clear plaque number was visualized post-washing and the virus titer in plaque-forming units (pfu) was calculated. The experiment was done in triplicate.

2.4. Treatment of virus with drugs

To analyze the effect of both drugs on virus *per se*, 1×10^4 pfu/ml viruses were treated with serially diluted drugs and incubated at 37 °C for 1 h. Then, Vero cells were infected by the viruses and subjected to virus plaque assays as above.

2.5. Treatment of infected cells

To analyze the inhibition of drugs to PrV-infected cells, the cells in 24-well plates were infected with the 1×10^4 pfu/ml viruses at 37 °C for 1 h. After washing three times with PBS, the cells were treated with serially diluted drugs at 37 °C for 1 h post-infection (hpi). The infectivity of the viruses was analyzed as above.

2.6. Treatment of cells prior to virus infection

To analyze the sensitivity of the cells to the drugs, the cells in 24-well plates were treated with serially diluted drugs at 37 °C for 1 h. The cells were infected with 1×10^4 pfu/ml viruses, after washing with PBS. Then, the cells were subjected to plaque assays.

2.7. Determination of the viral genome load by PCR amplifying PrV gD gene

To confirm the effect of the drugs on PrV infection *in vitro*, polymerase chain reaction, PCR was used to assess the viral load using PrV gD-specific primers. Both cells infected with drug-treated viruses and virus-infected cells treated with increasing concentrations of LiCl were subjected to DNA extraction with a commercial kit (Qiagen, Germany). According to the published sequence of PrV gD gene (GenBank No. AJ271966), the sense primer 5′-CCCGAATTCATGCTGCTCGCAGCGC-3′ and anti-sense primer 5′-GGGCTCGAGACGGACCGGGCTGCGC-3′ were used to amplify the PrV gD gene (about 1200 bp in length). The PCR system included 2 μl DNA template, 12.5 μl 2× GC PCR Buffer, 0.5 μl each primer, 4 μl dNTP, 0.25 μl rTaq polymerase, 5.25 μl sterile H_2O.

The PCR was performed at 94 °C for 1 min and 30 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 2 min. A final extension at 72 °C for 10 min. The PCR product was analyzed in 1% agarose gel electrophoresis. At the same time, GAPDH (573 bp in length) used as an internal standard was amplified with the sense primer 5′-ATCACCATCTTCCAGGAGCGAGA-3′ and the anti-sense primer, 5′-GCTTCACCACCTTCTTGATGTCA-3′. The temperature profile for PCR amplification of the gene was 50 °C for 30 min, 94 °C for 2 min and 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, followed by a final extension of 6 min at 72 °C.

2.8. Analysis of PrV gD gene amplification by RT-PCR

Cells infected with drug-treated viruses or PrV-infected cells treated with increasing concentrations of LiCl were subjected to RNA extraction with a commercial kit (Qiagen, Germany). Reverse transcription (RT)-PCR was performed using a RT-PCR kit (TaKaRa, Japan). The anti-sense primers for amplifying either gD or GAPDH gene were used for RT, and the subsequent PCR procedures were performed as above.

2.9. Cell apoptosis determined with fluorescence analysis

Cell apoptosis was analyzed with Annexin V-FITC kit (Nanjing Keygen Biotech. Co., Ltd.). Briefly, FITC-conjugated Annexin V

(50 μl/well) and propidium iodide, PI (50 μl/well) were added the cells infected by drug-treated viruses, or the virus-infected cells treated with LiCl in 24-well plates and incubated at room temperature for 15 min in the dark prior to fluorescence observation. The detection of cell early apoptosis was based on the observation that soon after initiating apoptosis, cells translocate the membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Thereafter, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. Cells stained by PI were detected as cellular late apoptosis. The results were analyzed by fluorescence microscope (Leica, Germany) at 12 hpi.

