Antiviral Research 85 (2010) 366–372

Contents lists available at ScienceDirect

Antiviral Research

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

Avoiding drug-resistance development by novel approach of combining anti-enteroviral substances against coxsackievirus B1 infection in mice

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ARTICLE INFO

Article history:
Received 14 May 2009
Received in revised form 29 August 2009
Accepted 3 November 2009

Keywords:
Coxsackievirus B1
Combinations
Mice
Resistance
Drug sensitivity

ABSTRACT

Current study presents a novel scheme for combined application of anti-enteroviral substances in coxsackievirus B1 neuroinfection in newborn mice. It consists of a consecutive alternating, not simultaneous, administration of the substances in combination. A triple combination showing good efficacy was selected as a result of a screening of double, triple and quadruple combinations of enteroviral inhibitors. Its effectiveness is expressed in lengthening of the mean survival time and about 50% reduction of mortality rate in infected newborns as compared both to the placebo group, individual compounds used alone every day, and to the same combination applied simultaneously every day. Chronology of alternation of the individual drug administration plays a key role in the efficacy of the combination. Studies of the drug sensitivity of viral brain isolates from mice, treated with the drug combination indicate that virus isolates from the group treated with the alternating combination not only preserve, but even increase their sensitivity to the drugs. MIC_{50} values of virus isolates from groups treated with monotherapies of the compounds manifested development of drug resistance. Obviously, the consecutive alternating administration of anti-enteroviral substances hinders the occurrence of drug resistance in the course of experimental coxsackievirus B1 infection in mice.

1. Introduction

Human enteroviruses, including polioviruses, coxsackieviruses, echoviruses and rhinoviruses represent a significant health problem due to the unusual diversity of diseases they cause. This wide spectrum ranges from mild enteritides, respiratory infections such as common cold and summer flu, manifested by pharyngitis, bronchitis and bronchiolitis, croup, pneumonia, to severe diseases of the central nervous system such as poliomyelitis, encephalitis, aseptic meningitis, neuritis and polyneuritis, including pleurodynia (Bornholm disease). Neurotropism of many of these viruses, especially coxsackieviruses determine their affinity to the pancreatic beta cells, may have paraneuronal origin (Fujita, 1977, 1989; Fujita et al., 1988). Enteroviral infection of these cells could lead to development of juvenile acquired insulin-dependent diabetes. One collaborative study shows that most children recently diagnosed with such severe form of diabetes had been infected with coxsackieviruses (Hyöty et al., 1995). Enteroviruses could replicate in the heart tissue and this cardiotropism could lead to serious heart disorders such as pericarditis and myocarditis, which often precede dilatative cardiomyopathy. For the rhinoviruses, recently included in the Enterovirus genus (announced during the 14th International Congress of Virology, organized by IUMS, August, 2008) there are more than 150 types and they are known as the causative agents of the common cold. They could contribute to the development of sinusitis and other chronic respiratory diseases, which could lead to chronic obstructive pulmonary disease, projected to be the third leading cause of death worldwide by 2020 (Rabe et al., 2007).

Fighting these viruses experiences significant difficulties because of their extreme contagiousness as well as the very high percentage of unapparent infections (more than 85%). Registered clinical cases caused by enteroviruses are only top of the iceberg—lying underneath is a great number of non-manifested infections that could lead to development of severe complications. When summarized, these facts result in limited opportunities for taking anti-epidemic steps.

All of the mentioned features of this virus genus show that application of chemotherapeutic agents is strongly indicated in the enterovirus-caused diseases. Despite the large number of in vitro effective compounds (Barnard, 2006; De Palma et al., 2008), so far anti-enteroviral chemotherapeutics for clinical use are not registered, and the results of clinical trials carried out with the most active antivirals could be considered as modest. Unsatisfactory effectiveness of enteroviral replication inhibitors in vivo

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doi:10.1016/j.antiviral.2009.11.003

has been ascribed to the rapid development of drug resistance of initially drug-sensitive viruses. This phenomenon is related to the quasispecies composition of enteroviral population (Eigen and Biebricher, 1988; Domingo et al., 2008) which is a consequence of an accumulation of resistant population of pseudospecies as a result of a countless number of point mutations due to errors introduced by the viral RNA-dependent RNA polymerase during viral replication. It was found on a model of poliovirus type 1 that each newly synthesized molecule of poliovirus RNA contains, on average, one mutation (Chumakov et al., 2007). As a result of a Darwinian type of selection, drug-resistant mutants have been developed to almost every specific enterovirus replication inhibitor.

The resistance occurring after monotherapy with a certain drug being the main obstacle to an effective therapy of enteroviral infections makes it reasonable to focus interest on combined administration of antiviral compounds. This approach has proven its efficacy against HIV and HCV infections and may be applied in a situation of pandemic flu as well. Use of drug combinations of antivirals might be one of the possible efficient approaches to overcome the disadvantages of monotherapy concerning the development of resistance to each or all of the partners in the combination. Using such combined therapy, a greater effect could be achieved at lower concentrations than those required if drugs were to be used alone and thus to prevent the so-called "pressure of the dose" effect, which favors the rapid development of virus-drug resistance.

Due to the multiple viral replication cycles in the presence of the different substances in combination, even the application of synergistic combinations with proven activity in vitro (Nikolaeva-Glomb and Galabov, 2004) does not guarantee that the occurrence of single or multiple resistance could be avoided.

