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Synergistic drug combinations against the in vitro replication of Coxsackie B1 virus

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Abstract

The existence of synergistic drug combinations against the in vitro replication of poliovirus type 1 (Mahoney) (PV-1) had been established in our previous work. The objective of the present study was to test the combined effects of the different drugs against another representative of the *enterovirus* genus, i.e. Coxsackievirus B1 (CBV-1). Dual combinations of enviroxime, disoxaril, arildone, PTU-23, HBB and S-7 were evaluated.

The susceptibility of CBV-1 to the individual effects of the inhibitors was compared to that of PV-1. CBV-1 was more sensitive to enviroxime, S-7, PTU-23 and HBB and less sensitive to the effects of disoxaril and arildone. The effect of most dual drug combinations tested against CBV-1 replication was additive or synergistic. Enviroxime and S-7, enviroxime and PTU-23, disoxaril and HBB, disoxaril and PTU-23, arildone and HBB, arildone and PTU-23, S-7 and HBB revealed a strong synergistic effect. Synergy against CBV-1 replication was stronger as compared to that noted for the same drug combinations against PV-1 replication.

Keywords: Coxsackievirus B1; Synergy; Drug combinations

1. Introduction

Human enteroviruses, the largest genus of the *Picornaviridae* family, are of great medical and economic importance causing a variety of clinical syndromes and diseases. Any enterovirus can cause any of the syndromes and, vice versa, any syndrome or disease could be the result of infection by any enterovirus. Coxsackieviruses, part of the *enterovirus* genus, can cause severe diseases of the heart, liver, eyes and pancreas, as well as acute infections of the central nervous system. Nowadays, Coxsackieviruses B are the major etiological agents of human myocarditis, causing between 25 and 35% of cases for which a cause is found (Martino et al., 1995). Transition from acute myocarditis to dilated cardiomyopathy has been suspected (Sole and Liu, 1993). So far, dilated cardiomyopathy is one of the major reasons for cardiac transplantation. Coxsackieviral RNA in the myocardium can be a marker of a poor clinical outcome and might influence prognosis after heart transplantation (Fujioka and Kitaura, 2001). Enteroviruses and Coxsackieviruses in particular, have been implicated in several chronic illnesses including juvenile onset diabetes mellitus, chronic fatigue syndrome, dermatomyositis and polymyositis, congenital hydrocephalus and amyotrophic lateral sclerosis (Dalakas, 1995; Rewers and Atkinson, 1995; Muir et al., 1996). Until now, there are no enterovirus-specific drugs available for clinical use (Rotbart, 2002). A great number of picornavirus replication inhibitors in vitro have been described but just few of them have shown effectiveness in vivo (Carrasco, 1994), and none has been approved for clinical use yet. Etiological therapy for enteroviral diseases remains still elusive. The main reason for that is the fast development of drug-resistant and even drug-dependent mutants (Loddo, 1980; Nikolova and Galabov, 2003). Use of synergistic combinations of antivirals might be one of the possible efficient approaches to overcome the disadvantages of monotherapy. The same or greater effect could be achieved at lower concentrations than those required if drugs were to be used alone. Combined chemotherapy may also restrict the emergence of resistance to either or both of the partners in the combination. Also, research on the antiviral effect of combinations of picornavirus inhibitors might contribute to the better understanding of their mode of action and, in general, the mechanism of picornavirus replication.

The existence of synergistic dual combinations of antivirals against the replication in vitro of poliovirus type 1 (Mahoney) (PV-1) has been established in our previous work

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(Nikolaeva and Galabov, 1999). The combined effect of enviroxime and disoxaril is synergistic against the replication in vitro and additive against the replication in vivo of another representative of the enterovirus genus, i.e. Coxsackievirus B1 (CBV-1) (Nikolaeva and Galabov, 2000).

