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Antiviral Effect of Triple Combination of VP1 Ligands against Coxsackievirus B3 Infection in Newborn Mice

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Abstract

The Coxsackie B viruses (CVB), are distributed worldwide and cause a broad spectrum of diseases. Currently, clinically effective antivirals for the treatment of these infections do not exist. It was found that the unsatisfactory effectiveness of enteroviral inhibitors *in vivo* has been associated with the rapid development of drug resistance and that explains why effective antivirals have failed in monotherapy trials. Combination therapy with two or more drugs has the potential to successfully inhibit viral infection more effectively than either drug alone. It could achieve greater effects and avoid side effects using lower drug concentrations, and thus, they could prevent the rapid development of drug resistance. In this work, we examine the combined treatment effect of three enterovirus replication inhibitors – pleconaril, pocapavir, and vapendavir, which are VP1 hydrophobic pocket ligands. All of them have passed clinical double-blind trials as monotherapy courses, but none of them have been approved for clinical application, because of slide effects. We evaluate the effectiveness of the combination pleconaril/pocapavir/vapendavir (PPV) applied via CAA treatment scheme on Coxsackievirus B3 (CVB3) infection in newborn mice.

Keywords: pleconaril, pocapavir, vapendavir, CAA, triple combination, mice, Coxsackievirus B3

Резюме

Коксаки В вирусите (CVB) са много разпространени и причиняват широк спектър от заболявания. Към момента не съществуват клинично ефективни антиентеровирусни средства за лечение на тези инфекции. Установено е, че ниската ефективност на ентеровирусните инхибитори *in vivo* е свързана с бързото развитие на лекарствена резистентност и това обяснява защо ефективните *in vitro* антивирусни лекарства се провалят при използването им като монотерапия. Комбинираната терапия включваща две или повече лекарства има потенциал да инхибира успешно вирусната инфекция по-ефективно, отколкото самостоятелното използване на лекарства. Тази терапия позволява да се постигне по-добър ефект и да се избегнат страничните ефекти, като се използват по-ниски концентрации на лекарството, по този начин те би могло и да се предотврати бързото развитие на лекарствена резистентност. В настоящето изследване проучваме ефекта на комбинирано лечение с три инхибитора на ентеровирусна репликация – плеконарил, покапавир и вапендавир, които са лиганди на VP1. Всички те са преминали клинични двойно-слепи изпитвания в монотерапевтичен курс, но нито един от тях не е одобрен за клинично приложение поради нежелани странични ефекти.

Introduction

The Coxsackie B viruses (CVB), members of the Enterovirus genus, are distributed worldwide and cause a broad spectrum of diseases, including severe illnesses that involve pathologies of the heart, central nervous system, skeletal muscles, pancreatic β-cells, etc. These viruses have been identified as the viral agents most frequently associated with viral myocarditis and pericarditis in humans (Smith, 1970). Susceptibility to viral infections varies widely among individuals and more than 85% of cases are inapparent infections. Morbidity and mortality rates are especially high in neonates, children, and people with immunodeficiency (Tan, 2005; Pallansch and Roos, 2006; Mallia *et al*., 2007).

Using specific anti-enteroviral drugs as ur-

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gent prophylaxis during disease latency periods could reduce the risk of acquired diabetes and severe forms of enterovirus-induced infections (Hyöty *et al*., 1995; Hyöty and Taylor, 2002; Galabov and Angelova, 2006).

Unfortunately, currently, clinically effective antivirals for the treatment of these infections do not exist. A large number of compounds are effective *in vitro* (Barnard, 2006; De Palma et al., 2008), but in clinical trials, the most active antivirals demonstrate modest effects. Previously was found that the unsatisfactory effectiveness of enteroviral inhibitors *in vivo* has been associated with the rapid development of drug resistance (Melnick *et al*., 1961; Loddo, 1980) by initially drug-sensitive viruses. The rapid development of drug resistance explains why effective antivirals have failed in monotherapy trials. Combination therapy with two or more drugs has the potential to successfully inhibit viral infection more effectively than either drug alone. It could achieve greater effects and avoid side effects using lower drug concentrations, and thus, they could prevent the rapid development of drug resistance.

