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In vitro antiviral efficacy of moroxydine hydrochloride and ribavirin against grass carp reovirus and giant salamander iridovirus

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ABSTRACT: Moroxydine hydrochloride (Mor) and ribavirin (Rib) have been reported to exhibit multi-antiviral activities against DNA and RNA viruses, but their antiviral activities and pharmacologies have seldom been studied in aquaculture. This paper has selected 3 aquatic viruses including a double-stranded RNA virus (grass carp reovirus, GCRV), a single-stranded RNA virus (spring viraemia of carp virus, SVCV) and a DNA virus (giant salamander iridovirus, GSIV) for antiviral testing. The results showed that Mor and Rib can effectively control the infection of GCRV and GSIV in respective host cells. Further study was undertaken to explore the antivirus efficiencies and pharmacological mechanisms of Mor and Rib on GCRV and GSIV *in vitro*. Briefly, compounds showed over 50% protective effects at 15.9 µg ml^{-1} except for the group of GSIV-infected epithelioma papulosum cyprinid (EPC) cells treated with Mor. Moreover, Mor and Rib blocked the virus-induced cytopathic effects and apoptosis in host cells to keep the normal cellular structure. The expression of VP1 (GCRV) and major capsid protein (MCP; GSIV) gene was also significantly inhibited in the virus-infected cells when treated with Mor and Rib. Cytotoxicity assay verified the 2 compounds had no toxic effects on grass carp ovary (GCO) cells and EPC cells at ≤96 µg ml^{-1}. In conclusion, these results indicated that exposing GCRV-infected GCO cells and GSIV-infected EPC cells to Mor and Rib could elicit significant antiviral responses, and the 2 compounds have been shown to be promising agents for viral control in the aquaculture industry.

KEY WORDS: Moroxydine hydrochloride · Ribavirin · GCO cell · EPC cell · GCRV · GSIV · Apoptotic response

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INTRODUCTION

Three different types of aquatic viruses — grass carp reovirus (GCRV), spring viremia of carp virus (SVCV) and giant salamander iridovirus (GSIV) — have been identified as important pathogens that cause severe hemorrhagic symptom and almost 85% mortality in a wide range of naturally infected aquatic animal and amphibians (Björklund et al. 1995, Daszak et al. 1999, Attoui et al. 2002, Dong et al. 2011, Padhi & Verghese 2012, Q. Wang et al. 2012). The family *Iridoviridae* currently comprises 5 genera: *Ranavirus*, *Lymphocystivirus*, *Megalocytivirus*, *Iridovirus* and *Chloriridovirus* (Chinchar et al. 2005). GSIV is a large DNA virus belonging to the genus *Ranavirus* in the family *Iridoviridae* and has been reported in farmed Chinese giant salamanders in recent years (Dong et al. 2011, Geng et al. 2011). The clinical signs of giant salamanders infected with GSIV include skin and internal organ hemorrhage, limb swelling, and ulceration (Geng et al. 2011). SVCV is a single-stranded RNA virus belonging to

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the genus *Vesiculovirus*, and it can cause an acute hemorrhagic and highly contagious disease in cyprinids (Padhi & Verghese 2012). GCRV, a double-stranded RNA virus belonging to the species *Aquareovirus* C in the family *Reoviridae*, is the most pathogenic aquareovirus that causes hemorrhagic disease with severe mortality in grass carp, and it has been known in China since 1983 (Attoui et al. 2002, Q. Wang et al. 2012). The biological characteristics and pathogenesis of GCRV, SVCV and GSIV have therefore been studied because all of these viruses cause severe economic losses in the aquaculture industry (Tidona et al. 1998, Koutná et al. 2003, Holopainen et al. 2009, Fan et al. 2013, Ma et al. 2014).

Moroxydine hydrochloride (Mor) is known to possess multi-antiviral efficacy against RNA and DNA viruses, but its antiviral activity and pharmacology has rarely been reported in aquaculture. Mor has been shown to be effective in the treatment of hepatitis C, influenza symptoms, varicella-zoster, measles, mumps disease, tobacco mosaic virus, etc. (Sheppard 1994, Z. Wang et al. 2012, Gasparini et al. 2014, Magri et al. 2015). Ribavirin (Rib) was selected as a positive control in this study because this compound has several activities against aquatic viruses, such as rainbow trout rhabdovirus (Marroquí et al. 2007), GCRV (Zhu et al. 2015), chum salmon reovirus (DeWitte-Orr & Bols 2007), infectious pancreatic necrosis virus and infectious hematopoietic necrosis virus (Hudson et al. 1988). In addition, Rib as an antiviral agent has many antiviral activities in the treatment of flaviviruses, paramyxoviruses, hepatitis virus, influenza-A virus, respiratory syndrome coronavirus, etc. (Leyssen et al. 2005, Kamar et al. 2010, Rowe et al. 2010, Chan et al. 2013). Although Mor and Rib exhibit excellent disease-resistant potency for targeting RNA and DNA viruses, little information exists on their efficacy and the mechanisms used by the 2 compounds on aquatic viruses. Therefore, further studies will be needed to close these knowledge gaps.