The objective of the present study was to test the combined effects of a panel of picornavirus inhibitors with known mechanisms of action against the replication in vitro of CBV-1. The following picornavirus replication inhibitors were used: enviroxime, disoxaril, arildone, PTU-23, HBB and S-7. All compounds mentioned target virus-specific proteins and are de facto viral protein ligands. Enviroxime, first described as a potent rhinovirus inhibitor (DeLong and Reed, 1980), targets entero- and rhinoviral protein 3A and prevents proper formation of the replication complex (Heinz and Vance, 1995, 1996). Disoxaril inhibits virus uncoating by direct insertion into the canyon of capsid protein VP1 (Fox et al., 1986; Smith et al., 1986; Zeichhardt et al., 1987). Arildone selectively inhibits the replication of some RNA and DNA viruses (Diana et al., 1977). Enterovirus replication is inhibited by arildone by direct interaction with the viral capsid and thus uncoating is blocked (Caliguiri et al., 1980; Eggers and Rosenwirth, 1988). PTU-23 inhibits the synthesis of viral 37S RNA as a result of suppression of the synthesis of a viral protein with regulatory functions in the replicative cycle (Galabov, 1979; Galabov and Dmitrieva, 1983; Galabov et al., 1983). HBB inhibits the activity of the virus-specific RNA polymerase, and thus the single-stranded RNA synthesis is suppressed (Eggers and Tamm, 1962; Dmitrieva and Agol, 1974). S-7 prevents uncoating by direct interaction with the virus particle (Lonberg-Holm et al., 1975).

In the present study the combined antiviral effects of dual combinations between enviroxime, disoxaril, arildone, PTU-23, HBB and S-7 against the in vitro replication of Coxsackievirus B1 are presented.

2. Materials and methods

2.1. Cells

All experiments were carried out on monolayer cultures of FL cells routinely subcultured once weekly. Growth medium consisted of equal parts of Medium 199 with Hanks' salts and Hanks' solution, supplemented with 10% heated calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin).

2.2. Virus

CBV-1 (Connecticut 5 strain) was produced in FL cells in maintenance medium of Eagle's Minimum Essential Medium with Hanks' salts (MEM), supplemented with 5% heated calf serum and antibiotics. Virus stock was titrated by the plaque procedure of Dulbecco (1952). Stock virus titer was 1.1×10^7 PFU/ml.

2.3. Compounds

Enviroxime (2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime), (Lilly Laboratories, Eli Lilly & Co., USA); disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)-phenoxy]-heptyl]-3-methyl-isoxazole; WIN 51 711), (Sanofi Winthrop, Inc., PA); arildone (4-[6-2-chloro-4-methoxy-phenoxy)-hexyl]-3,5-heptandione, (Sterling Research Group, Sterling Drug, Inc., USA); S-7 (ethyl 2-methylthio-4-methyl-5-pyrimidine, carboxylate) (a gift from Dr. P. La Colla, University of Cagliari, Cagliari, Italy) and HBB (2-α-hydroxybenzyl-benzimidazole), (a gift from Dr. T. Dmitrieva, Moscow State University, Moscow, Russia) were prepared as 30 mmol stock solutions in dimethylsulfoxide (DMSO) and then diluted in the maintenance medium to the required concentrations. PTU-23 (*N*-phenyl-*N*′-3-hydroxyphenylthiourea), originally synthesized by Prof. G. Vassilev, Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria, was first dissolved in ethyl alcohol and then diluted in the maintenance medium.

2.4. Plaque inhibition test

The antiviral effect of the compounds, alone or in combination, was determined by the plaque inhibition test of Herrmann (1961) and Siminoff (1961), accepted as the 'gold standard' in experimental anti-enteroviral chemotherapy. Briefly, monolayer cell cultures in scintillation glass vials (diameter 25 mm) were inoculated each with 80–100 PFU of the virus and were left for an hour at room temperature for virus adsorption. Then 1 ml per vial of an agar overlay (1% purified Difco agar in MEM with Hanks' salts supplemented with 10% heated calf serum, 1.65 mg/ml sodium bicarbonate and antibiotics) was laid over the cells. Test compounds in serial dilutions, alone or in combination, were included in the agar overlay. Following 48 h 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 h till visualization of the virus plaques. Plaques were counted and the titer was expressed in PFU per vial. Inhibition was calculated as percentage in comparison to the control (no drug in the agar overlay). Concentration that inhibited 50% of the plaques was considered as the 50% inhibitory concentration (IC_{50}). The experiments were carried out in triplicate with three parallels per sample.

