Research in Veterinary Science 117 (2018) 37–44

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effects of moroxydine hydrochloride and ribavirin on the cellular growth and immune responses by inhibition of GCRV proliferation

Xiao-Bo Yu[1], Kai Hao, Jing Li, Xiao-Hui Chen, Gao-Xue Wang*, Fei Ling*

College of Animal Science and Technology, Northwest A&F University, Xinong Road 22nd, Yangling, Shaanxi 712100, China

ARTICLE INFO

Keywords:
Grass carp
GCRV
Moroxydine hydrochloride
Ribavirin
Immune response

ABSTRACT

Moroxydine hydrochloride (Mor) and ribavirin (Rib) are known for their multi-antiviral activities against DNA and RNA viruses but little information is available about the pharmacological impact in aquaculture. The present study was undertaken to investigate the response of host cells to antiviral compounds during the anti-GCRV treatment. The scanning electron microscope results showed that *Ctenopharyngodon idella* kidney (CIK) cells have a higher death rate at 72 h post virus infection. At the concentration of 40 μg mL^{-1}, Mor and Rib had a significant protective effect on virus-infected cells. Moreover, the gene expressions of *vp5, vp6* and *NS66* were significantly inhibited by treatment with Mor or Rib, especially gene expression of the *vp5*. For the immunoregulatory action, no distinct induction of the expression of immune genes was observed after the addition of Mor and Rib to the virus-free cells. However, the compounds significantly decreased the virus-induced gene overexpression of Myd88, Mx1, IL-1β, IL-8, I-IFN and TNFα in CIK cells. Moreover, Mor and Rib significantly inhibited the immune genes upregulation which was induced by GCRV in kidney, liver, muscle and gill of grass carp, despite greater partial gene expressions were detected than the virus-free control group. Besides, Mor and Rib blocked cell cycle changes, cytopathic effects, cellular death and virus proliferation in CIK cells thereby maintaining normal morphological structure. Overall, Mor and Rib as antiviral compounds are effective for the control of GCRV replication and the indirectly regulation of cellular immune response.

1. Introduction

Grass carp is the largest freshwater aquaculture species in China and the fish farming is of significance in providing basic protein as food for human beings. However, GCRV as a dsRNA virus causes hemorrhagic disease with severe mortality in grass carps for many years (Wang et al., 2012). But the viral disease has been more difficult to control than other pathogenic factors, due to the insufficient evidences to reveal pathogenesis of viral infections and unclear resistance mechanisms in host cell (Kibenge et al., 2012; Meyer, 1991). Anyhow, the use of some herbal medicines or chemical agents can inhibit transcription of virus to reduce the replication in the host cells and regulate the innate immune system (An et al., 2011; Citarasu, 2010; Yu et al., 2014). Our previous studies have proved Mor and Rib were safe and effective inhibitors for the treatment of GCRV (Yu et al., 2016; Zhu et al., 2015).

Moroxydine hydrochloride (Mor) has emerged over sixty years and confirmed the effect in the treatment of a number of viral disorders, including hepatitis C, influenza symptoms, varicella-zoster, measles, mumps disease, tobacco mosaic virus, *etc.* (Gasparini et al., 2014; Magri et al., 2015; Sheppard, 1993). In addition, ribavirin (Rib) as a broad-spectrum agent showed antiviral activity against a variety of RNA viruses, such as flaviviruses, paramyxoviruses, hepatitis virus, influenza-A virus, alphaviruses, *etc.* (Briolant et al., 2004; Crotty et al., 2000; Leyssen et al., 2005; Sidwell et al., 1995). Our previous study has verified that Mor and Rib could inhibit grass carp reovirus replication and intervene mitochondrial apoptotic signals of CIK cells (Yu et al., 2016). Although they have wonderful disease-resistant potency, it is little known of the cellular growth and immunoregulation mechanism of Mor and Rib and needs further to study.