In previous studies, we developed and tested a combination treatment scheme applied via consecutive (not simultaneous) alternating administration (CAA) of enteroviral inhibitors. The obtained data demonstrate that this scheme prevented the rapid development of drug resistance and provided significant antiviral activity in mice with Coxsackievirus B infection (Vassileva-Pencheva and Galabov, 2010; Stoyanova *et al*., 2015a, 2015b, 2015c; Galabov and Stoyanova, 2016).

In this work, we examine the combined treatment effect of three enterovirus replication inhibitors – pleconaril (WIN 63843), pocapavir (V-073), and vapendavir (BTA 798), which are VP1 hydrophobic pocket binders that attack in the early phases of enteroviral replication. All of them have passed clinical double-blind trials as monotherapy courses, but none of them was approved for clinical application, because of side effects. We evaluate the effectivity of the combination pleconaril/pocapavir/vapendavir (PPV) applied via CAA treatment scheme on Coxsackievirus B3 (CVB3) infection in newborn mice.

Materials and Methods

Pleconaril (WIN 63843) was synthesized by Dr. Vadim Makarov (State Research Center for Antibiotics, Moscow, Russia). Pocapavir (V-073) was obtained from MedKoo Biosciences, Morrisville, NC, USA. Vapendavir (BTA-798) was synthesized by Dr. Georgi Dobrikov (Institute of Organic Chemistry with Center of Phytochemistry, BAS). The compounds were dissolved in polyethylene glycol 400 (PEG 400).

Coxsackievirus B3 (Woodruff strain) used for *in vivo* experiments was obtained through intracerebral passages (0.02 ml/mouse) in newborn albino mice and prepared as a 10% brain suspension in phosphate-buffered saline (PBS). The virus underwent at least three intracerebral passages in newborn mice (without intermediary passages in cell cultures).

The study was carried out on ICR random-bred newborn albino mice (obtained from the Experimental and Breeding Base for Laboratory Animals of the Bulgarian Academy of Sciences, Slivnitza, Bulgaria). Experiments were conducted in accordance with the national requirements for animal experiments in compliance with Council Directives 86/609/EEC. The animals were housed in a controlled environment (26±2°C, humidity approx. 50%) with a 12-h light/dark cycle, the mother mice had *ad libitum* access to food and water.

The newborn mice were divided into four main groups: uninfected and untreated or drug-treated mice, saline-treated CVB3-infected mice (placebo), drug-treated CVB3-infected mice (pleconaril, pocapavir, and vapendavir monotherapy groups), and CVB3-infected CAA-treated mice (triple combination group; tested compounds are applied via consecutive alternating administration, CAA).

Before treatment, newborn mice received subcutaneous inoculations of CVB3, 20 LD_{50} (median lethal dose). Drugs were administered per os (pleconaril) or by subcutaneous (pocapavir, vapendavir) injection in a volume of 0.05 ml.

CAA treatment groups received one compound per day, administered consecutively, and applied 1 hr post-inoculation (Day 1). Each compound in the combination was administered every third day, starting with pleconaril, followed by pocapavir, and ending with vapendavir (Table 1).

The dose of pleconaril (25 mg/kg) was selected as optimal based on our previous studies (Stoyanova *et. al.,* 2015). The doses of pocapavir were 12.5, 25, 37.5, or 50 mg/kg, vapendavir was applied in doses of 12.5, 25, 37.5, 50, or 100 mg/kg.

Cumulative mortality (percentage), mean survival time (days), and daily body weight were recorded. Statistical analysis was conducted using a 2-tailed unpaired Fisher's exact test, one-way ANOVA followed by Bonferroni's post-test. $P < 0.05$ was considered statistically significant. Survival curves were plotted according to the Kaplan–Meier method (log-rank test).

Table 1. Compound administration schedule.

Groups	Days								
	1	2	3	4	5	6	7	8	9
Consecutive PPV therapy	Ple	Poc	Vap	Ple	Poc	Vap	Ple	Poc	Vap
Monotherapy	Either Ple, Poc or Vap administered daily								
Placebo	Saline solution administered daily								

Ple (P) = pleconaril, oral; Poc (P) = pocapavir, subcutaneous; Vap (V) = vapendavir, subcutaneous. Compounds were administered once per day, beginning 1 hr post-inoculation.

