ANTIVIRAL EFFECT OF THE COMBINATION OF ENVIROXIME AND DISOXARIL ON COXSACKIEVIRUS B1 INFECTION

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Summary. – Effects of enviroxime and disoxaril, inhibitors of replication of some picornaviruses with known mechanisms of action, alone or in combination, on replication of coxsackievirus B1 (CVB1) in FL cells and on experimental CVB1 infection in newborn mice were tested. The combination of enviroxime and disoxaril resulted *in vitro* in a synergistic interaction. Both compounds were administered *in vivo,* alone or in combination, daily by subcutaneous (s.c.) route since the day of virus inoculation till the 5th day post inoculation (p.i.). Our findings about the *in vivo* antiviral effects of the individual compounds correlated with those of other authors, i.e. disoxaril significantly reduced the virus-induced death (the minimum 50% effective dose (ED_{50}) was 12.5 mg/kg; P = 0.0037), while enviroxime was not effective even when applied at a dose as high as 100 mg/kg (P = 0.264). However, when both the substances were combined, the same protective effect was achieved with concentrations of disoxaril two to four times lower than those of the drug administered alone. In this way a higher selectivity ratio was achieved. Namely, the combination of 50 mg/kg enviroxime and 3.125–6.25 mg/kg disoxaril was synergistic. Along with reduction in mortality a marked delay in the course of the disease was observed.

Key words: coxsackievirus B1; enviroxime; disoxaril; antivirals; in vivo; synergism

Introduction

Picornaviruses are the causative agents of the majority of viral upper respiratory tract infections (Gwaltney, 1989), of central nervous system infections, and of severe and sometimes lethal diseases of the heart, liver and pancreas (Baekkeskov and Hansen, 1990; Muir *et al.*, 1996). A great number of picornavirus replication inhibitors *in vitro* have been described but just few of them were effective also *in vivo* (Carrasco, 1994). The main reason for that is fast development of drug-resistant and even drug-dependent virus mutants (Loddo, 1980). To overcome the disadvantages of monotherapy synergistic antiviral combinations could be used. In this way an effect with a higher selectivity ratio could be achieved with lower concentrations of the drugs.

Enviroxime and disoxaril are potent inhibitors of replication of some picornaviruses *in vitro* with known but different mechanisms of action. Enviroxime blocks the replication of plus-strand viral RNA by targeting the 3A protein (Heinz and Vance, 1995). Disoxaril inhibits virus uncoating by direct insertion into the hydrophobic canyon within the VP1 capsid protein (Zeichhardt *et al.*, 1987). The combination of these two inhibitors results in a synergistic inhibitory effect *in vitro* on the replication of poliovirus 1 (PV1) (Nikolaeva and Galabov, 1995). Neither cross-resistance (Nikolaeva and Galabov, 1995) nor enhanced cytotoxicity (Nikolaeva and Galabov, 1999) have been observed. Disoxaril alone prevented the development of paralysis and subsequent death of mice infected with human

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Abbreviations: CVB1 = coxsackievirus B1; DMSO = dimethylsulfoxide; MEM = Minimum Essential Medium; MST = mean survival time; PC = protection coefficient; PI = protection index; p.i. = post inoculation; PV1 = poliovirus 1; s.c. = subcutaneous(ly)

poliovirus (McKinlay and Steinberg, 1986). In contrast, enviroxime did not show any promising therapeutic or preventive effect *in vivo* (Wyde *et al.*, 1988).

Data in the present paper on the effects of enviroxime and disoxaril, alone or in combination, on CVB1 infection revealed synergistic character of the combination both *in vitro* and *in vivo*.

Materials and Methods

Compounds. Enviroxime [2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime], kindly supplied by the Lilly Laboratories of Eli Lilly & Co. (USA), and disoxaril [5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methyl-isoxazole, WIN 51 711), kindly supplied by Sanofi Winthrop, Inc. (USA), were prepared as 1% stock solutions in dimethylsulfoxide (DMSO) and then *ex tempore* diluted in Minimum Essential Medium (MEM) with Hanks' salts (Gibco-BRL) for the *in vitro* assays and in saline for the *in vivo* experiments.