Immune regulation (Chen et al. 2013, Liu et al. 2014), vaccine development (He et al. 2011, Xue et al. 2013, Zhou et al. 2015) and antiviral agents (Yu et al. 2014) have been introduced to help control GCRV and GSIV in recent years. However, aquatic virus diseases are still difficult to efficiently control with vaccine in the aquaculture industry because the subtype specificity of vaccines has a relatively narrow antiviral spectrum (Hammond et al. 1999). Immune regulation has been widely accepted as a good method to improve the health of cultured aquatic animals and help them resist disease (Chen et al. 2013, Samanta et al. 2013), but it is not enough to effectively decrease mortality in severe outbreaks of virus disease. Furthermore, some antiviral agents directly inhibit transcription of the virus to reduce the replication in host cells and may regulate the immune system (Citarasu, 2010, Yu et al. 2014). However, there is a shortage of effective antiviral agents and lack of relevant research on the mechanisms that control GCRV and GSIV.

The present study was undertaken to determine the antiviral activity and potential mechanisms of 2 compounds on GCRV and GSIV *in vitro* by detecting the morphology, cell viability, virus titer, and viral gene expressions. The epithelioma papulosum cyprinid (EPC) cell line is known to be susceptible to infection with GSIV and SVCV (Koutná et al. 2003, Ma et al. 2014), and the findings in our study indicated that the grass carp ovary (GCO) cell line is susceptible to infection with GCRV, which is consistent with the findings of a previous study (Ke et al. 2013). The GCO cell line is known to be susceptible to viruses such as *Cyprinus carpio* spring viremia virus, *Rana grylio* virus, *Paralichthys olivaceus* lymphocystis virus (Zhang et al. 2003, Ou et al. 2014) and was used to replicate and propagate part of the virus. The EPC cell line represents a suitable *in vitro* model to study GSIV morphogenesis and characterize the GSIV replication cycle (Gao et al. 2012, Ma et al. 2014). Therefore, GCO and EPC cell lines can be used as stable *in vitro* models to perform some specific studies involving virus–host interactions and pharmacologic activity.

MATERIALS AND METHODS

Cells, virus, antiviral compounds

The GCO cell line, EPC cell line, GCRV and GSIV, were kindly provided by Prof. Ling-Bing Zeng of the Yangtze River Fisheries Research Institute, Wuhan, Hubei, China. SVCV was maintained in our laboratory. The EPC cells were cultured in MEM medium supplemented with 10% inactivated foetal bovine serum (FBS) (GIBCO BRL) and maintained in an incubator at 26°C. GCO cells were cultured in M199 medium supplemented with 10% inactivated FBS and maintained in an incubator at 28°C. The antiviral compounds, Mor (CAS No. 3160-91-6) and Rib (CAS No. 36791-04-5), were purchased from Aladdin Chemistry Co.

Virus infection and cell viability assays

The virus infection and viral titres of GCRV and GSIV were determined by the standard 50% tissue culture infective dose ($TCID_{50}$) method, as described previously (Yu et al. 2014). GCO cells were susceptible to GCRV with titers approaching 2.24 × 10^7 $TCID_{50}$ ml^{-1}, and EPC cells were susceptible to GSIV and SVCV infection with virus titers approaching 1.58 × 10^5 and 1.95 × 10^5 $TCID_{50}$ ml^{-1}, respectively. The protection efficiencies of the compounds on the viabilities of GCO and EPC cells were evaluated using the WST-8 Kit (Shanghai BestBio Biology) according to the manufacturer's protocol.

Briefly, cells were seeded in a 96-well plate (5 × 10^4 cells per well) containing 200 µl medium and placed in an incubator for about 24 h until cells reached approximately 90% confluence in each well. The medium in each well was then replaced with 100 µl of 100 $TCID_{50}$ virus and replaced again after 2 h using isopyknic MEM basal medium (3% FBS) containing Mor and Rib in a geometric dilution series with a common ratio of 2.51 covering concentration from 100 to 1 µg ml^{-1}. In addition, a toxicity test of the 2 compounds was performed in a geometric dilution series with a common ratio of 0.5 covering compound concentrations from 1000 to 15.6 µg ml^{-1}.

After 96 h of culture, the sample medium in each well was replaced by 190 µl MEM and 10 µl WST-8 test solution, and then 2 h later, the 96-well plate was read on a multi well scanning spectrophotometer at an optical density (OD) of 450 nm. The spectrophotometer was calibrated to zero absorbance using the same solution without cells. In a toxicity test of the compounds, the cell viabilities were calculated using an absorbance (A) test: $A_{test}/A_{control}$ × 100%. Each treatment was carried out in triplicate. The half toxic concentration (TC_{50}, 50% cell viability) and safe concentration (SC_{95}, 95% cell viability) were calculated by using probit analyses (Finney 1971).

Morphological effect of compounds for virus-infected cells

GCO cells and EPC cells were seeded into 6-well culture plates for about 24 h and infected by 100 $TCID_{50}$ GCRV and GSIV for 2 h, respectively. Samples were observed under an inverted microscope at 48 and 96 h post medication (40 µg ml^{-1}). Virus-induced apoptosis and drug-treated anti-apoptosis in host cells were studied to determine the morphological changes in the nucleus and cytoplasm. Briefly, normal cells, virus-infected cells and compound-treated (40 µg ml^{-1}) cells were collected at 48 h and stained using 1 µg ml^{-1} DAPI and 3.74 mg ml^{-1} 1,1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine (Dil) for 20 min, with phosphate-buffered saline cleaning 3 times in each step. These were mounted on glass slides and observed with an upright fluorescence microscopy (Leica-DM5000).

Antiviral efficiency of Mor and Rib

The antiviral efficiencies of compounds on the virus-infected cells were determined by the $TCID_{50}$ method. Identical methods of cell culture and virus infection were used for antiviral assay. Each well of a 96-well plate was seeded with 5 × 10^4 cells in 200 µl medium and incubated in a biochemical incubator for about 24 h under the appropriate conditions. The medium in each well was then replaced with 100 µl of 100 $TCID_{50}$ virus and replaced again after 2 h using isopyknic basal medium (3% FBS) containing 20 µg ml^{-1} Mor or Rib. Viral titres of GCRV and GSIV were determined by the standard $TCID_{50}$ method, as described previously (Yu et al. 2014). All samples were prepared in triplicate.