2.10. Flow cytometry analysis of cell apoptosis

In parallel, flow cytometry technique was used to further analyze cell apoptosis. Above-mentioned cell samples were trypsinized and then centrifuged at 2000 rpm for 5 min. The cells were re-suspended with 500 μl binding buffer at a concentration of 10^6 cells/ml, after washing two times with PBS at 2000 rpm for 5 min. Then, 5 μl FITC-conjugated Annexin V and 5 μl PI were added to the cells and incubated at room temperature for 15 min in the dark. The samples were analyzed within 1 h post-staining.

3. Results

3.1. Maximum non-toxic concentrations of drugs

The effects of DG and LiCl on Vero cell viability and proliferation were determined as above and the maximum non-toxic concentrations of DG and LiCl diluted in DMEM medium were 1250 μg/ml and 50 mM, respectively. Under above-mentioned drug concentrations, there were no difference between the drug-treated cells and mock-treated cells in terms of cell morphology. The cell proliferation was not significantly affected under the maximum non-toxic concentrations of both drugs as indicated in MTT assay (Fig. 2). Trypan blue staining showed that the cells were completely viable under above-mentioned concentrations of both drugs (Fig. 3).

3.2. Direct effect of drugs on the virus

The viruses were used to infect the cells, after they were treated with the drugs. The plaque-reduction assays showed that both drugs inhibited the virus infection in a dose-dependent manner. At the maximum non-toxic concentration of the drugs, the inhibition rate of LiCl and DG for PrV infection in cell culture reached 100% (Fig. 4).

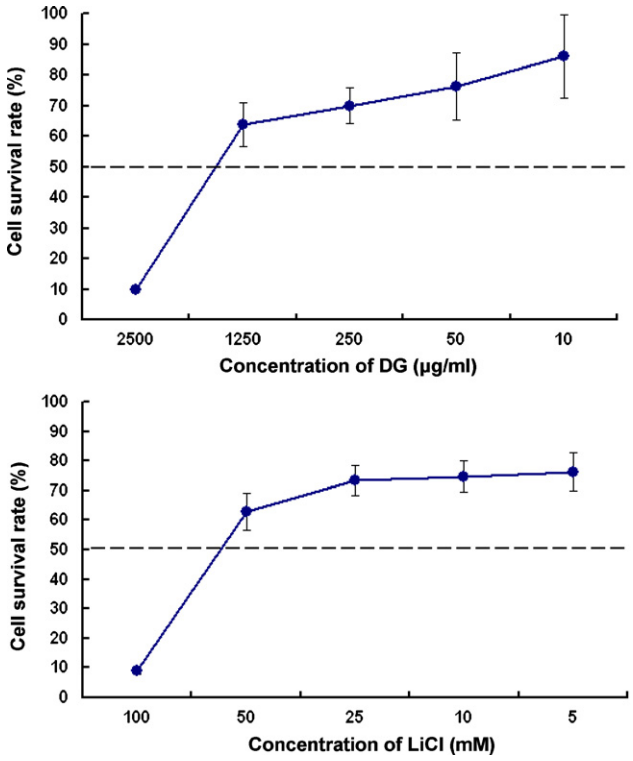

Fig. 2. MTT assay. The cell proliferation was analyzed by MTT colorimetric assays. The cell survival rates under different concentrations of drugs are given and 50% above cell survival rate (over broken line) is regarded as non-toxic concentration of drugs.

3.3. Effect of the drugs on virus-infected cells

The effect of both drugs on PrV-infected cells was analyzed using plaque-reduction assays. No reduction of the virus infectivity was observed at any of the different samples in the DG treatment group (data not shown). When the effect of LiCl was analyzed, the formation of virus plaques was inhibited in a dose-dependent manner (Fig. 5).

