Antiviral Research 144 (2017) 173–185

Contents lists available at ScienceDirect

Antiviral Research

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

Evaluation on antiviral activity of coumarin derivatives against spring viraemia of carp virus in epithelioma papulosum cyprini cells

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ARTICLE INFO

Article history:
Received 7 March 2017
Received in revised form
4 June 2017
Accepted 12 June 2017
Available online 15 June 2017

Keywords:
Spring viraemia of carp virus
Coumarin derivatives
Cytoskeleton damage
Antiviral activity

ABSTRACT

As one of the most serious pathogens in the freshwater aquatic environment, spring viraemia of carp virus (SVCV) induces a high mortality rate in several cyprinid fishes. In this study, we designed and synthesized a total of 44 coumarin derivatives to evaluate the anti-SVCV activity. By comparing the inhibitory concentration at half-maximal activity (IC_{50}), two imidazole coumarins (B4 and C2) were selected, with maximum inhibitory rates on SVCV more than 90%. Mechanistically, B4 or C2 did not affect viral adhesion and delivery from endosomes to the cytosol. Further, B4 and C2 could decline the apoptosis in SVCV-infected cells and the viral activated caspase-3, 8, 9 activities. Other results showed that SVCV induced the cytoskeletal structure to be a circumferential ring of microtubules near the nucleus, with occurring a disrupted microfilament organization. In comparison, cytoskeleton structure in drug-treated cells kept complete. In addition, the cellular microstructure in drug treatments showed no significant change; while SVCV-infected cells were seriously shrunk, and observed typical apoptotic features including cell shrinkage, volume reduction and cell blebbing. More importantly, B4 and C2 enhanced anti-oxidative enzyme gene expression and triggered the Nrf-2 pathway to keep balance of intracellular redox state. Therefore, the use of two imidazole coumarins (B4 and C2) could be a viable way of preventing and controlling SVCV infection.

1. Introduction

As the causative agent of spring viraemia of carp (SVC), spring viraemia of carp virus (SVCV) is one of nine piscine viruses recognized by the International Office of Epizootics as a notable animal disease (Ashraf et al., 2016). SVCV brings significant mortality to cyprinids, with case fatality rates of young fish up to 90% (Baudouy et al., 1980), which causes substantial economic losses to the aquaculture industry. In recent years, SVCV has been found in Europe, North America the Middle East and China (Fijan, 1999; Ahne et al., 2002; Dikkeboom et al., 2004; Garver et al., 2007; Teng et al., 2007; Phelps, 2012; Taylor et al., 2013; Petty et al., 2014; Yu et al., 2014). Since it is difficult to eradicate the virus from affected ponds, all aquatic life should be destructed when the infection is established (Ahne et al., 2002).

Up to now, there is no licensed treatments available for the control of SVCV infection, making them serious public health threats in aquaculture industry. Traditionally, vaccines as a basic prevention strategy for controlling viral disease are widely studied (Kanellos et al., 2007; Emmenegger and Kurath, 2008; Min et al., 2012; Zhu et al., 2015; Cui et al., 2015). However, vaccines must be administered prior to infection to allow for a robust immune response to develop and cannot be used to treat infected fish (Gotesman et al., 2015). Additionally, their use is still limited in aquaculture industry due to efficient vectors for delivery of vaccine antigens and the handling stress on the fish, as well as high labour and production costs (Life, 2008; Ashraf et al., 2016). Lately, RNA-mediated interference (RNAi) by small interfering RNAs (siRNAs) as a new technique in the field of drug discovery and development showed a promise for controlling SVCV replication *in vitro* (Gotesman et al., 2015), but needed future study using *in vivo* models. Overall, it is important to develop novel antiviral agents that can be used for prophylaxis or as antiviral agents against SVCV infection.

Coumarin compounds, known as benzopyran-2-ones, form an elite class of naturally occurring compounds that possess promising therapeutic perspectives (Sandhu et al., 2014). Due to the structural diversity, variety of biological activities of coumarins based on