Sample collection, RNA extraction and reverse transcription

The virus titer curve cannot be obtained when a 40 µg ml^{-1} drug concentration is used, because the drugs significantly inhibit the viruses. We found the 96 h titer of GCRV and GSIV far below the 72 h titer in a preliminary experiment, suggesting that the proliferation of viruses was restricted in the last phase of apoptosis. Therefore, the viral gene expressions and virus titer were detected at 12, 24, 48, and 72 h after medication with 20 µg ml^{-1} Mor and Rib, respectively. Total RNA was extracted from respective virus-infected GCO and EPC cells using TRIzon Reagent (CWBIO) applied according to the kit instructions. The concentration of the RNA in samples were tested on a 1.5% agarose gel, and RNA samples with $OD_{260\,nm}/OD_{280\,nm}$ ratios between 1.8 and 2.0 were stored at –80°C until use. All the samples had RNA concentrations greater than 600 ng μl^{-1}. First-strand complementary DNA was synthesized by using 3.25 µl of total RNA and the PrimeScript RT reagent Kit (TaKaRa) that uses oligo dT primers and PrimeScript RT enzyme mix I. Samples were incubated for 15 min at 37°C followed by 5 s at

85°C. The resulting first-strand complementary DNA was stored at –80°C.

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR method was used to quantify the mRNA of viral gene expression. All the qRT-PCR reactions were performed using a Bio-Rad icycler IQ5 Real-time PCR Detection System (Bio-Rad) and an UltraSYBR Mixture (Cwbio). The complementary DNA was used as a template for qPCR with primer designed for particular genes (Table 1), and β-actin was used as a housekeeping gene to normalize gene expression studies. A 13 µl set-up was used: 1 µl of cDNA sample, 4.5 µl nuclease-free water, 6.5 µl of SYBR Green PCR master mix, and 0.5 µl of each gene-specific primer (10 µM). The cycling conditions were 95°C for 10 min followed by 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The threshold cycle (CT) value was determined using the IQ5 Sequence Detection System and exported into a Microsoft Excel Sheet for subsequent data analyses where the fold changes of gene expressions were calculated by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

Data analysis

The results were expressed as the mean ± standard deviation (SD), and all data analyses were done using SPSS 16.0 software (SPSS). The least significant difference (LSD) test was used for between-group comparisons in the entire gene expression validations, viral titers and cell viability assay. The differences were determined by 1-way ANOVA with p-values less than 0.05 and 0.01 being considered as different significance levels.

Table 1. Primers used for the analysis of mRNA expression by qRT-PCR

Gene	GenBank acc. no.	Primer sequence (5′–3′)	Product size (bp)
VP1	JN967629	Fwd: TCAGTCGTCGGATAGGAGGC Rev: CGATTGGTCCCAGAACGAGA	84
β-actin (grass carp)	M25013	Fwd: GATGATGAAATTGCCGCACTG Rev: ACCGACCATGACGCCCTGATGT	135
MCP	FJ358611	Fwd: GCGGTTCTCACACGCAGTC Rev: ACGGGAGTGACGCAGGTGT	93
β-actin (common carp)	M24113	Fwd: AGACATCAGGGTGTCATGGTTGGT Rev: CTCAAACATGATCTGTGTCAT	352

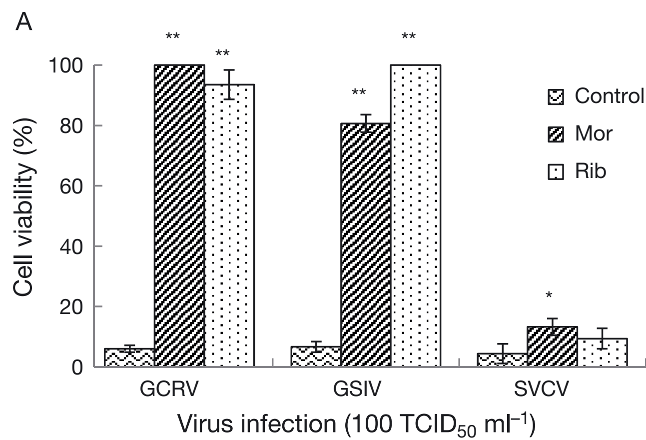
RESULTS

Protective efficiency of compounds against GCRV and GSIV

In the screening test, 100 µg ml^{-1} of Mor and Rib exhibited more than 80% protective efficiency to GCRV- and GSIV-infected host cells (Fig. 1A). However, the compounds showed no obvious activity against SVCV (less than 15%). Under the condition of 100 TCID$_{50}$ ml^{-1} viral infections, GCO cells and EPC cells exposed to Mor and Rib showed concentration- and time-dependent induction of cell viability (Fig. 1B). The 2 compounds showed a positive protective efficiency after medication. Briefly, Mor prevented over 40% apoptosis in virus-infected GCO cells and EPC cells at 6.3 µg ml^{-1}, which is better than the same concentration of Rib (less than 25% apoptosis). Moreover, cell viabilities of the Mor-treated GCO cells and Rib-treated EPC cells demonstrate no significant decrease at 100 µg ml^{-1}. When virus-infected cells were exposed to 100 µg ml^{-1} of Mor for 96 h, the cell viability of the EPC cells (74.55 ± 3.82%) was less than that of the GCO cells (100.43 ± 0.64%). The same concentration of Rib also offered excellent protection to GCRV-infected GCO cells (90.97 ± 1.49%) and GSIV-infected EPC cells (100.03 ± 1.88%). In addition, cell viabilities of all the virus-infected cells were found to have more than a 95% reduction within 96 h.