2.5. Estimation of the combined effects

A checkerboard design of the experiments was made and the following concentrations of each partner in the dual combinations were used in the plaque inhibition test: $2IC_{50}$, IC_{50}, $IC_{50}/2$, $IC_{50}/4$ and $IC_{50}/8$. Experiments were done in duplicate with three parallels per sample. Evaluation of the character of the combined effects was done according to the three-dimensional model of Prichard and Shipman (1990) using the MacSynergy™ II software

(Prichard et al., 1992). Briefly, theoretical additive interactions were calculated from the dose–response curves for each drug tested individually. The obtained theoretical additive surface was subtracted from the experimentally determined dose–response surface. The 95% confidence interval around the experimental dose–response surface was used to evaluate data statistically. Any peaks above the horizontal plane at 0% inhibition were indicative of synergy. Any depression in the plane indicated antagonism. The following guidelines were applied to volumes of synergy or antagonism expressed as $\mu M^2\%$: <25: insignificant, 25–50: minor but significant, 50–100: moderate, >100: strong synergy or antagonism (Prichard et al., 1993).

3. Results

3.1. Individual antiviral effects in vitro

As a first step 50% inhibitory concentrations (IC_{50}) of each compound tested alone were determined from the dose–response curves obtained by the plaque inhibition test (Fig. 1). The following mean values of IC_{50} for each compound alone were obtained: enviroxime, 0.03 μM, disoxaril, 3 μM, arildone, 8.9 μM, PTU-23, 10 μM, HBB, 22 μM and S-7, 50 μM.

3.2. Combined antiviral effects of dual combinations

Each compound in twofold serial dilutions starting from 2 IC_{50} was combined in dual combinations with the other test compounds using checkerboard design of the plaque inhibition test. The three-dimensional model of Prichard and Shipman (1990) evaluated raw experimental data. Graphic presentation of the results is shown in Figs. 2–12.

The following combinations provided a strong synergistic effect against the replication of CBV-1: enviroxime + S-7 (Fig. 2), enviroxime + HBB (Fig. 3), enviroxime + PTU-23 (Fig. 4), disoxaril + HBB (Fig. 5), disoxaril + PTU-23 (Fig. 6), arildone + HBB (Fig. 7), arildone + PTU-23 (Fig. 8), S-7 + HBB (Fig. 9) and PTU-23 + HBB (Fig. 10). The synergy shown for the combination of enviroxime and disoxaril (Fig. 11), although modest, was significant at the 95% confidence level. The only exception was the combination between enviroxime and arildone (Fig. 12), which showed an insignificant synergy and minor but significant antagonism. The numerical values of synergy or antagonism of the studied combinations are presented in Table 1. Absolute values over 25 $\mu M^2\%$ indicate significant values of synergy or antagonism. When examining the numerical values of synergy or antagonism in Table 1, a very slight insignificant antagonism can be observed in the case of the combination of enviroxime and disoxaril. That antagonism is present solely when only one definite dose of enviroxime (0.0075 μM) is combined with disoxaril. Following the guidelines for interpreting results obtained by MacSynergy™ II (Prichard et al., 1993) the observed values of antagonism at 95% confidence should be regarded as insignificant.

4. Discussion

The character of the dose–response curves (Fig. 1) and IC_{50} values obtained for the tested compounds are in accordance with those reported by other authors (Galabov, 1979; McSharry and Pancic, 1982; Siegl and Eggers, 1982;