Cells. All *in vitro* experiments were carried out on FL cells, which were routinely subcultured weakly. Growth medium consisted of a mixture of equal parts of Medium 199 with Hanks' salts (Gibco BRL) and Hanks' solution, supplemented with 10% heated calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Test animals. Newborn white mice of the randomly bred ICR line were used.

Virus. CVB1 was grown in FL cells kept in a maintenance medium (MEM with Hanks' salts, 5% heated calf serum and antibiotics). A stock CVB1 virus was obtained from newborn white mice intracerebrally infected with 2 x 10^5 PFU of CVB1 in a 0.02 ml volume. A 10% brain suspension in saline was thrice frozen and thawed, and then clarified by centrifugation at 6000 rpm for 30 mins. The supernatant underwent 5 blind serial passages and then the product (stock virus) was titered in newborn white mice by s.c. route. The LD_{50} titer was calculated according to Kaerber (1931).

In vitro evaluation of drugs. Antiviral effects of the drugs, alone or in combination, were determined by a plaque inhibition test. Monolayer FL cell cultures in scintillation glass vials with a diameter of 2.5 cm were inoculated with 80–100 PFU of the virus per vial and were left for an 1 hr at room temperature. Then 1 ml per vial of an agar overlay (1% purified Difco agar in MEM with Hanks' salts supplemented with 10% of heated calf serum, 1.65 mg/ml sodium bicarbonate and antibiotics) was laid over the cells. The tested drugs, alone or in combination, were included in the agar overlay. Following a 48 hrs incubation at 37°C a second agar overlay (1.5% agar in saline with 0.02% neutral red) was added and vials were kept at room temperature for 2–3 hrs till virus plaques were visible. Plaques were counted and the titer was expressed in PFU per vial. Reduction of the titer was evaluated in comparison to the control (100%, no drug in the agar overlay). The character of antiviral effect of a drug combination was estimated according to the three-dimensional model of Prichard and Shipman (1990) for analyzing drug-drug interactions. The additivity assumption equation for different-site inhibitors was used and the difference between the observed and expected effects was calculated. Positive values were indicative for synergism, while negative ones for antagonism.

In vivo evaluation of drugs. Virus challenge was carried out in one-day-old mice by s.c. inoculation of 5–10 LD_{50} of the virus in 0.02 ml (Galabov and Velichkova, 1974). Drug administration started 6 hrs p.i. Doses of drugs in 0.02 ml were administered s.c. daily for 5 days. The observation period lasted up to the day 7 p.i. when all placebo-treated animals were already dead.

Statistical analysis. The effects of the drugs, applied alone or in combination, were determined on the basis of the following indices: (1) the cumulative mortality rate; calculation of the protection index (PI),

$$PI = [(PC-1)/PC] \times 100$$

where PC (protection coefficient) is the ratio of the mortality percentage in the placebo group to that in the treated test group at the end of the observation period. The maximum error (Δp) of the mortality rate relative portion (p) was evaluated by the Fisher's φ-method. The Van der Warden's method was used in cases of p = 0 or 100% (Kendall and Stewart, 1973; Urbach, 1963; Ranchov, 1997); (2) mean survival time (MST). The standard error of the quadratic deviation of the MST (σ_x) was calculated as described by Karparov *et al.* (1985). The MedCalc 2.20 computer program was used to calculate associated P values.