In the current study we investigate a novel approach for combined administration of anti-enteroviral compounds with proven effect, which consists of a consecutive, not simultaneous application of the substances included in the combination in an experimental infection in vivo. We used an in vivo model of infection with neurotropic coxsackievirus B1 infection in newborn mice. Compounds used in combination were selected as specific inhibitors of enteroviral replication with a different mode of action and attacking different targets in the replicative cycle. Among them was disoxaril – a representative of one of the most active anti-enteroviral inhibitors – the WIN compounds (Diana et al., 1985; Smith et al., 1986; Rossmann, 1989). It is a VP1 hydrophobic pocket binder, which is active in the early steps of the viral replication. Guanidine hydrochloride is a "classic" enteroviral inhibitor that interferes with the function of 2C protein, and thus prevents the initiation of the synthesis of negative RNA strands (Loddo et al., 1962; Barton and Flanegan, 1997). Oxoglaucine is a compound of plant origin, specifically inhibiting replication of a broad spectrum of enteroviruses (Nikolaeva-Glomb et al., 2008). Its mechanism of action is not completely cleared, but it is assumed that it inhibits some of the early steps in the viral replication cycle (L. Nikolaeva, A.S. Galabov, unpublished data). PTU-23 is a wide-range picornavirus inhibitor attacking the synthesis of both viral 37S and 20S (RF) RNA (Galabov, 1979; Galabov and Dmitrieva, 1983; Galabov et al., 1983).

Selection of the compounds included in this study was based on the data for their efficacy in vivo, in experimental enteroviral infections in laboratory animals. Guanidine HCl is an exception: it is known that the only evidence for its activity in animals is given when the substance is applied in combination with another anti-enteroviral inhibitor—HBB (Eggers, 1976). The effect of oxoglaucine applied alone against enterovirus infection with coxsackievirus B1 in newborn mice is relatively modest (A.S. Galabov, S. Spassov, unpublished data).

2. Materials and methods

2.1. Virus

Coxsackievirus B1 stock for in vivo experiments was obtained through intracerebral passages (0.02 ml/mouse) of the standard laboratory strain (Connecticut 5) in newborn albino mice (ICR line) and was prepared as a 10% brain suspension in phosphate-buffered saline (PBS). The virus underwent multiple intracerebral passages in newborn mice (without intermediary passages in cell cultures).

2.2. Cells

FL cells (transformed human amnion cells) monolayer cultures in CELLSTAR 35 mm petri dishes (Greiner Bio-One GmbH, Frickenhausen, Germany)—for plaque purification of the viral isolates from mouse brains, and 6-well microplates CELLSTAR—for determination of MIC_{50}, grown at 37 °C in 5% CO_2 thermostate HERAcell (Kendro Laboratory Products GmbH, Langensbold, Germany) were used. Growth medium contained 10% fetal bovine serum (Gibco BRL, Paisley, Scotland, UK) and antibiotics (penicillin, 100 IU/ml, streptomycin, 100 μg/ml) in Dulbecco's MEM (minimal essential medium) (Gibco BRL, Paisley, Scotland, UK). Maintenance solution was 0.5% heated fetal bovine serum in Dulbecco's MEM.

2.3. Mice

ICR random-bred newborn albino mice (obtained from the Laboratory Animals Farm of the Bulgarian Academy of Sciences, Slivnitza, Bulgaria). This random-bred stock was originally developed at the Institute for Cancer Research (Fox Chase Cancer Center) by Dr. T.S. Hauschka beginning 1948.

2.4. Compounds tested

5-[7-[4(4,5-Dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole (disoxaril, WIN 51711) was supplied by Sanofi Winthorp, Inc. (Malverne, Pennsylvania).

Oxoglaucine, an aporphinoid alkaloid from *Glaucinum flavum* Cranz, was obtained from the Institute of Organic Chemistry, Bulgarian Academy of Sciences.

Guanidine hydrochloride was provided by Eastman Organic Chemicals (New York).

N-Phenyl-N′-3-hydroxyphenylthiourea (PTU-23), original Bulgarian compound, was synthesized by Dr. Boryana Hajieva from Faculty of Chemistry, University of Sofia, after the method of Prof. G. Vassilev, Institute of Plant Physiology, Bulgarian Academy of Sciences.

2.5. Coxsackievirus B1 infection in newborn mice

2.5.1. Testing of double, triple and quadriple combinations of anti-enteroviral compounds

Groups of newborn mice (ICR line) were inoculated subcutaneously (s.c.) with coxsackievirus B1, 20 LD_{50}. Animals were subjected to combined treatment course with two, three and four compounds, administered consecutively, starting on the day of virus inoculation. Each substance was injected via subcutaneous route every other day (double combinations—DC), every third day (triple combinations—TC) and every fourth day (quadruple combination—QC). The course lasted until day 12 after virus inoculation.

The general scheme of arrangement of the treatment courses is presented in Table 1.

Daily doses of each compound were selected as optimal, based on literature data and previous experiments in our laboratory and are as shown in Table 2.

Table 1
Scheme of daily administration of the used compounds in different combinations or applied alone.