3.4. Both drugs showed no inhibitory effect after pre-treatment of cells

After the cell monolayers were treated with LiCl or DG, the viruses were applied to the cells and the plaque-reduction assays

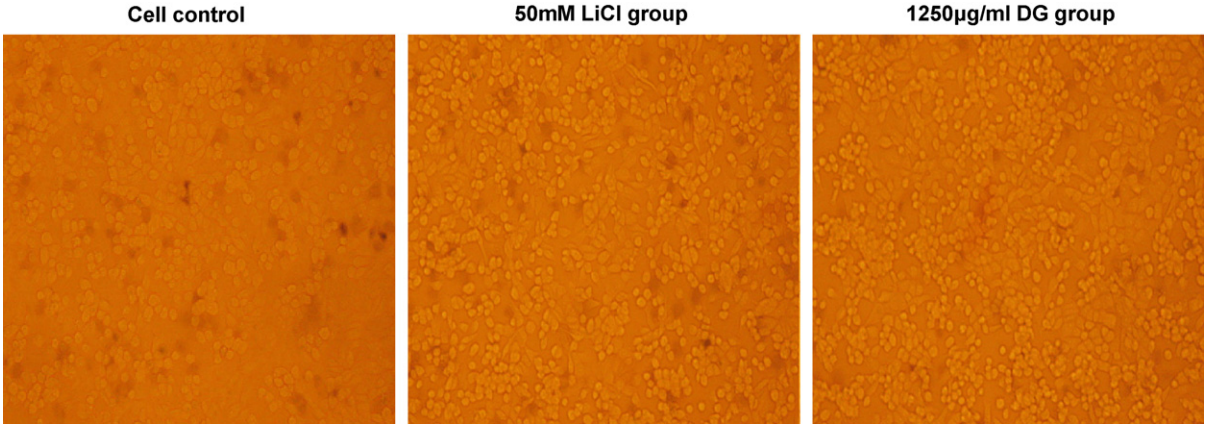

Fig. 3. Trypan blue staining. The mock-treated cells and cells treated with maximum non-toxic concentrations of drugs were subjected to trypan blue staining and a representative comparison is provided.

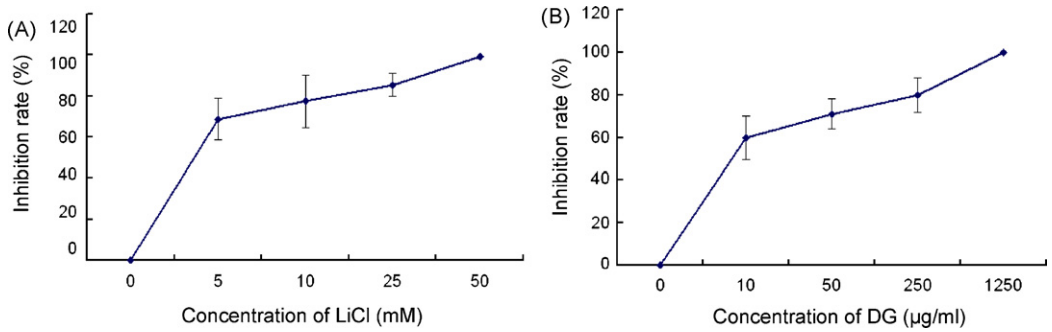

Fig. 4. Direct effect of the drugs on viruses. Increased concentrations of DG and LiCl were incubated with PrV for 1 h prior to cell infection. The cell infection was analyzed by virus plaque-reduction assays. The plaque inhibition rates of LiCl and DG to the virus infectivity are shown in Panels A and B, respectively.

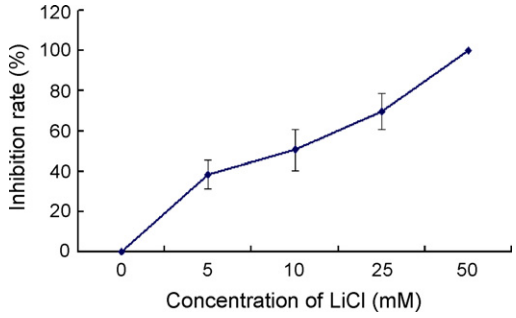

Fig. 5. Inhibitory effect of LiCl to virus-infected cells. Various concentrations of LiCl were applied to the PrV-infected cells at 1 hpi. The cell infection was analyzed by virus plaque-reduction assays and the plaque inhibition rate is shown.

done 48 h later showed that no inhibitory effect was observed for either of both drugs (data not shown).