Results

In vitro antiviral effects of enviroxime and disoxaril alone or in combination

As a first step, 50% inhibitory concentrations (IC_{50}) of enviroxime and disoxaril applied alone were determined from the dose-response set-up of the plaque inhibition test. They were 0.03 μmol/l for enviroxime and 3 μmol/l for disoxaril. Then a checkerboard set-up of the experiment for estimation of antiviral effect of the combination of these drugs in various concentrations ($2IC_{50}$, IC_{50}, $IC_{50}/2$, $IC_{50}/4$ and $IC_{50}/8$) was performed. The titer reduction data are presented in Table 1 as mean values of reduction in %. Fig. 1

Table 1. Inhibition of CVB1 replication in FL cells by the combination of enviroxime and disoxaril

Disoxaril	Enviroxime					
	0	IC50/8	IC50/4	IC50/2	IC50	2IC50
2IC50	63.17	88.63	63.11	89.56	95.8	95.65
IC50	44.98	65.12	51.82	84.94	94.75	92.65
IC50/2	39.8	65.02	58.74	76.19	88.02	94.66
IC50/4	30.24	53.89	71.93	68.83	80.33	90.66
IC50/8	26.8	48.94	67.86	77.73	73.63	71.79
0	0	25.43	32.31	39.06	57.38	63.19

The values represent the reduction of the virus titer of the control expressed in %.

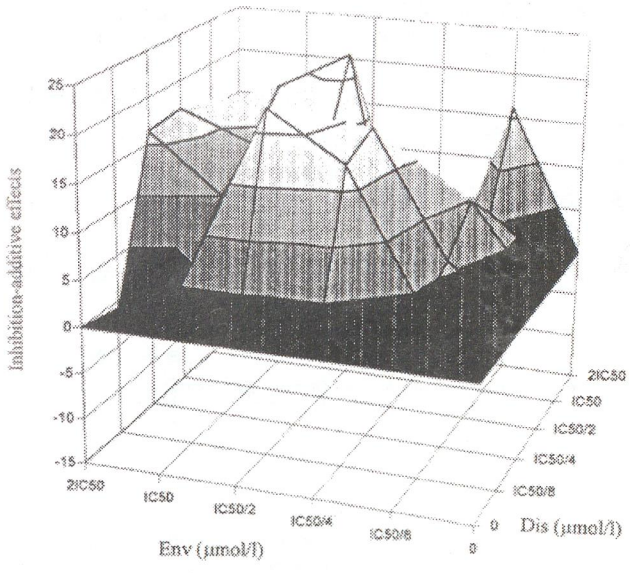

Fig. 1
Effect of the combination of enviroxime and disoxaril on CVB1 replication in FL cells
Env = enviroxime. Dis = disoxaril.

shows a three-dimensional dose-response surface of the combination according to the Prichard and Shipman's model and reveals that this combination led to a well manifested synergistic antiviral effect.

In vivo antiviral effects of enviroxime and disoxaril alone and in combination

First the antiviral effects of the drugs alone were determined. It can be seen that enviroxime alone was ineffective in preventing the virus-induced death in mice (Table 2). Only at the highest dose of 100 mg/kg a slight delay in the inevitable course of the disease was observed (Figs. 2 and 3). In contrast, disoxaril alone exerted a significant dose-dependent antiviral effect (Figs. 4 and 5). At the end of the test period all the placebo-treated mice were dead while survivors were registered in each group treated with disoxaril. The ED_{50} was 12.5 mg/kg ($P = 0.0037$). A significant delay in the course of the disease was observed too (Fig. 5).

Then different doses of enviroxime (6.25 mg/kg, 12.5 mg/kg, 25 mg/kg, and 50 mg/kg) were combined with different doses of disoxaril (3.125 mg/kg and 6.25 mg/kg, i.e. four times and twice lower than the ED_{50}, respectively) in a checkerboard manner. The results are presented in Table 2. The protective effect of the combination was almost the same as that of disoxaril alone at low doses of enviroxime (up to 25 mg/kg). But when enviroxime was applied at a concentration of 50 mg/kg an enhanced antiviral effect of the combination was observed as compared to the effect of the both drugs alone (Figs. 6–9). When the obtained results were evaluated according to the three-dimensional model an additive/synergistic dose-response surface was obtained (Fig. 10).