Compounds in combination or as monotherapies			Days											
			Compounds used in combination											
			1	2	3	4	5	6	7	8	9	10	11	12
Consecutive combination	DC	Dis/Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo
		Dis/Guan	Dis	Gua	Dis	Guan	Dis	Gua	Dis	Gua	Dis	Gua	Dis	Gua
		Dis/PTU	Dis	PTU	Dis	PTU	Dis	PTU	Dis	PTU	Dis	PTU	Dis	PTU
	TC	D/O/G	Dis	Oxo	Guan	Dis	Oxo	Gua	Dis	Oxo	Gua	Dis	Oxo	Gua
		D/G/O	Dis	Gua	Oxo	Dis	Guan	Oxo	Dis	Gua	Oxo	Dis	Gua	Oxo
	QC	D/O/G/PTU	Dis	Oxo	Gua	PTU	Dis	Oxo	Gua	PTU	Dis	Oxo	Gua	PTU
Simultaneous triple combination			Combination of Dis, guan and Oxo is simultaneously applied every day											
Monotherapies of the partner substances			Each drug applied every day h partner is applied every day											
Placebo			PBS every day											

Dis (D), disoxaril; Oxo (O), oxoglaucine; Gua (G), guanidine HCl; PTU (P), PTU-23.

Along with the placebo group the following control groups were present in each experiment—disoxaril, oxoglaucine, guanidine HCl and PTU-23, administered alone every day, the same administered every other day, and every third day.

In all these experiments two parameters were monitored—cumulative mortality (in percentage) and mean survival time (in days).

2.5.2. Evaluation of the influence of the arrangement of the partners in the combinations

Groups of newborn mice were inoculated s.c. with coxsackievirus B1, 20 LD_{50} per mouse. There were three sets of triple combinations, the first of which started with disoxaril, the second with oxoglaucine and the third with guanidine hydrochloride.

2.5.3. Preparation of virus brain isolates from treated mice

Groups of 40–50 newborn mice were inoculated s.c. with Coxsackie B1 virus (20 LD_{50}/mouse). Treatment with the combination disoxaril/guanidine HCl/oxoglaucine (DGO) and with the partners in the combination applied alone, started on the day of infection, and lasted till the 12th day. Virus samples (four brain samples from each of the treated and the placebo group) were taken every day since day 4 post-virus inoculation. These brain isolates (mixed sample of the brains of 4 animals) were prepared as a 10% brain suspension in phosphate-buffered saline (PBS) for testing.

2.5.4. Virus assay and plaque purification of virus isolates

The viral content in the brain isolates was determined by the plaque method (in PFU/ml). Monolayer FL cell cultures in 35 mm petri dishes CELLSTAR were inoculated with 10-fold dilutions of each virus stock, and left for 1 h at room temperature for virus adsorption. The agar overlay (1.75 ml per dish) was 1% purified Difco agar in Dulbecco's MEM, supplemented with 10% heated calf serum, 1.65 μg/ml sodium bicarbonate and antibiotics (penicillin, 100 IU/ml, streptomycin, 100 μg/ml). After 48-h incubation at 37 °C a second, neutral red containing agar overlay (1.5% agar with 0.02% neutral red Fluka, Buchs, Switzerland, in physiological saline) was added and petri dishes were kept at room temperature for at least 4 h. The virus titer in PFU/ml was evaluated. A single plaque from each viral stock, which was isolated from the appropriate dilution, was resuspended in 5% heated calf serum in Dulbecco's MEM and subjected to three further rounds of plaque purification.

2.5.5. Determination of sensitivity to disoxaril of virus isolates

The plaque-inhibition test of Herrmann (1961) and Siminoff (1961) was applied on virus progenies that had been previously plaque purified (3 rounds). Monolayer FL cell cultures in 6-well microplates CELLSTAR were inoculated with 35–40 PFU/ml of virus per well, and left for 1 h at room temperature for virus adsorption. The agar overlay (as described above) was laid over the cells. The test compound—disoxaril, was included in the agar overlay at the following concentrations: 0.032, 0.1, 0.32, 1, 3.2, 10 and 32 μM. Following a 48-h incubation period at 37 °C a second, neutral red containing agar overlay (as described above) was added and microplates were kept at room temperature for at least 4 h. The percentage of PFU inhibition was evaluated in comparison to the control (without test compound in the agar overlay). The 50% minimal inhibitory concentration (MIC_{50}) of compound that inhibited the plaque titer by 50% for each virus sample was determined.

2.5.6. Statistical analysis

The survival time includes the period from the day of virus inoculation until the day before the animals' death. The mortality was followed until day 12. PI (protection index) was evaluated by the equation $PI = [(PC - 1)/PC] \times 100$, where PC is protection coefficient (that is, % mortality in placebo group/%mortality in the drug-treated group). Fisher's exact test was applied to compare mouse survival rate between the experimental groups. Differences in MST were compared using a two-tailed Student's *t*-test. *P*-Values <0.05 were estimated as statistically significant. To determine the statistical differences (if any) between values of MIC_{50} of disoxaril alone, the combination and the placebo one-way ANOVA with Bonferroni's post-test was used.

Table 2
Optimal daily doses of the used compounds in different combinations or applied alone.

Compound	Effective daily dose (mg/kg)	Animals	References
Disoxaril	25	Newborn mice	McKinlay and Steinberg (1986) and Nikolaeva and Galabov (2000)
Oxoglaucine	25	Newborn mice	A.S. Galabov and S. Spasov (unpublished data)
Guanidine HCl	48	Newborn mice	Herrmann et al. (1982)
PTU-23	130	Newborn mice	Galabov and Velichkova (1974) and Galabov (1979)

Table 3
Effect of consecutively applied double, triple and quadruple combinations of anti-enteroviral inhibitors against neurotropic coxsackievirus B1 infection in newborn mice.

