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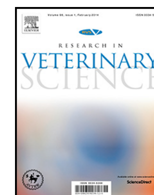

In vitro inhibition of caprine herpesvirus 1 by acyclovir and mizoribine

G. Elia [1,*], M. Camero [1], N. Decaro, A. Lovero, V. Martella, M. Tempesta, C. Buonavoglia, G. Crescenzo

Department of Veterinary Medicine, University of Bari, Valenzano, Bari, Italy

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ABSTRACT

Caprine herpesvirus 1 (CpHV-1) infection in goats induces genital vesicular-ulcerative lesions that strictly resemble the lesions induced by herpesvirus 2 in the human host. The immunosuppressive drug Mizoribine (MIZ) was found to increase the antiviral activity of Acyclovir (ACV) against herpesvirus infections, raising interesting perspectives on new combined therapeutic strategies. In this study the anti-CpHV-1 activity in vitro of ACV alone or in combination with MIZ was characterized. When applied alone at non-toxic concentrations, ACV had a slight effect on CpHV-1 replication while in combination with MIZ a dose-dependent inhibition of the virus yield was observed with an IC_{50} of ACV of 28.5 μM. These findings suggest that combined therapy of ACV and MIZ is potentially exploitable in the treatment of genital infection by herpesviruses.

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1. Introduction

Caprine herpesvirus 1 (CpHV-1) is an alphaherpesvirus widespread in goat population worldwide with a seroprevalence of 30–40% in the Mediterranean countries. CpHV-1 is responsible for recidivous genital disease in adult goats, characterized by confluent vesicles evolving to ulcers and crusts on the vulvar rima and vaginal mucosa (Tempesta et al., 1999a). As shown in Table 1, there are several biological similarities between CpHV-1 and human genital herpesvirus type 2 (HSV2), such as the preferential tropism for the genital tract, the vesicular-ulcerative nature of the topical lesions, and the tendency to become latent in the sacral ganglia (Tempesta et al., 1999a, 1999b, 2002). Developing and evaluating antiviral agents for treatment and/or prevention of CpHV-1 infection in the goat model is therefore of interest for the close parallelism with the human genital herpesvirus.

Nucleoside antiviral medications for treatment of herpesvirus infection became available in the early 1980s. Acyclovir (ACV, 9-2-hydroxyethoxymethyl guanine) represents the first-line therapy for management of herpesvirus infections due to its efficacy and safety (Suzuki et al., 2006). ACV exhibits anti-herpetic activity after phosphorylation by viral thymidine kinase (TK). ACV triphosphate competes with the natural nucleotide, dGTP, interfering with viral DNA polymerization and preventing continued extension of the DNA chain (Biron and Elion, 1980; Coen and Schaffer, 1980; Elion et al., 1977).

Although ACV and other nucleoside analogues are used as standard mono-therapy for human infections by Herpes simplex virus (HSV) and Varicella zoster virus (VZV), combined treatment with ACV and with antiviral drugs exploiting a different mechanism of action is expected to trigger synergistic antiviral activity (Suzuki et al., 2006). Also, multi-drug therapy could allow decreasing the therapeutic dose of each molecule, maintaining the same efficacy but minimizing adverse effects. Marked enhancement of ACV antiviral activity has been demonstrated in conjunction with inhibitors of inosine monophosphate dehydrogenase (IMPDH) (Neyts and De Clercq, 1998; Pancheva et al., 2002), a key enzyme in *de novo* synthesis of purine nucleotides.

Mizoribine (MIZ) is an imidazole nucleoside used as immunosuppressant because of its antiproliferative activity against lymphocytes (Ishikawa, 1999). MIZ is known to inhibit replication of some DNA and RNA viruses, such as vaccinia virus (Mizuno et al., 1974), influenza virus types A and B (Hosoya et al., 1993) and herpesviruses in combination with ACV (Pancheva et al., 2002). Although the antiviral mechanisms of MIZ remain to be elucidated, antiviral activity could be exerted by inhibition of IMPDH, as observed for ribavirin (Yanagida et al., 2004). The compound is phosphorylated by adenosine kinase and converted into its active form that inhibits IMPDH triggering depletion of the intracellular dGTP pool. It has been demonstrated that depletion of the intracellular dGTP pool may potentiate the effect of ACV, facilitating the interactions with the target enzyme DNA polymerase (Pancheva et al., 2002).

* Corresponding author. Department of Veterinary Medicine of Bari, S.p. per Casamassima km 3, 70010 Valenzano, Bari, Italy. Tel.: +39 08 04679833; fax: +39 08 04679843.
E-mail address: gabriella.elia@uniba.it (G. Elia).

[1] Elia G. and Camero M. contributed equally to this work.

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Table 1
Analogies between CpHV-1 and HSV2.

	HSV2	CpHV-1
Genus	Simplexvirus	Varicellovirus
Natural host	Humans	Goats
Site of latency	Sacral ganglia	Sacral ganglia
Primary target cell	Mucoepithelial	Mucoepithelial
Lesion features	Papulo-vesicular, ulcers, crusts	Papulo-vesicular, ulcers, crusts
Tropism	Vulvo-vaginal mucosa, oro-nasal mucosa	Vulvo-vaginal mucosa, oro-nasal mucosa
Means of spread	Close contact (oral or sexually transmitted infection)	Close contact (oral or sexually transmitted infection)

In this study, the antiviral activity of ACV in combination with MIZ was evaluated against CpHV-1 replication in cell cultures as a propedeutical approach for *in vivo* trials.