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http://dx.doi.org/10.1016/j.antiviral.2017.06.007
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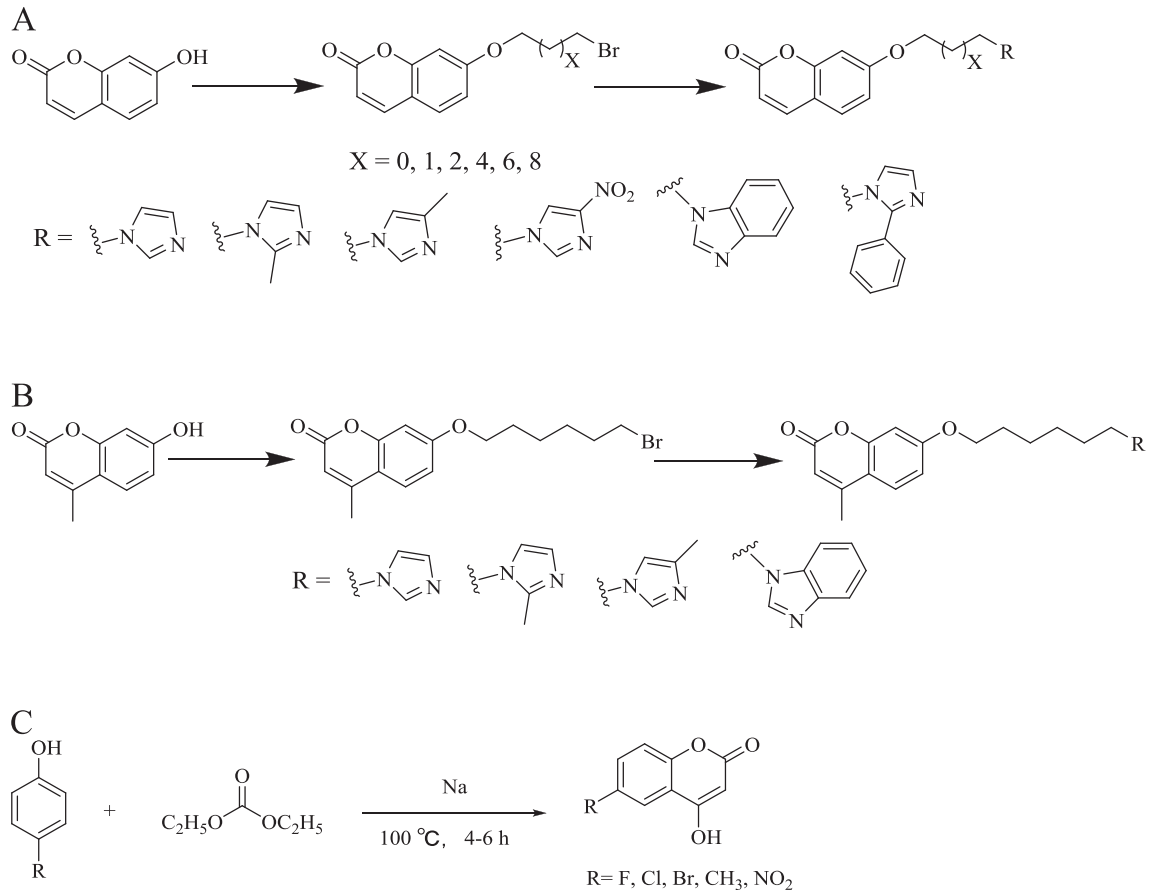

Fig. 1. Synthetic route of derivatives of 7-hydroxy-2H-chromen-2-one (A), 4-methyl-7-hydroxy-2H-chromen-2-one (B) and 4-hydroxy-2H-chromen-2-one (C).

natural and synthetic derivatives were found, like anticancer (Riveiro et al., 2008), antimicrobial (Ostrov et al., 2007), antioxidant and anti-inflammatory (Bansal et al., 2012; Manvar et al., 2011), and antiviral efficacies (Kashman et al., 1992; Shikishima et al., 2001), usually associated with low toxicity. In an attempt to address the urgent need for therapeutics for prophylaxis and treatment on SVCV infection, 44 novel coumarin-imidazole hybrid derivatives were designed and synthesized in this study. We identified these active compounds against SVCV by using Quantitative Real-time PCR (qRT-PCR) to detect the expression of glycoprotein (G) of SVCV. The antiviral activities of 7-(3-(1H-benzo[d]imidazol-1-yl) propoxy)-2H-chromen-2-one (B4) and 7-(4-(4-methyl-1H-imidazol-1-yl)butoxy)-2H-chromen-2-one (C2) were further validated in secondary assays, including apoptosis assay, microtubule structure and nucleus damage test, and cell surface ultrastructure observation, which indicated that both drugs could prevent SVCV damaging the host cells.

2. Materials and methods

2.1. Cell and virus

Epithelioma papulosum cyprini cells (kindly provided by Prof. Ling-bing Zeng in Yangtze River Fisheries Research Institute, Wuhan, Hubei, China) were maintained in Medium 199 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; ZETA LIFE, USA) penicillin (100 IU/mL), and streptomycin (0.1 mg/mL) at 25°Cin humidified atmosphere with 5% CO_2. SVCV (strain 0504) was kindly provided by Professor Qiang Li, Dalian Ocean University, and titrated on EPC cells as described previously (Adamek et al., 2012).

2.2. Synthetization of coumarin derivatives

According to the study of Liu (2016), 44 derivatives were synthesized by substitution reaction, Wiiliamson etherification reaction and Fries Rearrangement using three coumarins as the initial material (Fig. 1). The details are described in the Supplemental materials and methods available online. Based on these reactions, each intermediate product or end-product yield was ranged from 60% to 75%. All the intermediates and products were identified by EI-MS, 1H-NMR and 13C-NMR. Stock solutions were prepared at a concentration of 50 mg/mL in dimethyl sulfoxide (DMSO) and stored at −20 °C during the experiment. Due to the structural changes affecting the solubility of these derivatives, the medium containing each compound was sonicated for approximately 10 min to ensure that the compounds were fully dissolved and mixed before treating EPC cells.

2.3. In vitro antiviral and cell viability assays

For the screening assay, 47 compounds were divided into three groups (initial concentration: 1, 10 or 25 mg/L) based on trypan blue exclusion dye staining test and compound solubility. Cytotoxicities on initial concentration of compounds used herein showed no significantly detectable (cell death was less than 10%). A DMSO-control (v/v, the highest percentage of DMSO in treatment groups) was set 0.02, 0.2 and 0.5‰ for 1, 10 and 25 mg/L groups,