Compounds	Survivors/total	Mortality, %	MST ± SE, days[a]	PC	PI %
DO	0/32	100	5.13 ± 0.1[*]	1	0
DG	4/28	85.7	7.6 ± 0.8[*,¶,§]	1.17	14.53
DP	0/12	100	3.7	1	0
DOG	5/34	85.2	5.6 ± 0.4[*,¶]	1.17	14.53
DOGP	0/25	100	4.0	1	0
Disoxaril	0/35	100	3.5 ± 0.5	1	0
Oxoglaucine	0/32	100	4.7 ± 1	1	0
Guanidine HCl	0/30	100	4.5 ± 0.4	1	0
PTU-23	0/26	100	3.6	1	0
Placebo (PBS)	0/30	100	3.9 ± 0.2	1	0

The data in Table 1 are from three independent experiments. MST, mean survival time; PBS, phosphate-buffered saline; PC, protection coefficient; PI, protection index; se, standard error. DO, double combination of disoxaril and oxoglaucine; DG, disoxaril and guanidine HCl; DP, disoxaril and PTU-23; DOG, triple combination of disoxaril, oxoglaucine and guanidine HCl; DOGP, quadruple combination of disoxaril, oxoglaucine, guanidine HCl and PTU-23.

[a] Two-tailed Student's *t*-test.

[*] ANOVA: $P < 0.01$ compared to placebo group.

[¶] ANOVA: $P < 0.01$ compared to disoxaril monotherapy group.

[§] ANOVA: $P < 0.05$ compared to guanidine hydrochloride alone and oxoglaucine alone groups.

3. Results

3.1. Testing of the effects of combinations of consecutively applied anti-enteroviral compounds on experimental infection with coxsackievirus B1 in newborn mice

For these experiments four compounds with different modes of anti-enteroviral action were selected: disoxaril, oxoglaucine, guanidine HCl and PTU-23. The effect of the alternation of two, three and four substances was determined. Disoxaril, as a representative of one of the most active antipicornavirus inhibitors—WIN compounds (from Winthrop-Sanofi), was present in all of the tested combinations.

The data in Table 3 demonstrate some antiviral activity only of the double combination DG and the triple combination DOG (disoxaril, oxoglaucine and guanidine HCl). The other double drug combinations (DO and DP), as well as the quadruple combination DOGP proved to be ineffective. Monotherapy of the tested substances was also unsuccessful. The favorable effect of the first two mentioned combinations is expressed mostly in lengthening of the mean survival time compared to the placebo group. Therefore, it was of some interest to examine the effect of a triple combination, consisting of disoxaril, guanidine hydrochloride and oxoglaucine.

Table 4
Effect of the combination of disoxaril, oxoglaucine and guanidine hydrochloride during the treatment of experimental neuroinfection with coxsackievirus B1 in newborn mice.

Compounds	Survivors/total	Mortality, %	MST, days	PC	PI %
DOG	2/11	81.8	6.5	1.22	18.03
DGO	4/9	55.5	8.8	1.8	44.44
ODG	0/7	100	4.0	1	0
OGD	2/8	75	7.8	1.33	24.81
GOD	0/8	100	6.5	1	0
GDO	0/10	100	4.2	1	0
Placebo (PBS)	0/9	100	5.0	1	0

For abbreviations (MST, PC and PI), see legend of Table 3.

3.2. Evaluation of the impact of the arrangement of the partners in the combinations

The results presented in Fig. 1 clearly show that the most suitable arrangement for the DGO combination when the first partner

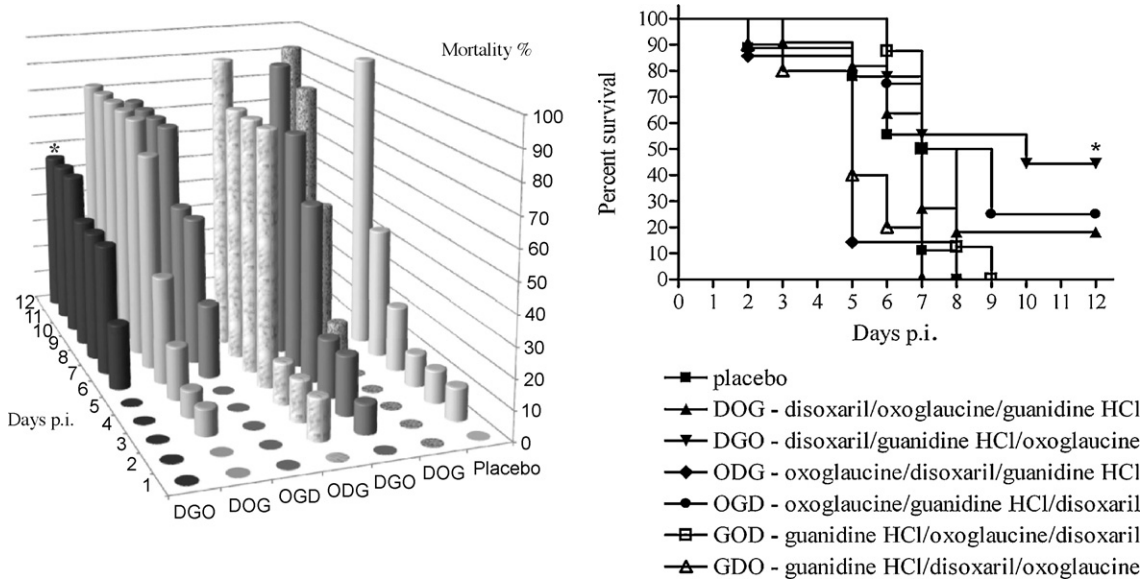

Fig. 1. Effect of the combination of disoxaril, oxoglaucine and guanidine hydrochloride when the order of consecutive administration of the partner compounds was changed. [*] $P < 0.0001$ compared to the placebo group. Statistical data are based on survivor numbers from three experiments by logrank test.