3.5. PCR and RT-PCR analysis

The viral genome load assessed by PCR analysis of the gD gene was inhibited partially at 25 mM LiCl or 50 μg/ml DG and was inhibited completely at 50 mM LiCl or 1250 μg/ml DG. The gD gene amplification was also inhibited in PrV-infected cells treated by LiCl. There was no positive PCR product in the 50 mM LiCl treatment group. The amplification of housekeeping gene GAPDH was positive in all PCR analyses (Fig. 6). To confirm the effect of both compounds on gD gene, RT-PCR was performed to analyze the amount of amplified DNA from the gD gene. As shown in Fig. 7, the RT-PCR analysis gave rise to a similar result with that of PCR analysis.

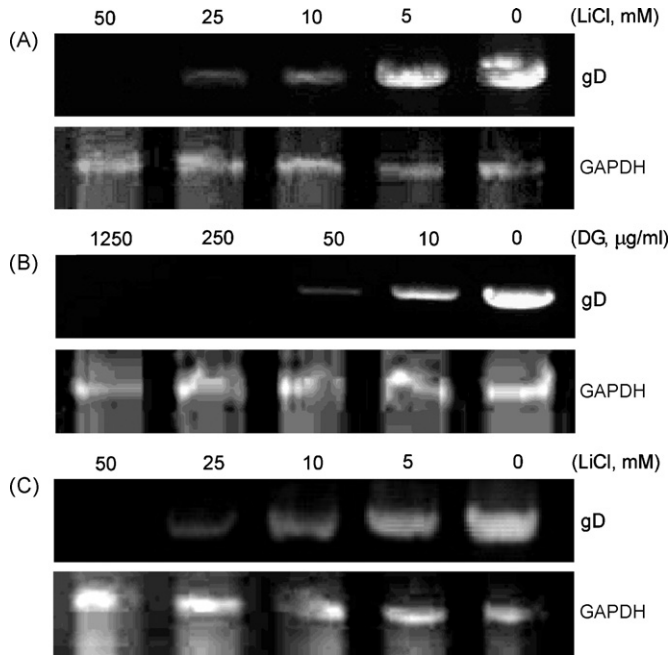

Fig. 6. PCR targeting the amplification of PrV gD gene. The cells infected by drug-treated viruses were subjected to PCR using PrV gD-specific primers. The effect of LiCl and DG on the gene amplification is shown in Panels A and B, respectively. PrV-infected cells were treated with LiCl and then subjected to PCR for detecting the gD gene. The PCR result is shown in Panel C. Housekeeping gene GAPDH is served as an internal reference.

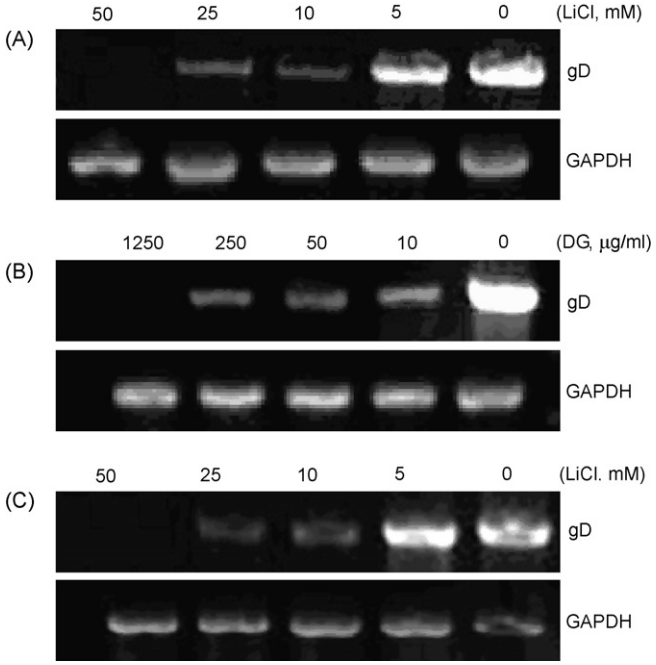

Fig. 7. RT-PCR analysis for the PrV gD gene. The cells infected by drug-treated viruses were subjected to RT-PCR. The effect of LiCl and DG on the amount of amplified DNA from PrV gD gene is shown in Panels A and B, respectively. PrV-infected cells were treated with LiCl and then subjected to RT-PCR for detecting the gD gene. The RT-PCR results are shown in Panel C. Housekeeping gene GAPDH is served as an internal reference.