Table 2. Effect of enviroxime and disoxaril, alone and in combination on CVB1 infection in newborn mice

Drug	Daily dose (mg/kg)	Mortality rate (day 7 p.i.)	p± Δp	PI (%)	MST±σ_x
Placebo		13/13	0.740<0.930<1	0	3.69+0.30
Enviroxime	6.25	11/11	0.703<0.923<1	0	3.36+0.32
	12.50	10/10	0.679<0.917<1	0	3.60+0.38
	25.00	11/11	0.703<0.923<1	0	3.19+0.30
	50.00	9/9	0.650<0.909<1	0	3.56+0.42
	100.00	6/8	0.415<0.750<0.969	24.81	4.00+0.53
Disoxaril	3.125	14/15	0.757<0.933<0.9999	6.54	3.47+0.24
	6.25	7/9	0.468<0.778<0.973	22.48	4.89+0.58
	12.50	5/13	0.150<0.385<0.653	61.54	4.92+0.39
	25.00	1/12	0.0001<0.083<0.295	91.67	6.75+0.59
	50.00	5/16	0.116<0.313<0.553	68.75	5.75+0.37
Enviroxime + Disoxaril	Env 6.25 + Dis 3.125	12/12	0.724<0.929<1	0	2.75+0.24
	Env 6.25 + Dis 6.25	11/12	0.704<0.917<0.9999	8.26	4.08+0.36
	Env 12.50 + Dis 3.125	15/15	0.771<0.941<1	0	2.80+0.19
	Env 12.50 + Dis 6.25	11/13	0.609<0.846<0.982	15.25	4.69+0.38
	Env 25.00 + Dis 3.125	11/11	0.703<0.923<1	0	2.91+0.28
	Env 25.00 + Dis 6.25	11/12	0.706<0.917<1	8.26	4.67+0.41
	Env 50.00 + Dis 3.125	6/12	0.233<0.500<0.768	50	5.42+0.47
	Env 50.00 + Dis 6.25	5/9	0.242<0.556<0.846	44.44	6.00+0.71

Env = enviroxime. Dis = disoxaril.

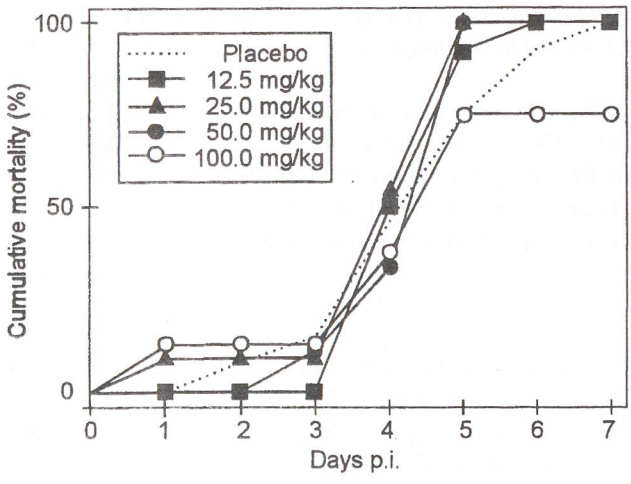

Fig. 2
Effect of s.c. administered enviroxime on CVB1 infection in newborn mice
For significantly different from the placebo curve can be taken the curves with $P < 0.01$.