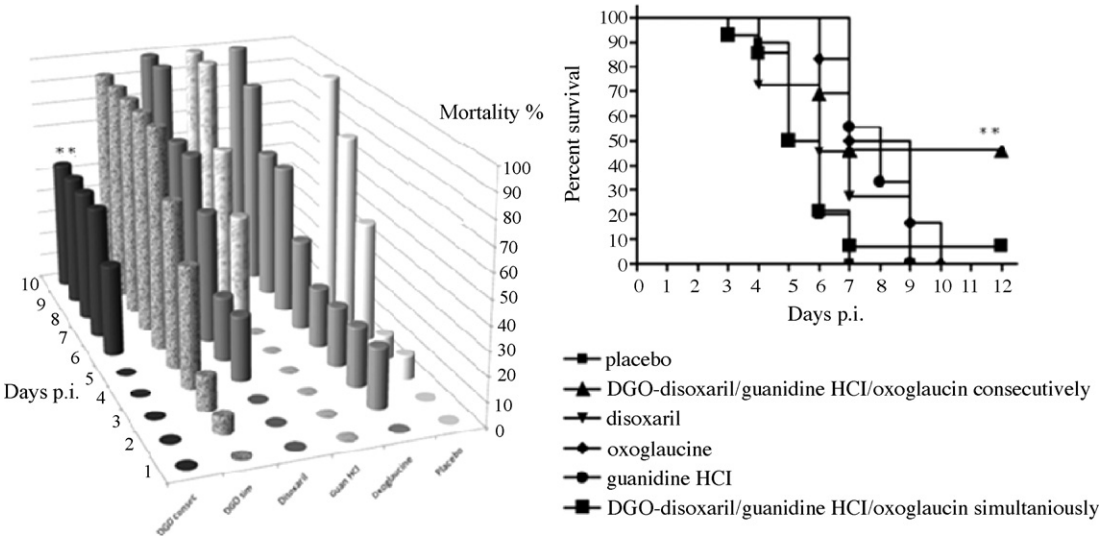

Fig. 2. Survival proportions of triple combination DGO vs. the effect of individual monotherapies of the partners and the effect of the same combination DGO in which the inhibitors are given simultaneously. $^{**}P < 0.005$ compared to placebo. Statistical analysis was determined 7 by logrank test.

Table 5
Comparison of the effects of the triple combination disoxaril, guanidine HCl and oxoglaucine administered at consecutive and simultaneous treatment course.

Compounds	Survivors/total	Mortality, %	MST ± SE, days[a]	PC	PI %
DGO consecutively	15/34[**]	55.8	8.3 ± 0.3	1.79	44.13
DGO simultaneously	1/33	96.9	5.7 ± 0.15	1.03	2.91
Disoxaril	0/28	100	5.5 ± 0.15	1	0
Oxoglaucine	0/29	100	4.9 ± 0.3	1	0
Guanidine HCl	0/30	100	5.5 ± 0.6	1	0
Placebo (PBS)	0/32	100	5.1 ± 0.2	1	0

Data are collected from three experiments. For abbreviations (MST, PC and PI), see legend of Table 3.
[a] Two-tailed Student's *t*-test.
[**] $P < 0.005$—statistical analysis was performed by logrank test.

is disoxaril, followed by guanidine HCl and oxoglaucine (DGO). The protective effect of this combination is very well expressed by lengthening of the MST with almost 4 days—MST for the DGO treated group is 8.8 days compared to 5 days for the placebo group (Table 4).

The results shown in Fig. 2 demonstrate the strong antiviral effect of consecutively applied DGO combination expressed in lengthening of the MST with almost 4 days and survival rate of nearly 50% compared to the control (Table 5).

3.3. Effect of the combination of disoxaril, guanidine HCl and oxoglaucine (DGO) on the infectious virus content in brains of treated mice

When the infectious virus content in the mouse brain isolates, infected with coxsackievirus B1 and treated with the combination DGO applied consecutively was determined, a distinct reduction of the viral titer (>10^3 reduction) was found, especially on day 4 after virus inoculation (v.i.). As demonstrated in Table 6 monotherapy with disoxaril showed marked inhibition of the infectious virus load on day 4 after v.i.

The combination of DGO applied consecutively, clearly demonstrated an inhibitory effect on the virus content. There was a 100% mortality rate in the placebo and the groups treated with monotherapies of the partners from day 8 onwards. Presumably, the increasing age of the suckling mice influences their sensitivity to the coxsackievirus B1 (Kunin, 1964; Teisner and Haahr, 1974).