3.6. Effective drugs decreased the number of apoptotic cells during PrV infection

The cell apoptosis analyzed with Annexin V-FITC and PI dual staining kit showed that PrV-infected cells displayed both early and late apoptosis. The late apoptosis rate of cells infected by drug-treated viruses was decreased in a drug dose-dependent manner. The late apoptosis of virus-infected cells treated with LiCl was inhibited significantly. In contrast, DG did not prevent virus-infected cells from apoptosis. These results were agreement with virus infection analysis. However, as early as 12 hpi, the early apoptosis of the cells was affected lightly. A representative staining picture regarding the effect of the drugs at maximum non-toxic concentration on the cell apoptosis is shown in Fig. 8A. The inhibition rate of late apoptosis is shown in Fig. 8B and C.

To confirm the cell apoptosis induced by PrV, flow cytometry technique was utilized. As shown in Fig. 9, the maximum non-toxic concentration of drugs had an inhibitory activity against PrV-induced cell apoptosis.

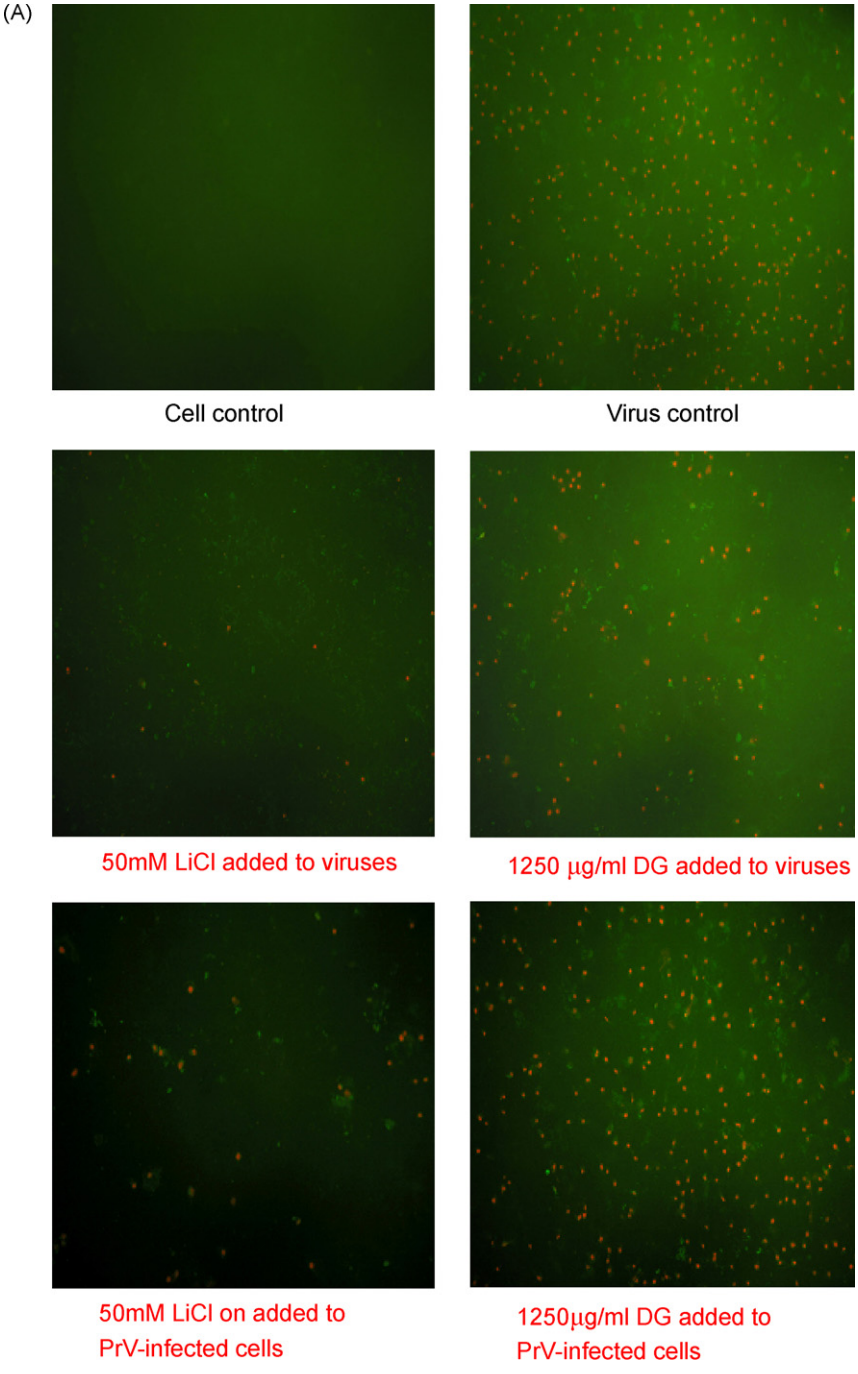

Fig. 8. Effect of the drugs on cell apoptosis analyzed by fluorescence staining. The cell apoptosis was analyzed after the cells were infected by drug-treated viruses or virus-infected cells were treated with LiCl. The red fluorescence signals are designated as the index of late apoptosis and green fluorescence signals are designated as the index of early apoptosis. A representative staining picture regarding the effect of the drugs with maximum non-toxic concentrations on the cell apoptosis is shown in Panel A. The corresponding apoptosis inhibition rates by LiCl are indicated Panel B (1: the apoptosis inhibition rate cells infected by LiCl-treated viruses; 2: the apoptosis inhibition rate of virus-infected cells treated by LiCl). The apoptosis inhibition rate of cells infected by DG-treated viruses is shown in Panel C.

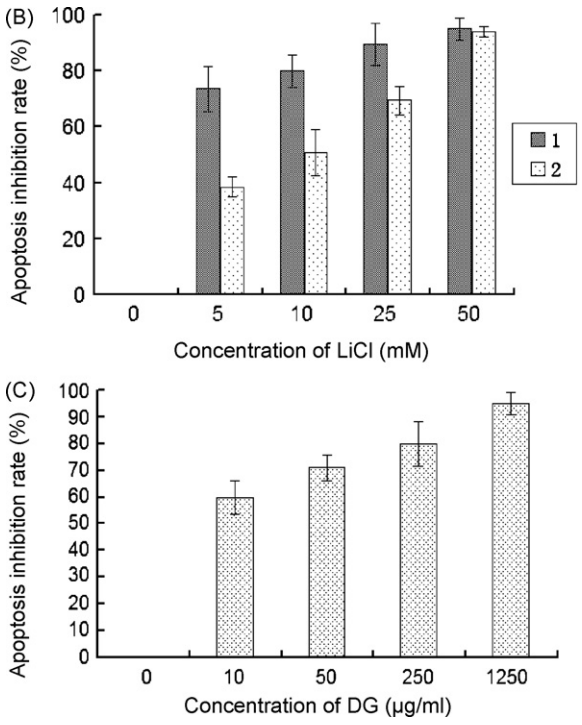

Fig. 8. (*Continued*).