Cytotoxicity of compounds in GCO cells and EPC cells

Under experimental conditions, the cytotoxicity of Mor was lower than Rib, as shown in Fig. 2. No significant decrease in cell viability was observed at 96 h until the concentration of compounds increased to 250 µg ml^{-1} in GCO cells and to 125 µg ml^{-1} in EPC cells. At the same concentration of compounds, cell viabilities of Mor-treated GCO cells and EPC cells were equal or greater than those of the Rib-treated cells. The TC$_{50}$ (660.3 ± 12.4 µg ml^{-1}) and SC (96.1 ± 16.0 µg ml^{-1}) of Rib at 96 h are lower than Mor (TC$_{50}$, 1117.2 ± 23.5 µg ml^{-1}; SC, 140.9 ± 25.7 µg ml^{-1}) in the GCO cells. Similarly, the 96 h TC$_{50}$



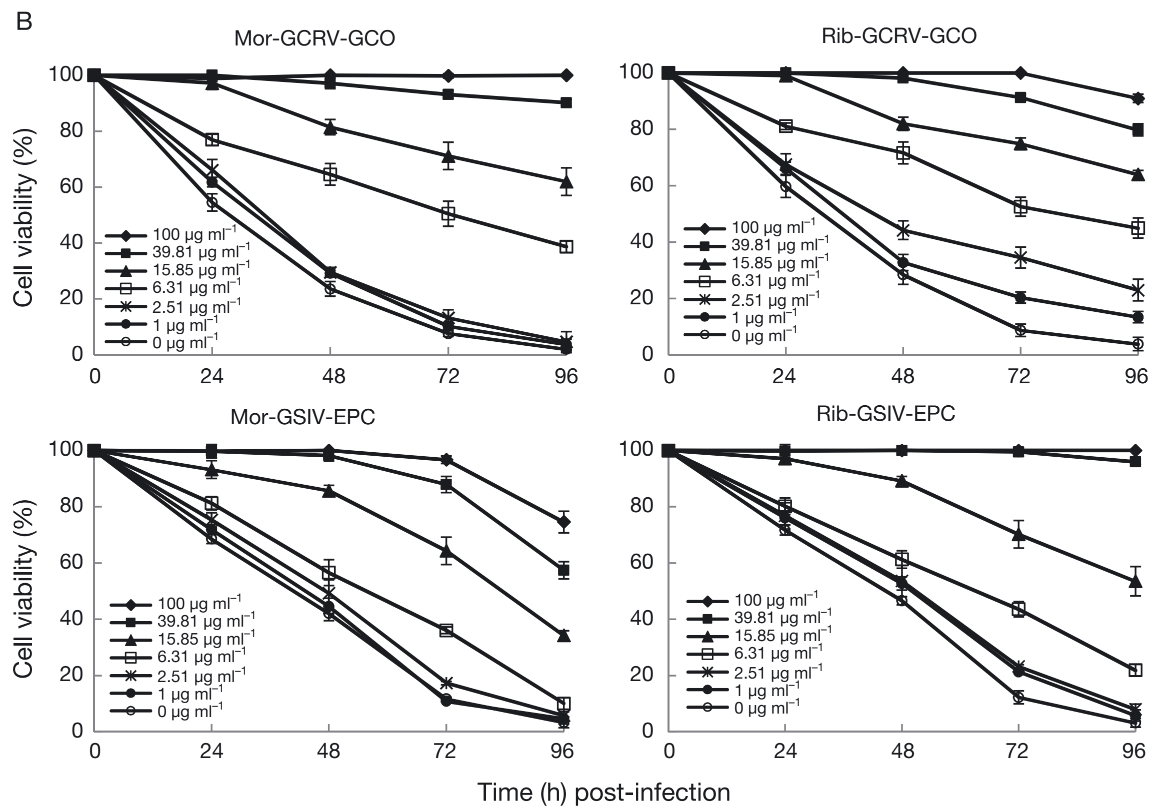


Fig. 1. Protective efficiency of moroxydine hydrochloride (Mor) and ribavirin (Rib) against different viruses *in vitro*. (A) The 96 h screening test of compounds against grass carp reovirus (GCRV), giant salamander iridovirus (GSIV) and spring viraemia of carp virus (SVCV) *in vitro*. Data are means ± SD. *$p < 0.05$; **$p < 0.01$. $TCID_{50}$: 50% tissue culture infective dose. (B) Protective efficiency of compounds against GCRV and GSIV at different concentrations and times. GCO: grass carp ovary; EPC: epithelioma papulosum cyprinid

(1162.1 ± 9.7 µg ml^{-1}) and SC (154.8 ± 16.8 µg ml^{-1}) of Rib are less than Mor (TC_{50}, 1486.3 ± 24.0 µg ml^{-1}; SC, 209.7 ± 17.4 µg ml^{-1}) in the EPC cells.