Results

Mice infected with CVB3 began to show physical signs of illness soon after infection. Deaths began occurring on day 2 but peaked on days 4-7. By day 10 there are no alive animals, except the pleconaril monotherapy group (Fig. 1a, b). The combination of pleconaril, pocapavir, and vapendavir, in different doses, applied via CAA course, demonstrated increased MST in CVB3-infected mice, reaching up to 3.5 days compared to placebo, but were without protective effect (Table 2 and Fig. 1). CAA combination of pleconaril 25mg/kg, pocapavir 50 mg/kg and vapendavir 100 mg/kg demonstrate low protective effect (PI=12.5%). Similar to our previous study, pleconaril in a dose of 25 mg/kg, applied in a monotherapeutic course, demonstrates a protective effect and increased MST.

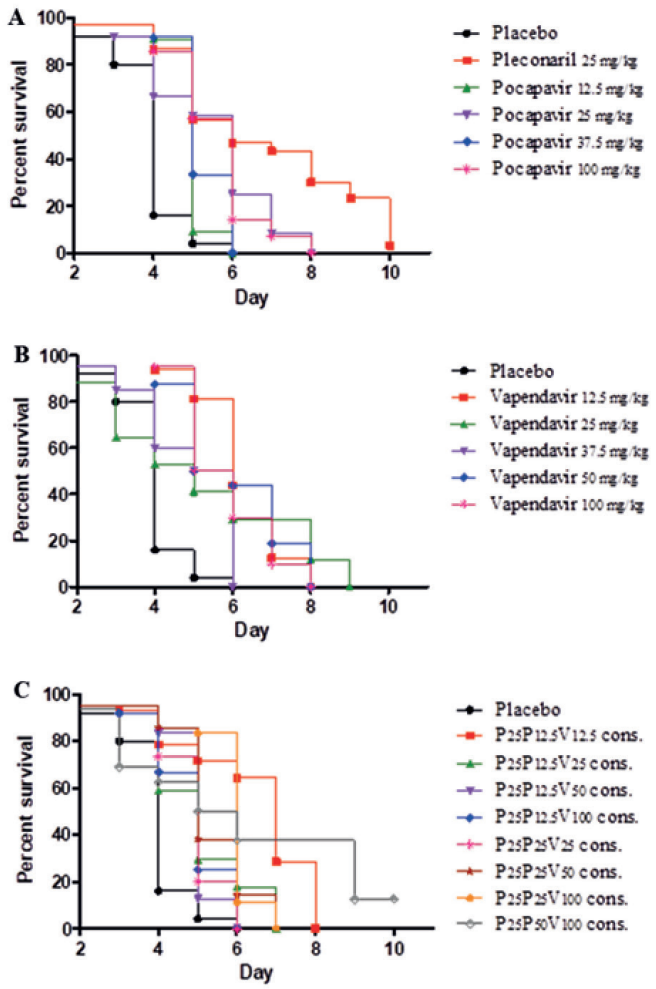

Fig. 1. Survival animals in groups with daily administration of (A) pocapavir monotherapies at different daily doses, (B) vapendavir monotherapies at different daily doses, and (C) CAA combination of PPV at different daily doses, applied consecutively, against experimental infection with Coxsackievirus B3 in newborn mice.

Pleconaril-only (25 mg/kg), given every third day, was ineffective (Table 2). Monotherapies of pocapavir and vapendavir in different daily doses also with no protective effect but increased MST in infected mice.

Uninfected and drug-treated (as monotherapy) mice showed no signs of increased toxicity based on the daily weight of the compounds used at the lower tested doses (12.5 and 25 mg/kg) compared to mice that did not receive the tested compounds (not illustrated).

Discussion

CVB3, like other RNA viruses, demonstrates an increased risk of rapid development of drug resistance. Combination therapy is one of the preferred options for limiting these processes. There are many studies on the combination effect *in vitro* of anti-enterovirals (Stoyanova and Galabov, 2021), but a limited number of combinations are explored in laboratory animals (Eggers, 1976; Herrmann *et al*., 1982; Vassileva-Pencheva and Galabov, 2010, 2016; Stoyanova *et al*., 2015b, 2015c, 2020, 2021).

In previous studies, the CAA treatment scheme provided significant activity and prevented the development of drug resistance in mice infected with neurotropic Coxsackievirus B1 (Vassileva-Pencheva and Galabov, 2010; Galabov *et al*., 2012; Stoyanova *et al*., 2015b, 2015c; Galabov and Stoyanova, 2016).