3.4. Sensitivity to disoxaril of virus brain isolates following treatment course with DGO combination, applied consecutively

The virus progeny in brain samples from mice, infected with coxsackievirus B1 and then treated with disoxaril and consecutively applied combination DGO was tested for determination of

Table 6
Effect of DGO treatment and the courses with the individual drugs on coxsackievirus B1 content in the mouse brains.

Test group	Infectious titer (PFU/ml) of the brain samples and % inhibition							
	Days after virus inoculation							
	4		5		6		7	
	PFU/ml	% Inhibition	PFU/ml	% Inhibition	PFU/ml	% Inhibition	PFU/ml)	% Inhibition
DGO	2.9×10^2	99.92	4.6×10^3	96.84	3.3×10^4	94.82	3.3×10^3	98.73
Disoxaril	<10	>99.99	3.7×10^5	No effect	2.8×10^6	No effect	3.4×10^4	No effect
Guan HCl	5.7×10^4	83.7	3.8×10^5	No effect	100% mortality		100% mortality	
Oxoglaucine	1.5×10^5	58.7	9.6×10^5	No effect	6.5×10^5	No effect	100% mortality	
Placebo	3.5×10^5		1.5×10^5		6.5×10^5		2.6×10^5	

Table 7
Sensitivity to disoxaril in the plaque-inhibition test of virus brain isolates from DGO treated and treated with disoxaril alone newborn mice, infected with coxsackievirus B1.

Brain sample	Test group	Disoxaril IC_{50} values (μM) of viral brain samples						
		Taken on day (after viral inoculation)						
		4	5	6	7	8	9	10
Plaque purified	Placebo	0.38	0.4	0.5	0.57	–[a]	–[a]	–[a]
	Disoxaril	–[b]	1.71	3.03	11.1[*]	–[a]	–[a]	–[a]
	DGO consecutively	0.55	0.52	0.64	0.09[¶]	0.12	0.08	0.13
Native	Placebo	0.45	0.42	0.55	0.59	–[a]	–[a]	–[a]
	Disoxaril	–[b]	2.53	5.95	14.7	–[a]	–[a]	–[a]
	DGO consecutively	0.54	1.5	0.19	0.08	0.1	0.09	0.14
	DGO simultaneously	1.7	1.64	5.82	16.3	–[a]	–[a]	–[a]

The statistical analysis was performed with one-way ANOVA with Bonferroni's post-test.
[a] After day 7 there was no animal alive, 100% mortality rate.
[b] Due to 100% inhibitory effect of disoxaril on day 4 there is no infectious virus in the brain isolate.
[*] $P < 0.05$ compared to the placebo group.
[¶] $P < 0.05$ compared to the group treated with disoxaril alone.

Table 8
Sensitivity to oxoglaucine in the plaque-inhibition test of virus brain isolates from DGO treated and treated with oxoglaucine alone newborn mice, infected with coxsackievirus B1.

Brain sample	Test group	Oxoglaucine IC_{50} values (μM) of viral brain samples						
		Taken on day (after viral inoculation)						
		4	5	6	7	8	9	10
Plaque purified	Placebo	0.2	0.26	0.39	0.43	–[a]	–[a]	–[a]
	Oxoglaucine	3.79	3.19	3.2	–[a]	–[a]	–[a]	–[a]
	DGO consecutively	0.37	0.34	0.12	0.06	0.09	0.1	0.12
Native	Placebo	0.34	0.22	0.38	0.8	–[a]	–[a]	–[a]
	Oxoglaucine	3.52	3.73	3.77	–[a]	–[a]	–[a]	–[a]
	DGO consecutively	0.5	0.6	0.91	0.73	0.34	0.23	0.31
	DGO simultaneously	3.12	3.53	3.45	3.4	–[a]	–[a]	–[a]

[a] After day 7 there was no animal alive, 100% mortality rate.

its sensitivity to disoxaril by using the plaque-inhibition test of Hermann–Siminoff in FL cells. It is clear from the results shown in Table 4 that the virus isolated from the group treated with disoxaril alone became resistant to the compound after day 5 p.i., as the values of MIC_{50} on the 5th day increase 4 times, on the 6th day—6 times, and on the 7th day—almost 20 times. These data correlate with the results obtained from Nikolova and Galabov (2003), concerning the treatment-emerging resistance of coxsackievirus B1 after monotherapeutic treatment with disoxaril.

The data presented in Table 7 demonstrate that when the combination DGO was applied consecutively, in addition to the obtained inhibitory effect there was a full preservation of sensitivity to disoxaril of coxsackievirus B1 isolated from the mice brains. Moreover, a 4–6-fold decrease of MIC_{50} values was observed from day 7 onwards, i.e. virus isolates taken on day 7 p.i. and onwards demonstrated significant increase of their sensitivity to disoxaril compared to the placebo group and the group treated with disoxaril. The same tendency was observed with the third compound, oxoglaucine (Table 8).

As seen in Tables 7 and 8, approximately identical IC_{50} values were recorded when plaque-purified or native (not plaque-purified) virus brain samples were evaluated.

4. Discussion

All literature data so far indicate that the development of effective chemotherapy against enteroviral infections is hampered by the extremely high mutational rate of these pathogens (10^{-4}). This results in rapid emergence of resistant viral populations to most of the antiviral inhibitors, when applied as monotherapy. Previous research in our laboratory, designed to determine the sensitivity of infected with coxsackievirus B1 mice to chemotherapy with one of the WIN compounds—disoxaril, proved the rapid occurrence of resistance to the inhibitor just 5 days after the beginning of treatment. After day 6 the brain isolates from the group treated with disoxaril every day contained a completely resistant viral population. Moreover, when the phenotype characteristics of this population were examined, a slight increase of pathogenicity was established (Nikolova and Galabov, 2003).