Morphological effect of compounds against GCRV and GSIV

Cytopathic effect (CPE) and cell death were observed in virus-infected GCO and EPC cells under an inverted microscope at 48 and 96 h (Fig. 3). Severe CPE appeared in GCO cells at 48 h post infection with 100 $TCID_{50}$ GCRV (Fig. 3A). The control cells maintained the normal growth state without any CPE or cell death. GCRV-induced CPE and cell death were effectively reduced in Mor- and Rib-treated GCO cells. In EPC cells, the GSIV-induced CPE was observed at 48 h, and severe CPE appeared at 96 h. These CPE and cell deaths were effectively inhibited in Mor- and Rib-treated EPC cells. Moreover, Rib was found more effective than Mor in the treatment of GSIV because CPE appeared in the Mor-treated EPC cells at 96 h. Thus, both Mor and Rib played an important role in controlling apoptosis induced by in this study.

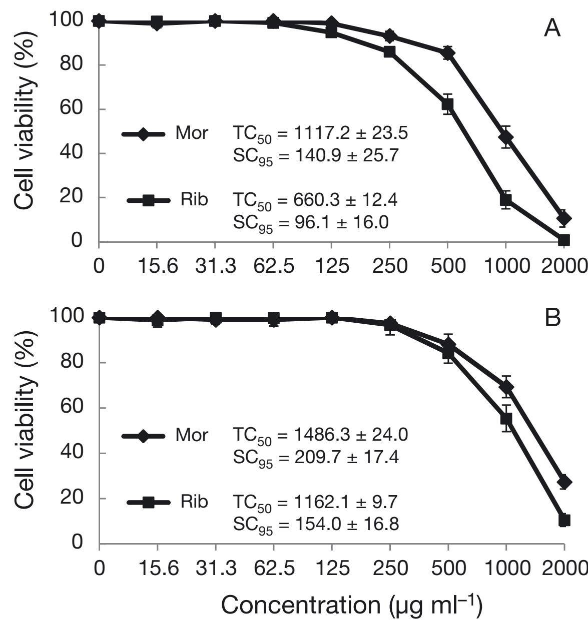


Fig. 2. Cytotoxicity of moroxydine hydrochloride (Mor) and ribavirin (Rib) in (A) grass carp ovary and (B) epithelioma papulosum cyprinid cells at 96 h post treatment. All data are means ± SD. Toxic (TC_{50}; 50% cell viability) and safe (SC_{95}; 95% cell viability) were calculated using probit analyses

Anti-apoptosis effects of compounds on the viruses-infected host cells

The GCRV-infected GCO cells and GSIV-infected EPC cells showed typical apoptotic features at 48 h, including nuclear and cytoplasmic degradation (Fig. 4). Mor and Rib provided significant protection to the virus-infected cells so that they could keep their normal morphology and cellular structure, as indicated in Fig. 4. Therefore, Mor and Rib could help infected cells maintain their normal morphology and structure and prevent the nuclear and cytoplasmic dissolution of the GCRV-infected cells.

Antiviral efficiency of Mor and Rib against GCRV and GSIV

The virus titers of GCRV and GSIV were calculated using the standard $TCID_{50}$ method. Following the initial infection conditions of 100 $TCID_{50}$ ml^{-1} for 2 h, the virulence of GCRV and GSIV in respective host cells showed a time-dependent increase from 12 to 72 h (Fig. 5). Mor and Rib significantly inhibited virulence of GCRV and GSIV compared to the respective control group, and the virus titer in each treatment showed a time-dependent increase. The lower virus level (ln value less than 2) could not be detected. Meanwhile, Mor and Rib presented a consistent antiviral activity when the treatment concentration was 20 μg ml^{-1}.

Effects of compounds on the expression of viral genes

Virus-infected cells exposed to Mor or Rib (20 μg ml^{-1}) showed a significant inhibition of viral gene expression (Fig. 6). In GCRV-infected GCO cells, expressions of all viral genes were significantly suppressed at all time points post medication. In particular, VP1 expression in Mor-treated GCO cells were significantly less than in Rib-treated cells at 36 and 48 h, which suggested that Mor had better antiviral activity than Rib in the treatment of GCRV. Our study also found that MCP gene expression in Mor- and Rib-treated cells were inhibited at all detected times. In GSIV-infected EPC cells, Mor and Rib showed no significant difference, and the relative expressions of the MCP gene were less than 0.5 at all detected times. Briefly, Mor and Rib presented a continuous antiviral activity post medication at 20 μg ml^{-1}.

DISCUSSION

Aquatic virus infections directly influence the aquaculture industry and have attracted much attention in recent years because these pathogens cause severe disease in aquatic animals. Consequently, controlling aquareovirus and iridovirus has become more important in aquaculture, and some studies have made advances in viral characterization (Kim et al. 2004, Williams et al. 2004). GCRV is the most pathogenic aquareovirus and to some extent has been suppressed by vaccine administration, immunoregulation or pharmacologic inhibition (Rangel et al. 1999, Samanta et al. 2013, Xue et al. 2013, Yu et al. 2014). GSIV can cause severe hemorrhagic disease in wild and farmed giant salamander (Williams et al. 2005). Moreover, this virus can induce the EPC cell lesion effect, and in some correlational research (Ma et al. 2014) its ultrastructure and morphogenesis was studied. However, it is still difficult to control GCRV and GSIV infection at present. One of the reasons for this is that there is no effective drug for use. Moreover, immunoregulation and vaccine administration is not enough to reduce the high mortality caused by many aquatic viral diseases, because the subtype specificity of vaccines show a relatively narrow anti-