Strain of GCRV 104 was found in recent years and the full genome contained 11 segments encoding 12 proteins (Fan et al., 2013). The genes expressions of *vp*5, *vp*6 and NS66 were significantly inhibited by Mor and Rib. The core protein VP5 was predicted to be encoded by GCRV segment 4 and was a nucleoside triphosphate hydrolase (NTPase). Non-structural protein NS66 and major inner capsid protein VP6 were encoded by GCRV segment 6 and 8, respectively (Fan et al., 2013). Anyhow, the viral proteins play a key role in the viral replication and the viral gene expressions were significantly inhibited in Mor and

* Corresponding authors.
E-mail addresses: wanggaoxue@126.com (G.-X. Wang), feiling@nwsuaf.edu.cn (F. Ling).
[1] Present address: College of Animal Science and Technology, Southwest University, Tiansheng Road 2nd, Beibei, Chongqing 400715, China.

https://doi.org/10.1016/j.rvsc.2017.11.007
Received 13 September 2016; Received in revised form 13 November 2017; Accepted 18 November 2017

Rib treated cells.

The components derived from pathogens trigger MyD88-dependent TLRs signaling cascades (Chettri et al., 2011). And the virus-induced interferons (IFNs) have been identified in various fish species and display antiviral activities by the known IFN-induced antiviral mechanisms (Yu et al., 2010). Meanwhile, Mx pathway is one of the major pathways among the known IFN-induced antiviral mechanisms, which showed antiviral activities of Mx genes against GCRV in transgenic cells (Peng et al., 2012). Furthermore, innate and adaptive immunity constitutes a powerful defense in the protection of fish by producing cytokines to destroy invading pathogen (Wang and Secombes, 2013; Yu et al., 2010).

The kidney as an immune tissue plays an important role in pathogenic infection (Chabardes-Garonne, 2003), and many studies focused on the immunoregulation in head kidney of teleosts (Chettri et al., 2011). It has been proved that kidney is one of the major immune organs involved in B lymphoid cells developing (Rauta et al., 2012). Some studies have provided evidence that GCRV infection both *in vivo* and *in vitro* induced the innate and adaptive immune response (Chen et al., 2013; Zhang et al., 2007). Therefore, the stable cell line of *C. idella* kidney cell as an appropriate material can be used to perform some specific studies involving virus-host interaction, immunoregulation, pharmacologic action, *etc.*

However, the addition of antiviral agent is effective in virus inhibition, because the immunoregulation is not enough to effectively suppress virus when GCRV causes severe hemorrhagic disease (Zhu et al., 2015). And the viral replication, cell growth and immune response are likely to be regulated after medication (Feld and Hoofnagle, 2005; Yu et al., 2014). Therefore, the present study was undertaken to research the antiviral response of host cells to compounds and evaluate the interaction between anti-GCRV property and immune response.

2. Materials and methods

2.1. Cell, virus, grass carp and compounds

GCRV104 and CIK cell line were obtained from Prof. Ling-bing Zeng, Yangtze River Fisheries Research Institute, Wuhan, China. The GCRV104 was isolated from a grass carp with severe hemorrhagic disease in Hubei province, China. The CIK cells were cultured in MEM medium containing 10% inactivated foetal calf serum (GIBCO/BRL) and incubated in a humidified 5% CO_2 incubator at 28 °C for use. GCRV were collected using CIK cell after virus infection and the viral titres were calculated as 2.24×10^7 $TCID_{50}$ mL^{-1} using the standard of 50% tissue culture infective dose ($TCID_{50}$) method, as our described previously (Yu et al., 2014). Grass carp (2.36 ± 0.26 g) was purchased from Hua Du fry breeding base (Guangzhou, China) and placed in a 250 L glass aquarium with > 6 mg L^{-1} oxygen content saturation at 28 ± 0.5 °C temporary breeding for a week. The grass carp care was maintained in conformity with the general recommendation of Chinese experimental animal administration legislation. The compounds Mor (CAS No. 3160-91-6) and Rib (CAS No. 36791-04-5) were purchased from Aladdin Chemistry Reagent Co., Ltd. (Shanghai, China), and the purities were > 99.9%.