Many inhibitors of enteroviral replication cycle are found in vitro and some of them reached a stage of clinical trials—disoxaril, pleconaril (WIN compounds) (Diana et al., 1985; Rossmann, 1989; Smith et al., 1986; Pevear et al., 1999), pirodavir and its analogues (R compounds) (Barnard et al., 2004; Hayden et al., 1995), enviroxime (DeLong and Reed, 1980; Miller et al., 1985), but they all failed due to poor bioavailability, side effects or some other reasons. In the case of disoxaril the clinical trials were stopped (Hadfield et al., 1999) and pleconaril was not approved by FDA due to increased levels of liver enzyme, known as CYO3A4 (Beer and Geduldig, 2002). Obviously, the applied doses of these compounds in monotherapeutic regimens are close to toxic levels.

One of the most prominent characteristics of enteroviruses is that almost 80% of enteroviral infections are unapparent. This is complemented by another feature of these pathogens—their population consists of a great number of pseudospecies, which leads to manifestation of many different clinical pictures, i.e. acquired insulin-dependent diabetes, myocarditis, pericarditis, meningitis, etc.

Severe forms of enteroviral infections pass through a viremic stage, which results in longer incubation period. In these cases a preservation of the activity of specific anti-enteroviral therapy for at least 12–14 days is required. This type of chemotherapy, applied

in the incubation period of the enterovirus infections (chemoprophylaxis) could be effective in children, especially when epidemic outbreaks occur.

Data presented in the current paper for the first time show that the preservation of the activity of the used drug partners for the time necessary (for survival of the infected animals) is possible when a triple combination of anti-enteroviral inhibitors is applied not simultaneously, in which case multiple resistance could emerge, but consecutively. We developed a model of such combination, the treatment with which, unlike monotherapies, totally prevents the occurrence of resistance. That is in full contrast to the established multiple drug-resistance regimens when the compounds are administered simultaneously every day.

The results from our study demonstrate that the way of ordering the drug partners plays a key role for the effectiveness of the combination. The therapy must start with an inhibitor of an early step of the viral cycle, e.g. disoxaril, which has a pronounced effect in the first day of infection before the development of resistance. It must be followed by an RNA replication inhibitor, such as guanidine HCl, and then one that targets an early step again, oxoglaucine.

We assume that other successful models of triple drug combinations could be developed that could be the basis for new therapeutic strategies.

Acknowledgements

We thank Mme Ivana Roeva, DVM, for the preparation of cell cultures used in our study and Mme Monica Czeuz, MD, for the technical assistance in animal experiments.

References

Barnard, D.L., 2006. Current status of anti-picornavirus therapies. Curr. Pharm. Des. 12, 1379–1390.

Barnard, D.L., Hubbard, V.D., Smee, D.F., Sidwell, R.W., Watson, K.G., Tucker, S.P., Reece, P.A., 2004. In vitro activity of expanded-spectrum pyridazinyl oxime ethers related to pirodavir: novel capsid-binding inhibitors with potent antipicornavirus activity. Antimicrob. Agents Chemother. 48, 1766–1772.

Barton, D.J., Flanegan, J.B., 1997. Synchronous replication of poliovirus RNA: initiation of negative-strand RNA synthesis requires the guanidine-inhibited activity of protein 2C. J. Virol. 71, 8482–8489.

Beer, K., Geduldig, L., 2002. ViroPharma Press Releases, http://www.irconnect.com?vpharm/pages/news_release.html.

Chumakov, K., Ehrenfeld, E., Wimmer, E., Agol, V.I., 2007. Vaccination against polio should not be stopped. Nat. Rev. Microbiol. 5, 952–958.

DeLong, D.C., Reed, S.E., 1980. Inhibition of rhinovirus replication in organ culture by a potential antiviral drug. J. Infect. Dis. 141, 87–91.

De Palma, A.M., Vliegen, I., De Clercq, E., Neyts, J., 2008. Selective inhibitors of picornavirus replication. Med. Res. Rev. 28, 823–884.

Diana, G.D., McKinlay, M.A., Otto, M.J., Akullian, V., Oglesby, C., 1985. [[(4,5-Dihydro-2-oxazolyl)phenoxy]alkyl]isoxazoles. Inhibitors of picornavirus uncoating. J. Med. Chem. 28, 1906–1910.

Domingo, E., Martin, V., Perales, C., Escarmis, C., 2008. Coxsackieviruses and quasispecies theory: evolution of enteroviruses. Curr. Top. Microbiol. Immunol. 323, 3–32.

Eggers, H.J., 1976. Successful treatment of enterovirus-infected mice by 2-(alpha-hydroxybenzyl)-benzimidazole and guanidine. J. Exp. Med. 143, 1367–1381.

Eigen, M., Biebricher, C.K., 1988. Sequence space and quasispecies distribution. In: Domingo, E., Holland, J.J., Ahlquist, P. (Eds.), RNA Genetics, vol. 3. CRC Press, Boca Raton, FL, pp. 211–245.

Fujita, T., 1977. Concept of paraneurons. Arch. Histol. Jpn. 40, 1–12.