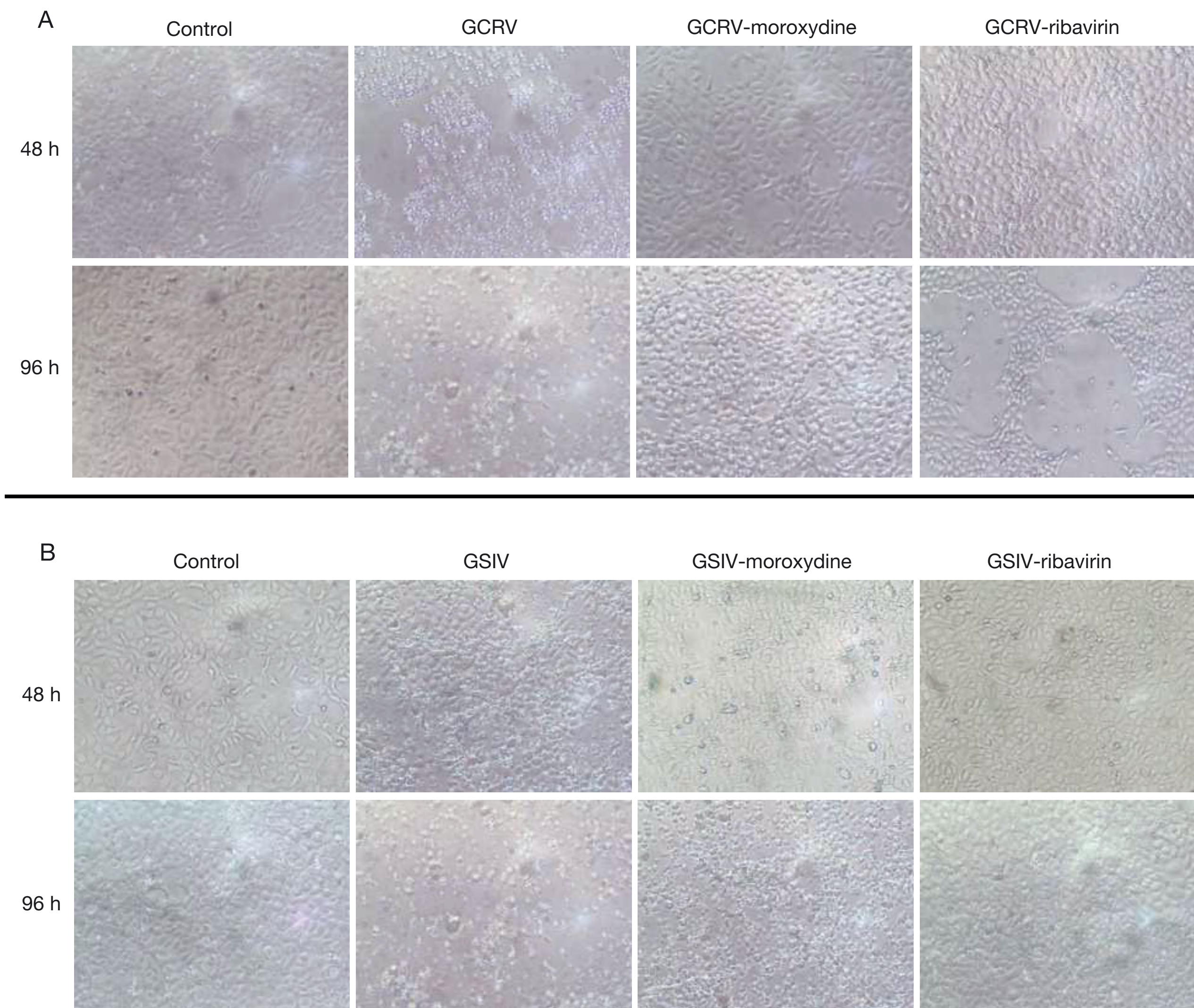


Fig. 3. Morphological effect of compounds (40 µg ml^{-1}) on virus-infected (100 × 50% tissue culture infective dose) (A) grass carp ovary and (B) epithelioma papulosum cyprinid cells. GCRV: grass carp reovirus; GSIV: giant salamander iridovirus

viral spectrum, and the complexity of the immune response which determines immunoregulation needs further research and development (Hammond et al. 1999, Citarasu, 2010). Therefore, the addition of an antiviral agent may lead to effective improvements in controlling virus pathogenicity (Feld & Hoofnagle 2005).

Mor was first developed in the 1950s and has been shown to have a number of antiviral efficacies against various human DNA and RNA viruses (Sheppard 1994, Gasparini et al. 2014, Magri et al. 2015), but its antiviral effects and pharmacological mechanisms have rarely been reported in aquaculture. It therefore is an antiviral substance which needs to be more fully studied and utilized in aquaculture. Likewise, Rib as an antiviral agent has many antiviral activities in the treatment of a number of aquatic viruses including rainbow trout rhabdovirus (Marroquí et al. 2007), GCRV (Zhu et al. 2015), and chum salmon reovirus (DeWitte-Orr & Bols 2007). Therefore, the broad-spectrum antiviral agent Rib was used as a positive control for a screening test against GCRV, SVCV and GSIV. The screening results showed that Mor and Rib can effectively control GCRV- and GSIV-induced cell apoptosis *in vitro*.