4. Discussion

Many antiviral agents have been tested for their effect on human herpes simplex viruses, the counterparts of PrV in the subfamily *Alphaherpesvirinae* in *Herpesviridae* family (De Clercq, 2004; Naesens and De Clercq, 2001; Sergerie and Boivin, 2008; Cheng et al., 2008). Due to the potential threat of PrV infection to the animal husbandry, it is necessary to develop effective antiviral agents against PrV infection. It was reported that LiCl was able to inhibit the replication of the DNA virus herpes simplex and interfered with the herpes simplex virion-associated inhibition of host protein synthesis (Skinner et al., 1980; Ziaie and Kefalides, 1989). In this study, we further investigated the effect of this reagent on PrV infection *in vitro* and its mode of action. The results showed that the PrV infectivity was decreased in a dose-dependent manner after the treatment of LiCl either to the virus-infected cells or to the virus *per se*. LiCl has been known for its inhibitory effect on the RNA coronavirus, infectious bronchitis virus (IBV) in cell culture, and such inhibition was due to a cellular, rather than virucidal, effect (Harrison et al., 2007). Therefore, LiCl may be a perspective and broad-spectrum antiviral.

Our apoptosis analysis indicated that PrV infection initiated cell apoptosis. Both drugs used in this study inhibited the virus-induced cell apoptosis, although some cells with early apoptosis were not affected by the drug treatment. We also investigated the cell apoptosis at 48 hpi, which gave the similar results with that at 12 hpi. It has been reported that point mutations in the ATP binding site of PrV US3 protein kinase prevented Bad phosphorylation and cell survival after apoptosis appearance and p38 MAPK and JNK/SAPK signaling were involved in PrV apoptosis (Deruelle et al., 2007; Yeh et al., 2008). The mechanisms regarding the effect of both drugs on apoptosis pathways are under investigation. Our flow cytometry analysis also suggested that LiCl had a more significant effect than DG in terms of their inhibitory activity against cell apoptosis. The microscopic scoring of fluorescent samples and the flow cytometric data indicated a similar reduction trend in cell apoptosis by the addition of the drugs, however, there was difference in the degree of reduction of apoptosis. This discrepancy might be attributed to the difference from the both methods *per se*, such as criterion, cell status, system error, etc. The PCR results showed that the amplified amount of PrV gD gene was decreased with the increasing drug concentration, manifesting that both drugs appear to reduce the viral genome load via an unresolved mechanism. The RT-PCR results suggested that the compounds interfered with the viral gD gene. It is possible that the observed anti-apoptotic effect of DG and LiCl during PRV infection is indirect and due to the reduced virus replication caused by the drugs. Our future work will further try to elucidate whether the inhibition of PrV infection by LiCl is attributed to its direct virucidal effect or to its inhibitory activity against the replication of PrV.

DG is a salt derived from GL, and it may have a similar antiviral activity with GL. In this study, we were especially interested in understanding its mode of action regarding its inhibitory activity against PrV infection *in vitro*. It was reported that GL was active against herpes viruses such as HSV-1, VZV, EBV (Lin, 2003). However, in above-mentioned experiment, EBV was incubated with 2.4 mM GL at 37 °C for 4 h, and then the EBV infectivity was analyzed. No significant reduction in viral infectivity was observed (Lin, 2003). The author assumed that the antiviral activity of GL was not attributed to a direct inactivation of the virus. In another report, it was found that 8 mM GL could inactivate herpes simplex virus particles irreversibly in 15 min (Pompei et al., 1979). Our data showed that the pre-treatment of the viruses with DG inhibited the cell infection by PrV in a dose-dependent manner. We suppose that the difference in the reported modes of action of the DG/GL may be attributed to either the different nature of individual virus in the herpes virus family or the discrepant concentrations of the drugs applied in each assay.

Compared with LiCl, DG did not inhibit the PrV infection after virus adsorption, and it seems that this drug is only virucidal to PrV. This finding would be interesting, because the effects of GL on cellular protein kinase C and P were reported (Abe et al., 2008; Ito