Fujita, T., 1989. Present status of paraneuron concept. Arch. Histol. Cytol. 52, 1–8.

Fujita, T., Kanno, T., Kobayashi, S., 1988. The Paraneuron. Springer-Verlag, Tokyo/Berlin/Heidelberg, pp. 163–184.

Galabov, A.S., 1979. Thiourea derivatives as specific inhibitors of picornaviruses. Arzneimittelforschung 29, 1863–1868.

Galabov, A.S., Velichkova, E.H., 1974. Antiviral activity of N-phenyl-N′-aryl- or alkylthiourea derivatives in Coxsackie virus infections in mice. Antimicrob. Agents Chemother. 5, 1–18.

Galabov, A.S., Dmitrieva, T.M., 1983. Inhibitory effect of N-phenyl-N′-3-hydroxyphenylthiourea (PTU-23) on the reproduction of encephalomyocarditis virus in Krebs-II cells. Zentralbl. Bacteriol. Microbiol. Hyg. A 254, 291–305.

Galabov, A.S., Ginevskaya, V.A., Svitkin, Yu. V., 1983. Effect of N-phenyl-N′-3-hydroxyphenylthiourea (PTU-23) on the protein synthesis in Krebs-II cells infected with encephalomyocarditis virus. Zentralbl. Bacteriol. Microbiol. Hyg. A 254, 306–317.

Hadfield, A.T., Diana, G.D., Rossmann, M.G., 1999. Analysis of three structurally related antiviral compounds in complex with human rhinovirus 16. Proc. Natl. Acad. Sci. U.S.A. 96, 14730–14735.

Hayden, F.G., Hipskind, G.J., Woerner, D.H., Eisen, G.F., Janssens, M., Janssen, P.A., Andries, K., 1995. Intranasal pirodavir (R77,975) treatment of rhinovirus colds. Antimicrob. Agents Chemother. 39, 290–294.

Herrmann, E.C., 1961. Plaque-inhibition tests for detection of specific inhibitors of DNA containing viruses. Proc. Soc. Exp. Biol. 107, 142–145.

Herrmann Jr., E.C., Herrmann, J.A., Delong, D.C., 1982. Prevention of death in mice infected with coxsackievirus A16 using guanidine HCl mixed with substituted benzimidazoles. Antiviral Res. 2, 339–346.

Hyöty, H., Hiltunen, M., Knip, M., Laakkonen, M., Vähäsalo, P., Karjalainen, J., Koskela, P., Roivainen, M., Leinikki, P., Hovi, T., et al., 1995. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. Diabetes 44, 652–657.

Kunin, C.M., 1964. Cellular susceptibility to enteroviruses. Bacteriol. Rev. 28, 382–390.

Loddo, B., Ferrari, W., Brotzu, G., Spanedda, A., 1962. In vitro inhibition of infectivity of polio viruses by guanidine. Nature 193, 97–98.

McKinlay, M.A., Steinberg, B.A., 1986. Oral efficacy of WIN 51711 in mice infected with human poliovirus. Antimicrob. Agents Chemother. 29, 30–32.

Miller, F.D., Monto, A.S., DeLong, D.C., Exelby, A., Bryan, E.R., Srivastava, S., 1985. Controlled trial of enviroxime against natural rhinovirus infections in a community. Antimicrob. Agents Chemother. 27, 102–106.

Nikolaeva-Glomb, L., Galabov, A.S., 2004. Synergistic drug combinations against the in vitro replication of Coxsackie B1 virus. Antiviral Res. 62, 9–19.

Nikolaeva, L., Galabov, A.S., 2000. Antiviral effect of the combination of enviroxime and disoxaril on coxsackievirus B1 infection. Acta Virol. 44, 73–78.

Nikolaeva-Glomb, L., Philipov, S., Galabov, A.S., 2008. A new highly potent antienteroviral compound. In: Georgiev, V. St. (Ed.), National Institute of Allergy and Infectious Diseases, NIH, Frontiers in Research, vol. I. Humana Press Inc., Totowa, NJ, pp. 199–202.

Nikolova, I., Galabov, A.S., 2003. Development of resistance to disoxaril in Coxsackie B1 virus-infected newborn mice. Antiviral Res. 60, 35–40.

Pevear, D.C., Tull, T.M., Seipel, M.E., Groarke, J.M., 1999. Activity of pleconaril against enteroviruses. Antimicrob. Agents Chemother. 43, 2109–2115.

Rabe, K.F., Hurd, S., Anzueto, A., Barnes, P.J., Buist, S.A., Calverley, P., Fukuchi, Y., Jenkins, C., Rodriguez-Roisin, R., Van Weel, C., Zielinski, J., 2007. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am. J. Respir. Crit. Care Med. 176, 532–555.

Rossmann, M.G., 1989. The structure of antiviral agents that inhibit uncoating when complexed with viral capsids. Antiviral Res. 11, 3–13.

Siminoff, P., 1961. A plaque suppression method for the study of antiviral compounds. Appl. Microbiol. 9, 66–72.

Smith, T.J., Kremer, M.J., Luo, M., Vriend, G., Arnold, E., Kamer, G., Rossmann, M.G., McKinlay, M.A., Diana, G.D., Otto, M.J., 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science 233, 1286–1293.

Teisner, B., Haahr, S., 1974. Poikilothermia and susceptibility of suckling mice to Coxsackie B1 virus. Nature 247, 568.