GCRV (dsRNA virus) and GSIV (large DNA virus) can cause systemic hemorrhagic disease in different aquatic animals and have been used as models for research (Rangel et al. 1999, Gao et al. 2012). Grass carp is the largest freshwater aquaculture species in China in terms of biomass production, and fish farm-

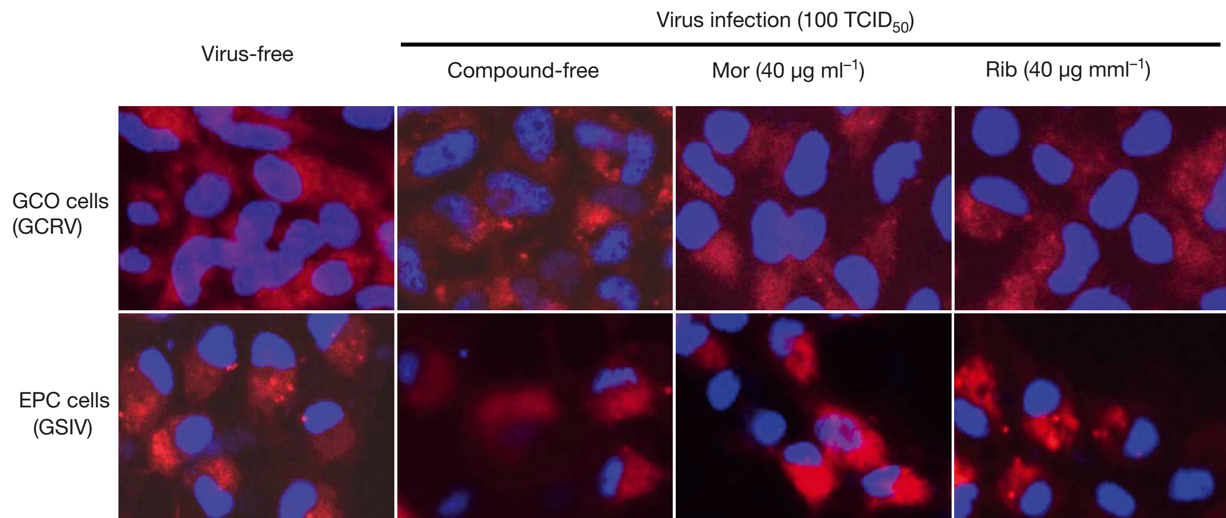


Fig. 4. Virus-induced apoptosis and the anti-apoptosis efficacy of moroxydine hydrochloride (Mor) and ribavirin (Rib) on virus-infected cells 48 h after infection. $TCID_{50}$: tissue culture infective dose 50%; GCO: grass carp ovary; EPC: epithelioma papulosum cyprinid; GCRV: grass carp reovirus; GSIV: giant salamander iridovirus

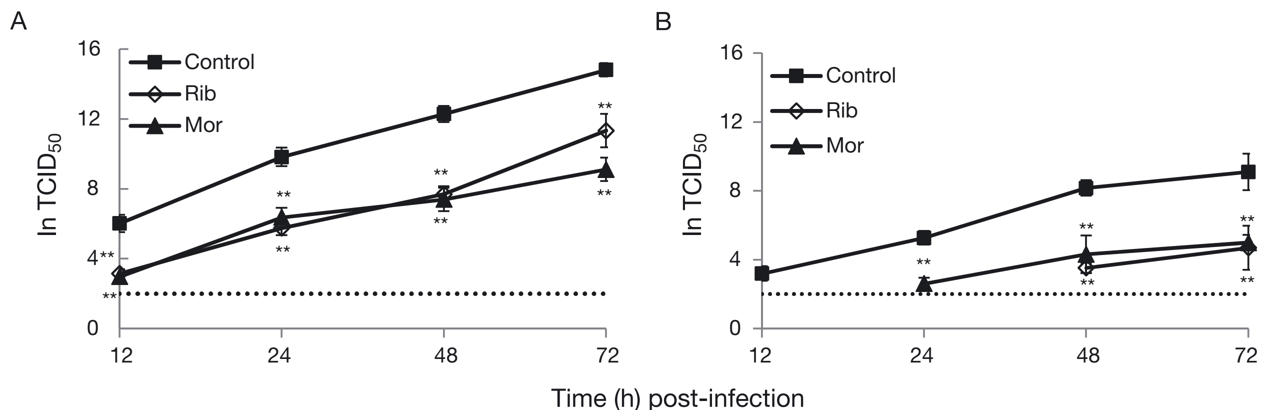


Fig. 5. Antiviral activity of moroxydine hydrochloride (Mor) and ribavirin (Rib) against (A) grass carp reovirus and (B) giant salamander iridovirus in respective host cells. $TCID_{50}$: 50% tissue culture infective dose. Data are means ± SD. $^{**}p < 0.05$. Dotted lines represent the detection threshold ($lnTCID_{50} \leq 2$)

ing is of significance in providing food protein for human beings (Wu et al. 2012). GCRV, known as the most pathogenic aquareovirus in the last 30 yr, causes severe hemorrhagic disease with almost 85% mortality in fingerling and yearling populations of grass carp (Q. Wang et al. 2012). The giant salamander is one of the world's largest amphibian species, and it was listed as a Class II protected animal in China. However, authorities have allowed farmed second filial generation giant salamander to be used as food for human beings in this country due to an increasing population of these salamanders. GSIV was frequently reported in farmed Chinese giant salamanders in recent years, and it causes pathologic changes causing up to 95% of mortality in major population areas including in Shaanxi, Sichuan, and Henan Province (Dong et al. 2011, Geng et al. 2011). However, there is a shortage of potent antiviral agents and lack of research on how to control GCRV and GSIV. For our study, GCRV-infected GCO cells and GSIV-infected EPC cells appeared to have a typical CPE within 96 h. The GCRV 104 virus strain was isolated from diseased grass carp in 2009, and the gene sequence contains 11 segments encoding 12 proteins (Fan et al. 2013). Among them, the conserved domain VP1 belonged to the reovirus L2 superfamily and was predicted to encode the guanylyl transferase protein with putative functions as an