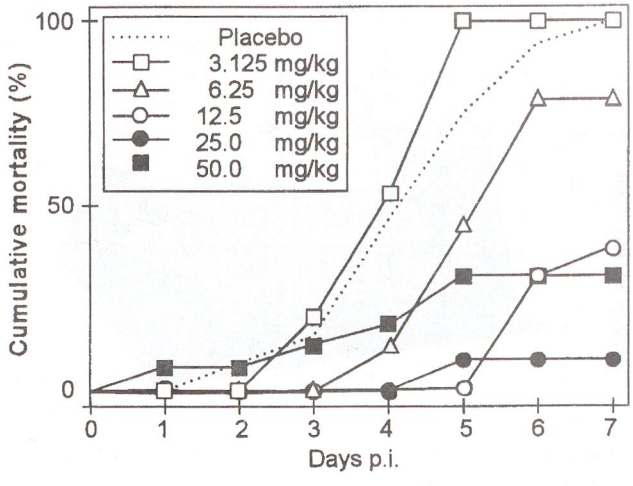

Fig. 4
Effect of s.c. administered disoxaril on CVB1 infection in newborn mice

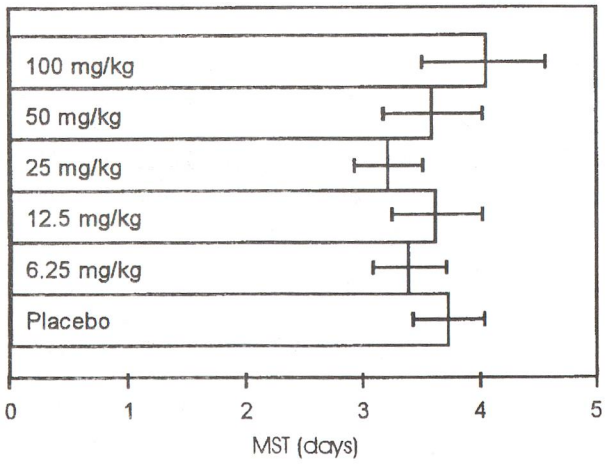

Fig. 3
MST of newborn mice infected with CVB1 and treated with s.c. administered enviroxime

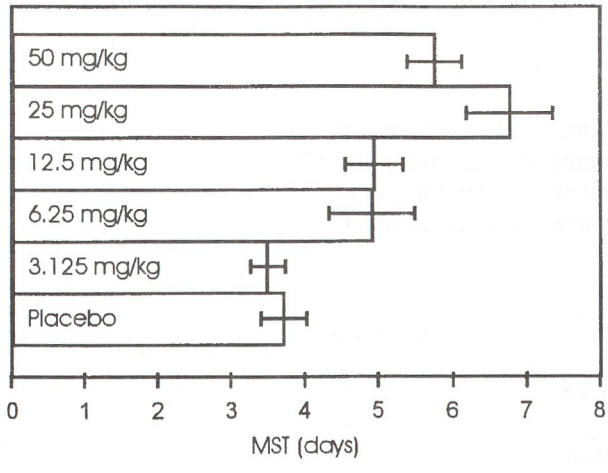

Fig. 5
MST of newborn mice infected with CVB1 and treated with s.c. administered disoxaril

The summarized results of all the *in vivo* experiments are presented in Table 2.

Discussion

As the obtained data show, the antiviral effect of the combination of enviroxime and disoxaril was synergistic. The synergistic character of the combination was very well illustrated in the *in vitro* experiments. The observed synergism in the CVB1 model correlates with our earlier data on the *in vitro* synergism in the PV1 model (Nikolaeva and Galabov, 1995). Moreover, a similar antiviral synergism of the drug combination was found also with another representative of the enterovirus genus, namely ECHO-13 virus (L. Nikolaeva and A.S. Galabov, unpublished data). It may be concluded that the observed synergism could be manifested in most enterovirus models. The synergistic antiviral effect of this combination may be explained by the fact that the partners attack different targets at even different steps of virus replication. Enviroxime targets the 3A viral protein and blocks the replication of plus-strand viral RNA (Heinz and Vance, 1995, 1996). Disoxaril targets capsid protein VP1, thus blocking the virus uncoating (Zeichhardt *et al.*, 1987).