2. Experimental/materials and methods

2.1. Cells, virus and compounds

Madin Darby bovine kidney (MDBK) cells were used in the experiments. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum. The CpHV-1 strain BA.1 (Buonavoglia et al., 1996) was cultivated on MDBK cells, titrated and used as stock virus. The virus titre was $10^{7.00}$ Tissue Culture Infectious Doses ($TCID_{50}$)/50 µl. For the experiments, MIZ (Sigma-Aldrich Co., St Louis, MO, USA) and ACV (Sigma-Aldrich Co.) were initially dissolved separately in Saline Solution 0.9% to a stock concentration of 10 mg/ml, then serially diluted in D-MEM to obtain working concentrations.

2.2. Cytotoxicity assay

Each compound was tested for cytotoxicity using an XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)-based method (Sigma-Aldrich Co.). Briefly, confluent monolayers of MDBK cells in 96-well plates were treated with serial twofold dilutions of the tested compound (from 4.4 to 440 µM for ACV and from 77 µM to 1.2 mM for MIZ), then incubated at 37 °C, using the same experimental conditions as those used to evaluate drug antiviral activity. Untreated cells served as control. After 72 hours of incubation, 1 mg/ml XTT was added to each well with a 20% of total cell volume. The cells were then incubated for an additional 4 hours in order to allow for the production of formazan. Optical densities were determined with an automatic spectrophotometer (microtitre plate reader, Biorad 550) at a wavelength of 450 nm. Percentage cytotoxicity was calculated by using the formula: % Cytotoxicity = [(OD of control cells–OD of treated cells)X100]/ OD of control cells.

The CC_{20} were defined as the concentrations at which viability of the treated MDBK cells decreased to 20% of the control cells.

The observed maximum non-cytotoxic concentration of MIZ was then used for the combined experiments with different concentrations of ACV by using the XTT protocol here described.

2.3. Antiviral activity assays

The antiviral efficacy of ACV alone or in combination with MIZ against CpHV-1 replication was assessed on 1-day-old confluent monolayers of MDBK cells by two different experiments.

In the first experiment, confluent MDBK cells in 24-well plates were used. The medium was replaced with 1 ml DMEM containing different concentrations of ACV alone (4.4, 22, 44, 220, 440 µM) or in combination with MIZ. MIZ was always used at the maximum non-cytotoxic concentration of 154 µM. After 6 h of incubation the cells were inoculated with serial dilutions (from 10^{-1} to 10^{-8} TCID/50) of CpHV-1, using two wells for each viral dilution.

Virus-induced cytopathic effect (CPE) was observed microscopically at 3 days post-infection and the apparent viral titres of the samples were calculated by end-point dilution according to the method of Reed and Muench (1933).

The inhibitory concentration 50% (IC_{50}) was determined as the concentration required to reduce virus titer by 50% in the drug-treated cells compared with the untreated infected (CV) wells.

The same experiment was performed three times independently for ACV and for ACV with MIZ, and the IC_{50} values were calculated as the average ± standard deviation (SD) of the three experiments.

In order to understand whether ACV alone or in conjunction with MIZ was able to affect the production of new virions, a second experiment was set up. At the end of the third day of incubation, the plates were frozen and thawed three times and pools of duplicate cryolisates from each virus dilution (from 10^{-3} to 10^{-8}) were serially 10-fold diluted in DMEM (without drug). The pools were inoculated in quadruplicate onto 24-hour-old MDBK cells in 96-wells microtitres plates to determine again the viral titre.

2.4. Data analysis

After logarithmic conversion of drug concentrations, the data obtained in the antiviral activity assay were analysed by non-linear curve fitting procedure (GraphPad Prism version 6.03 software). Goodness of fit was tested by r^2 from non-linear regression and by the runs test. From the fitted dose–response curves obtained in each experiment, the potency, expressed as the concentration required to inhibit virus replication by 50% (IC_{50}) of the drug was inferred.

3. Results

3.1. Cytotoxicity assay

Cells viability was assessed by the XTT assay after exposing the cells to various concentrations of ACV alone or in combination with MIZ for 72 h using the same experimental conditions as those used to evaluate drug antiviral activity. Acyclovir did not cause a significant decrease of viability of uninfected MDBK cells with a $CC_{20} > 440$ µM. MIZ was toxic at a concentration > 154 µM with a CC_{20} value of 220 µM (Fig. 1).

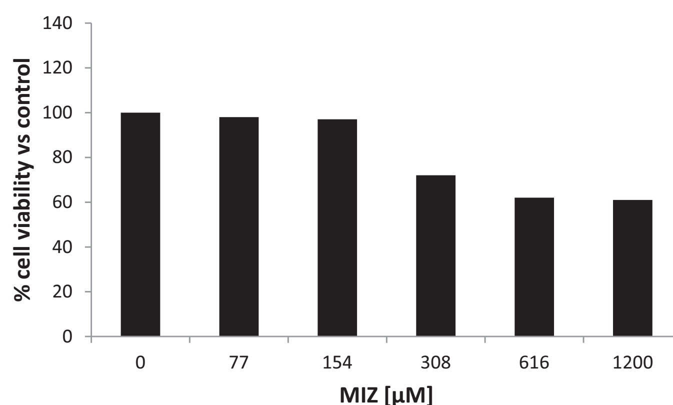

Fig. 1. XTT cytotoxicity assay: percentage of viable cultured after exposure to MIZ at different concentrations. Data are expressed as mean values.

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