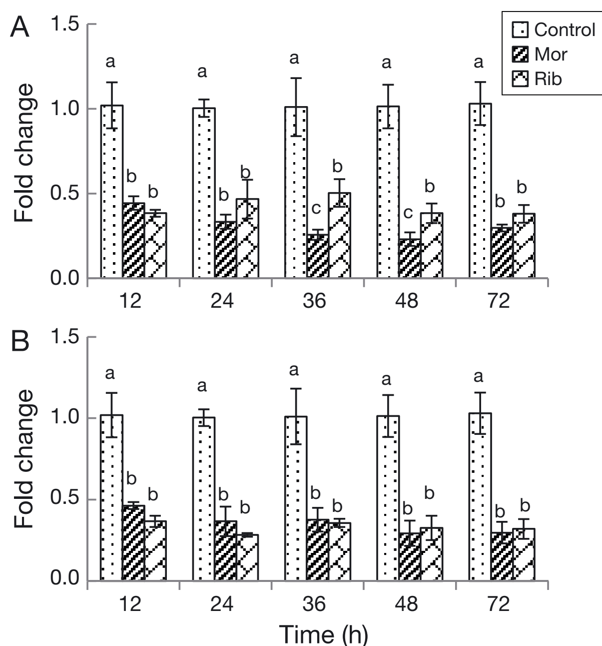


Fig. 6. Effects of antiviral compounds on viral protein gene expression in (A) grass carp reovirus-infected grass carp ovary cells and (B) giant salamander iridovirus-infected epithelioma papulosum cyprinid cells. Data are means ± SD; different letters represent significant differences between treatment groups at each timepoint ($p < 0.05$). Mor: moroxydine hydrochloride; Rib: ribavirin

mRNA capping enzyme (Fan et al. 2013). The GSIV strain has been isolated from Chinese giant salamander *Andrias davidianus*, and the MCP has been used for viral quantification (Zhao et al. 2007, Meng et al. 2013). The MCP is the major structural component of iridovirus particles, accounting for up to 45% of all virus proteins and is required for the assembly and release of virus progeny (Tidona et al. 1998, Hyatt et al. 2000). The conserved MCP coding regions successfully amplified from 9 iridovirus isolates and the expression levels correlate with viral production; this suggests that the MCP may be the key to developing PCR assays for iridovirus identification (Mao et al. 1997, Zhao et al. 2007). In this study, the MCP gene expression in GSIV-infected EPC cells and the VP1 gene expression in GCRV-infected GCO cells were found significantly inhibited after medication with Mor or Rib.

To measure cytotoxicity and antiviral activity, the WST-8 assay (which depends on the conversion of tetrazolium salt WST-8 to highly water-soluble formazan by viable cells) was used to measure changes in cell viability. It is a perfect method for use in cell viability assays and has been widely utilized in drug sensitivity tests (Tominaga et al. 1999, Yamawaki & Iwai 2006). The amount of produced formazan was determined with a spectrophotometer at 450 nm and is directly proportional to the number of living cells (Tominaga et al. 1999). The SC values of Mor and Rib indicated that compounds are safe for GCO and EPC cells up to 96 µg ml^{-1}, making it possible to rule out a toxic influence on antiviral activity assays. Therefore, the cell viability in antiviral activity assays directly reflects the protective activity of the compounds. Results of this study confirmed that virus-infected cells exposed to Mor and Rib result in different degrees of protection of cell viability. The results also indicate that Mor and Rib had a better *in vivo* anti-GCRV activity than herbal medicines or isolated chemicals (Yu et al. 2014).

The emergence of deadly iridovirus diseases in aquatic animals has led to a growing interest in the mechanisms of iridovirus-induced host cell death (Imajoh et al. 2004, Pham et al. 2012, Chen et al. 2016). Most iridoviruses induce typical apoptosis or trigger non-apoptotic-programmed cell death in fish cells (Gibson-Kueh et al. 2003, Chen et al. 2016). A similar phenomenon appeared in the GCRV-infected cells, and some studies suggested that the trigger of apoptosis and oxidative stress might be associated with the death receptor pathway. In this study, 3 aquatic viruses were propagated in respective host cells with a typical CPE. More importantly, Mor and Rib as broad-spectrum antiviral agents exhibited prominent morphological protection to different virus-infected GCO and EPC cells. Due to the direct inhibitory effect of compounds on virion replication, GCRV- and GSIV-induced CPE and cell death in respective host cells was effectively blocked, which contributed to the maintanence of the normal growth situation and intact cell structure.

In this study, GCO and EPC cells showed differential CPEs after inoculation of GCRV, SVCV or GSIV and were indicative of high sensitivities to respective viruses. Similar results appeared in other research which determined that the isolated *Pseudobagrus ussuriensis* skin cell line showed differential CPEs after inoculation of SVCV, GCRV and *Rana grylio* virus (Ou et al. 2014). In addition, the virus titers of GCRV and GSIV were significantly inhibited after medication with a low concentration of Mor or Rib.

In summary, Mor and Rib as broad-spectrum agents exhibit excellent antiviral activity and show low cytotoxicity to GCRV-infected GCO cells and GSIV-infected EPC cells. This study demonstrates that exposure of virus-infected cells to antiviral compounds could inhibit the virus replication, CPE and apoptosis and help cells maintain a normal morpho-

logical structure. Therefore, Mor and Rib are regarded as antiviral agents exhibiting high anti-GCRV and anti-GSIV activity and have proved to be appropriate inhibitors for controlling virus in aquaculture.

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