Based on these results, we considered testing in the CAA course of compounds that passed clinical trials because of the good knowledge of their pharmacological dynamics and effects. Three of these compounds that have been evaluated in phase I and II clinical trials were pleconaril, pocapavir,

Table 2. Effects of PPV (pleconaril/pocapavir/vapendavir) administered consecutively, as monotherapies, and placebo on induced Coxsackievirus B3 infection in newborn mice.

Compounds	Survivors/Total[a]	Mortality (%)	MST (days)[b]	D, days	PI (%)
$P_{25}P_{12.5}V_{12.5}$ consecutively	0/21	100	7.7**	+3.4	0
$P_{25}P_{12.5}V_{25}$ consecutively	0/24	100	7.4*	+3.1	0
$P_{25}P_{12.5}V_{50}$ consecutively	0/20	100	6.5ns	+2.2	0
$P_{25}P_{12.5}V_{100}$ consecutively	0/23	100	5.9 ns	+1.6	0
$P_{25}P_{25}V_{25}$ consecutively	0/21	100	4.1 ns	-0.2	0
$P_{25}P_{25}V_{50}$ consecutively	0/26	100	7.8**	+3.5	0
$P_{25}P_{25}V_{100}$ consecutively	0/20	100	5.9 ns	+1.6	0
$P_{25}P_{50}V_{100}$ consecutively	2/16	87.5	6.1 ns	+1.8	12.5
Pleconaril 25 mg/kg	9/38^^	76.3	7.4**	+3.1	23.7
Pleconaril 25 mg/kg 2 days apart	0/23	100	4.4	+0.1	0
Pocapavir 12.5 mg/kg	0/22	100	5.0	+0.7	0
Pocapavir 25 mg/kg	0/24	100	4.8	+0.5	0
Pocapavir 37.5 mg/kg	0/24	100	5.3	+1.0	0
Pocapavir 50 mg/kg	0/28	100	5.8	+1.5	0
Vapendavir 12.5 mg/kg	0/27	100	5.4	+1.1	0
Vapendavir 25 mg/kg	0/33	100	5.6	+1.3	0
Vapendavir 37.5 mg/kg	0/12	100	3.8	-0.5	0
Vapendavir 50 mg/kg	0/15	100	6.4	+2.1	0
Vapendavir 100 mg/kg	0/20	100	6.1	+1.8	0
Placebo PBS	0/34	100	4.3	–	0

See Table 1 for treatment regimens. MST = mean survival time; PBS = phosphate-buffered saline; $P_{\#}P_{\#}V_{\#}$ = tritherapy of pleconaril dose (mg/kg), pocapavir dose (mg/kg), and vapendavir dose (mg/kg); PI = protection index.

[a] Two-tailed Fisher's exact test.

^^ $p < .001$ vs Placebo

[b] Statistical analyses performed with one-way ANOVA and Bonferroni's multiple comparison post-test

** $p < 0.001$ vs Placebo

* $p < 0.01$ vs Placebo

ns $p > 0.05$ vs Placebo

and vapendavir – hydrophobic pocket binders. The tested compounds are VP1 ligands and share a similar mechanism of action – block the early stages of the viral replicative cycle by binding to the VP1 protein in the viral capsid, thereby preventing the binding of the virus to the target and its subsequent process into the cell.

Despite their promising *in vitro* potencies, they demonstrated insufficient efficacy and unwanted side effects in clinical trials. An advantage of the CAA treatment course is that the compounds are administered once per day and each day the substance is different from the previous one, this allows to avoid the compound's potential toxic effect and also prevents rapid development of drug resistance (Stoyanova *et al*., 2020, 2021).

Pleconaril holds a leading position among WIN enterovirus replication inhibitors (Pevear *et al*., 1999; Groarke and Pevear, 1999; Schiff and Sherwood, 2000). In their 2001 study, Aradottir *et al*. (2001) found a marked efficacy of pleconaril in 2 of 3 neonates with enteroviral hepatitis, while Rotbart and Webster (2001) found that pleconaril treatment for chronic meningoencephalitis resulted in 78% improvement, with minimal adverse effects. However, it was not effective in a double-blind trial in children with enteroviral meningitis, where twice as many adverse effects were observed (Abzug *et al*., 2003).

In a phase III double-blind placebo-controlled trial, Hayden *et al*. (2003) found that pleconaril had some efficacy against Coxsackievirus A21-induced respiratory infections (common cold) when the treatment started within 24 hours of symptom onset. However, in 2002 the U.S. Food and Drug Administration (FDA) stopped further trials due to drug-increased levels of the CYP3A4 liver microsomal enzyme.