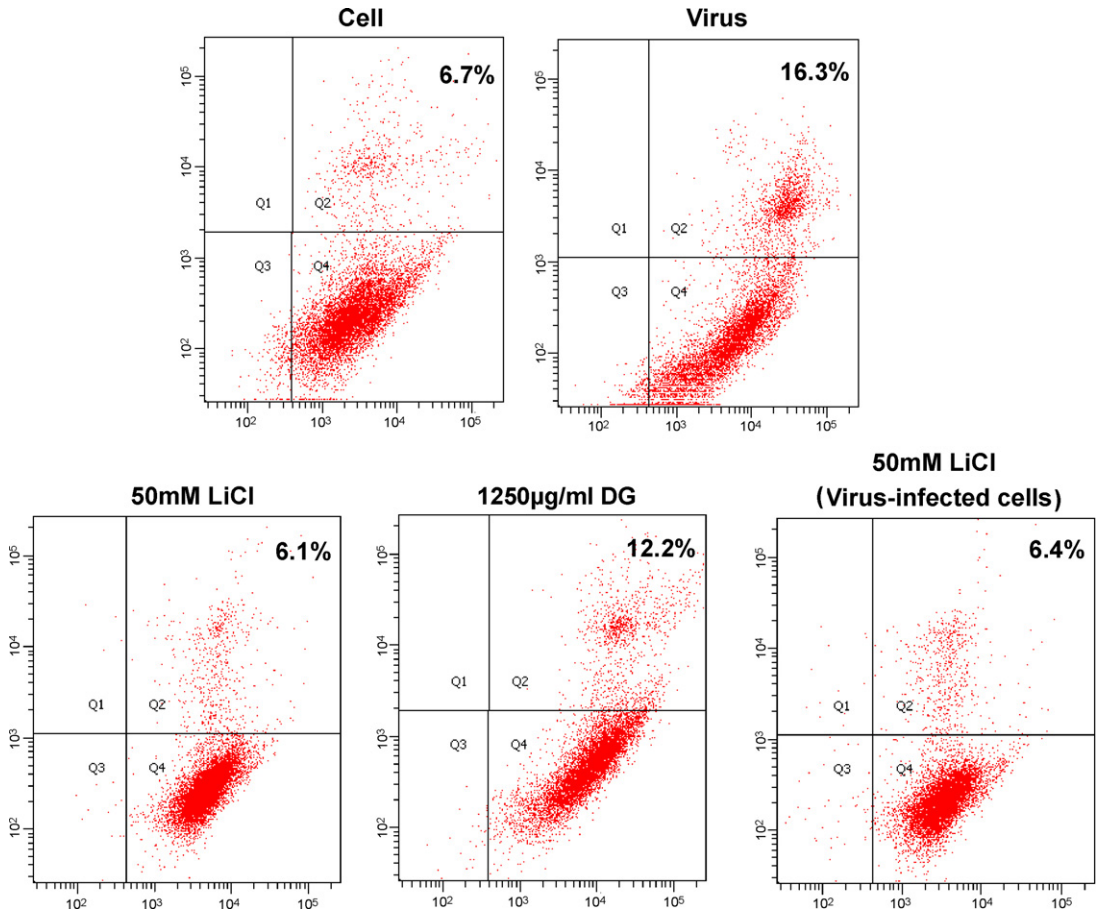

Fig. 9. Cell apoptosis analysis by flow cytometry. The cells were stained with Annexin V-FITC and PI, after either they were infected by maximum non-toxic concentration of drug-treated viruses or virus-infected cells were treated with LiCl at maximum non-toxic concentration. The cell apoptosis was analyzed by flow cytometry. Data are presented as dual-parameter FL1 of Annexin V versus FL2 of PI. The upper right areas are apoptotic cells. The apoptosis rates of cells are indicated.

et al., 1987; Ohtsuki and Iahida, 1988) and it was proposed that the anti-HIV effects of GL may be attributed to inhibitory effects on the cellular protein kinases. In our study, whilst DG has no observable effect on cell morphology or virus-infected cells, it has effects on cell signaling which could easily lead to changes in virus replication (Hsiang et al., 2002). Therefore, we will further investigate the effects of the DG.

Our data showed that pre-treatment of the cells with the both drugs did not result in a decrease of PrV infectivity, suggesting that cellular factors including cellular receptors for PrV might be not sensitive to the drugs. Like LiCl, DG showed a good inhibitory effect on cell apoptosis, after incubation of the drug with viruses. These data indicate that PrV infection induces cell apoptosis and addition of drugs also results in fewer apoptotic cells during PrV infection.

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