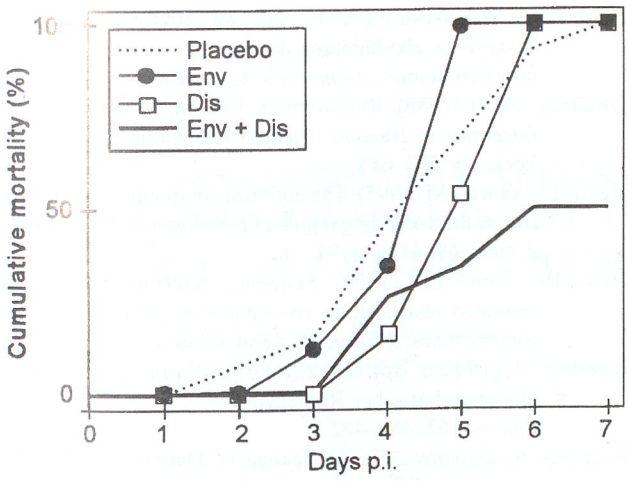

Fig. 6
Effect of s.c. administered combination of 50 mg/kg enviroxime and 3.125 mg/kg disoxaril on CVB1 infection in newborn mice

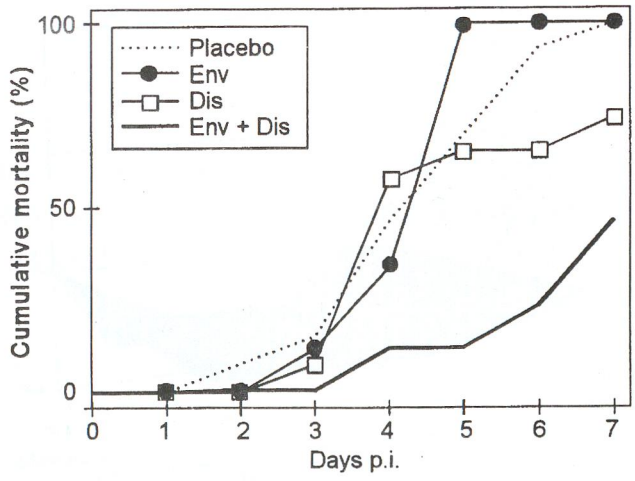

Fig. 8
Effect of s.c. administered combination of 50 mg/kg enviroxime and 6.25 mg/kg disoxaril on CVB1 infection in newborn mice

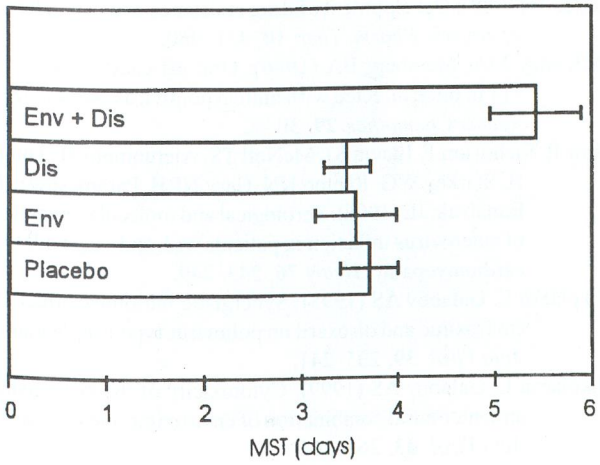

Fig. 7
MST of newborn mice infected with CVB1 and treated with s.c. administered combination of 50 mg/kg enviroxime and 3.125 mg/kg disoxaril
For the legend see Fig. 1.

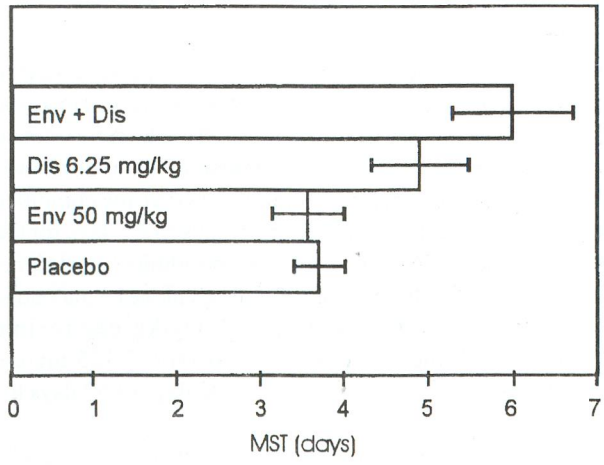

Fig. 9
MST of newborn mice infected with CVB1 and treated with s.c. administered combination of 50 mg/kg enviroxime and 6.25 mg/kg disoxaril
For the legend see Fig. 1.