2.2. Ultrastructural protection of compounds on virus-infected cell

Morphological protection of Mor and Rib were detected using scanning and transmission electron microscopy assay. Briefly, cells were seeded in 6-well plate containing 2 mL medium (MEM with 10% foetal calf serum) and incubated at 28 °C for about 24 h until cells reached approximately 90% confluence in each well. The medium in each well was then replaced with 100 μL 100$TCID_{50}$ GCRV and replaced again after 2 h using 2 mL fresh MEM basal medium (3% foetal calf serum) containing Mor and Rib at concentration of 40 μg mL^{-1}, respectively. Samples collected at 72 h post medication and prepared in the same manner as our previous described (Yu et al., 2016). Briefly, cells were collected and fixed with 2.5% glutaraldehyde at 4 °C for about 12 h. Then washed with PBS for 3 times, dehydrated in a series of ethanol solutions (50%, 70%, 80%, 90% and 100%), replaced with acetone and isoamyl acetate, successively (each step for about 15 min). Samples were fully dried with a CO_2 critical-point dryer and coated with gold-palladium, then observed and recorded with scanning electron microscopy at the 1×10^4 magnification (SEM-6360 LV, HITACHI, JAPAN). For the transmission electron microscope detection, cells samples were also fixed with 2.5% glutaraldehyde and fixed in 1% osmium tetroxide buffer. Then dehydrated with a series of ethanol solutions (30%, 50%, 70%, 80%, 90% and 100%) and embedded with white glues. Ultrathin sections were prepared with a microtome and stained with uranyl acetate and lead citrate, then observed and photographed using transmission electron microscope at the 1×10^5 magnification (TEM-HT7700, HITACHI, JAPAN).

Table 1
Primers used for the analysis of mRNA expression by RT-qPCR.

Genes	Accession no.	Primer sequences (from 5′ to 3′)	Product size (bp)
β-actin	M25013	Fwd: GATGATGAAATTGCCGCACTG Rev: ACCGACCATGACGCCCTGATGT	135
TNFα	EU047718	Fwd: TGTGCCGCCGCTGTCTGCTTCACGCT Rev: GATGAGGAAAGACACCTGGCTGTAGA	285
IL-1β	EU047716	Fwd: GGAGAATGTGATCGAAGAGCGT Rev: GCTGATAAACCATCCGGGA	448
Myd88	FJ843088	Fwd: GAAATGATGGACTTTACCTACCTG Rev: ACATCTTTCCTTTCGGCTTTT	149
Mx1	HQ245104	Fwd: CTGGGGAGGAAGTAAAGTGTTCT Rev: CAGCATGGATTCTGCCTGG	392
IFN-I	AB196166	Fwd: GGTGAAGTTTCTTGCCCTGACCTTAG Rev: CCTTATGTGATGGCTGGTATCGGG	173
IL-8	EU047717	Fwd: AGGTCTGGGTGTAGATCCACGCTG Rev: TTAGTGTGAAAACTCACATGATCTCT	137
VP5	JN967633	Fwd: GCAGCGATACAAGTTCTAAGCG Rev: GGAGTTATGAACAGAGCAGGCG	439
NS66	JN967634	Fwd: CAGACGAGGCGTTGGAGGTA Rev: GTGCCAGCGAGTCATAAGAAA	282
VP6	JN967636	Fwd: GAACTACCTGGTCAGATGTGGG Rev: TGCTGGTTATGGACCTGCC	166

2.3. Cell cycle analysis

CIK cells in six-well plates containing 2 mL medium were incubated at 28 °C for 24 h. Two milliliter 100$TCID_{50}$ GCRV were exchanged into each well for 2 h infection and then replaced by isopyknic MEM basal medium containing 20 μg mL^{-1} Mor and Rib. The control groups were prepared in the absence or presence of GCRV. Seventy-two hours later, the cells were harvested and immobilized into 70% precooling ethanol at 4 °C for at least 12 h. Then obtain the cells by centrifugation at 1000 rpm and wash cell with PBS containing 3% FBS. After immobilization samples were uniformly distributed and incubated in PBS containing 50 mg mL^{-1} PI, 10 mg mL^{-1} RNaseA 1% and Triton X-100 at room temperature for about 30 min. Cell cycle were detected by flow cytometry using a FACSCalibur (Becton Dickinson, USA) at 488 nm. Data of cell cycle were obtained and analyzed using CellQuest software (Becton Dickinson). All samples were prepared in triplicate.

2.4. Gene expression validation by quantitative real-time PCR

qRT-PCR method was established to quantify the mRNA of immune gene expression *in vitro* and *in vivo*. For the former, CIK cells were seeded in 6-well culture plates 24 h and then infected by 1000