More recently, pleconaril showed virucidal properties in hand treatments to prevent rhinovirus infection (Turner and Hendley, 2005). In 2006–2007, *Schering-Plough* conducted a placebo-controlled study of the effects of nasal spray on common cold symptoms and asthma exacerbations

following rhinovirus exposure (Hayden *et al*., 1995; www.Clinical Trials.gov; NIH, 2007).

Pocapavir (V-073, SCH 48973), is a small-molecule capsid inhibitor that blocks virus uncoating and viral RNA release into cells, an investigational drug candidate being developed for poliovirus indications, but also has variable antiviral activity against nonpolio enteroviruses Pocapavir is a potent, selective, anti-enterovirus molecule with *in vitro* and *in vivo* activities (Buontempo *et al*., 1997).

Torres-Tores *et al*. (2015) described the first use of pocapavir for the treatment of 33 33-week-old female infants with neonatal enteroviral sepsis with hepatic necrosis with coagulopathy (HNC) syndrome. Pocapavir used in a once-daily enteral regimen was associated with no significant adverse drug-related events in a premature infant with severe enteroviral sepsis. In another randomized blinded placebo-controlled study, immunodeficient individuals were challenged with a monovalent oral poliovirus type 1 vaccine (mOPV1) and subsequently treated with pocapavir or a placebo. Treatment with pocapavir was safe and significantly accelerated virus clearance. The emergence of resistant virus and transmission of the virus were seen in the context of a clinical isolation facility (Collett *et al*., 2017).

Vapendavir (BTA-798) belongs to oxime ether analogs of pirodavir developed by Biota Pharmaceuticals and displays a broader spectrum of *in vitro* activity against virus isolates than pleconaril (Watson *et al*., 2003). The compound mechanism of action rendered the susceptible virus serotypes non-infectious by direct contact, and the neutralized viruses became stabilized to acid and heat, strongly suggesting a direct interaction of the compound with the capsid protein. Drug-resistant mutants were shown to exhibit cross-resistance to WIN compounds and other capsid-binding agents. Indeed, studies have shown that the compound binds to the capsid protein in the same hydrophobic pocket, underneath the canyon floor as the WIN compounds (De Palma *et al*., 2008).

A phase I clinical trial was completed with a vapendavir, which was developed for the treatment and prevention of HRV infections in high-risk chronic obstructive pulmonary disease (COPD) and asthma patients. Recently, Tijsma *et al*. (2014) reported that the compound failed to reduce asthma exacerbations in a phase II clinical trial.

The CAA-PPV treatment manifested lengthened MST in newborn mice with CVB3 infection. Unfortunately, unlike a previous study of ours, in this combination of antivirals, the CAA treatment course had no protective effect on infected mice. The data shows lengthened MST in monotherapy-treated mice groups (pleconaril, pocapavir, or vapendavir monotherapy groups). Similar to the results obtained from our previous studies, pleconaril monotherapy demonstrated well well-expressed protective effect, and almost one-third of infected animals were alive at the end of treatment. This result is not surprising considering the data on the antiviral activity of this compound. On the other hand, based on the protection index (PI) percentage, pocapavir and vapendavir applied in different daily doses did not show an effect (PI=0).

In summary, our results show that CAA therapy, using pleconaril, pocapavir, and vapendavir, was without significant protective effect against CVB3 infection. The CAA-treated mice with pleconaril in a dose of 25mg/kg, pocapavir in a dose of 50 mg/kg, and vapendavir in a dose of 100 mg/kg demonstrate low protective effect (PI=12.5%). All tested combinations result in increased MST in infected mice. A possible reason for this result is the similar mechanism of action of the tested compounds. In our previous studies, we used compounds with different mechanisms of action and thereby proved the key role of the arrangement of partner compounds in the combinations. In the investigated combination, the mechanism of action of partner compounds is similar, so we can assume that in this case, the arrangement is not essential for the ultimate antiviral effect. To clarify this question, further studies of the viral population are needed to elucidate the effect that the combination has on the development of resistance (including cross-resistance) by the viral progeny to the test compounds.

Anyway, by following this approach, researchers can identify and develop successful models with other therapy combinations, the results of which could be the basis for new therapeutic strategies.

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