As far as the *in vitro* effects of these drugs alone on the replication of CVB1 are concerned we may conclude that CVB1 is more sensitive than PV1 (Mahoney) to enviroxime, the respective IC_{50} values being 0.03 µmol/l, and 0.2 µmol/l (Nikolaeva and Galabov, 1995). An opposite situation was observed for disoxaril. Its IC_{50} for CVB1 was 3 µmol/l and 0.3 µmol/l for PV1.

Our experimental data for the *in vivo* effects of the both drugs are in correlation with those of other authors, i.e. a well manifested prophylactic and therapeutic effect of disoxaril (McKinlay and Steinberg, 1986) and no promising effect of enviroxime (Wyde *et al.*, 1988). A dose-dependent *in vivo* effect of disoxaril alone was observed in our experiments. The observed higher mortality at the 50 mg/kg concentration of disoxaril could be a result of a toxic effect. McKinlay and Steinberg (1986) have reported no significant toxicity of this drug when administered to intact mice for a month at a daily dose of 100 mg/kg. However, influence of the drug on the immune system has not been monitored.

When the combination enviroxime and disoxaril was applied *in vivo* the same protective effect was achieved with concentrations of disoxaril two to four times lower than those of the drugs administered alone. Combining the 50 mg/kg

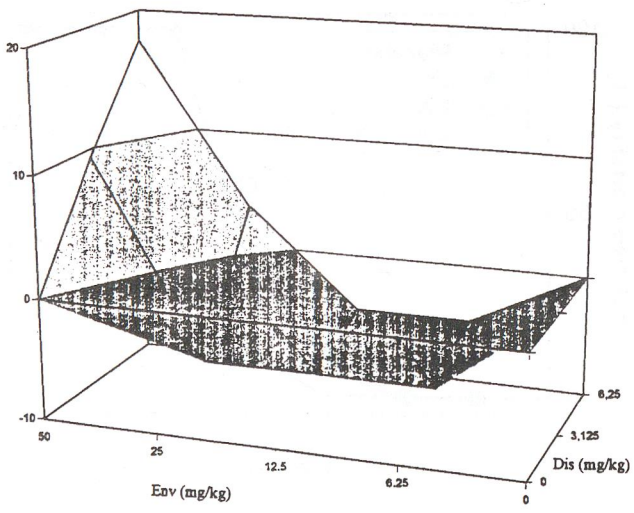

Fig. 10
Effect of the combination of enviroxime and disoxaril on CVB1 infection in newborn mice
For the legend see Fig. 1.

enviroxime and the 3.125 mg/kg or 6.25 mg/kg disoxaril resulted in a synergistic antiviral effect *in vivo*. However, along with reduction in mortality in this obligatory lethal infection a marked delay in the course of the disease was observed. The MST for the 50 mg/kg enviroxime combined with the 6.25 mg/kg disoxaril was 6 days (3.69 days for the placebo group, 3.56 days for enviroxime administered alone, and 4.89 days for disoxaril administered alone). Analogous results were obtained with the 50 mg/kg enviroxime combined with the lower dose of disoxaril, 3.125 mg/kg. The MST for this combination was 5.42 days (3.47 days for disoxaril alone).

In summary, combining enviroxime with disoxaril results in antienteroviral synergism both *in vitro* and *in vivo*. The established synergism, especially along with the lack of cross-resistance between both the drugs (Nikolaeva and Galabov, 1995) allows this combination to be classified as a very promising one.

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