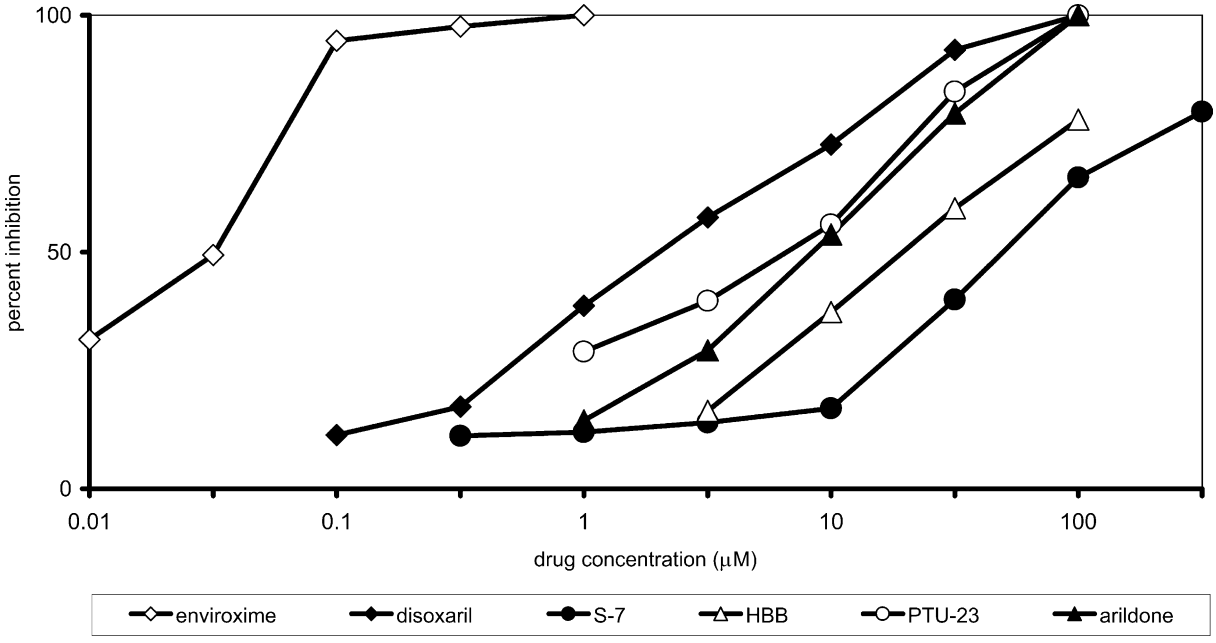

Fig. 1. Effects of the inhibitors on Coxsackievirus B1 replication in FL cells (dose–response curves).

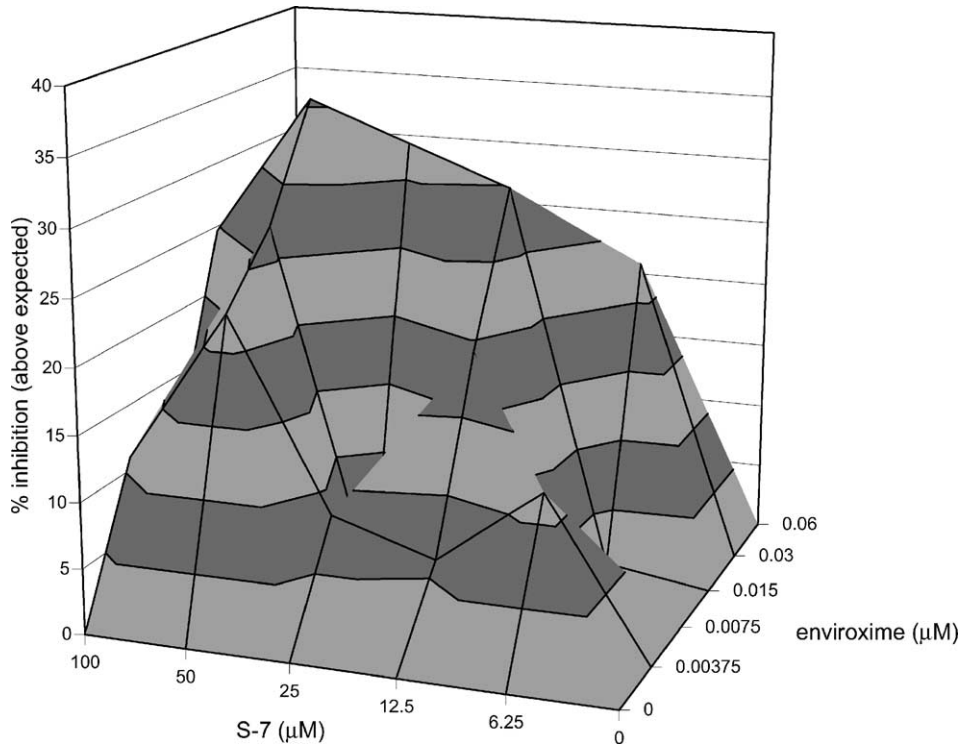

Fig. 2. Combined effects of enviroxime and S-7 on the replication of CBV-1 in FL cells (dose–response surface).

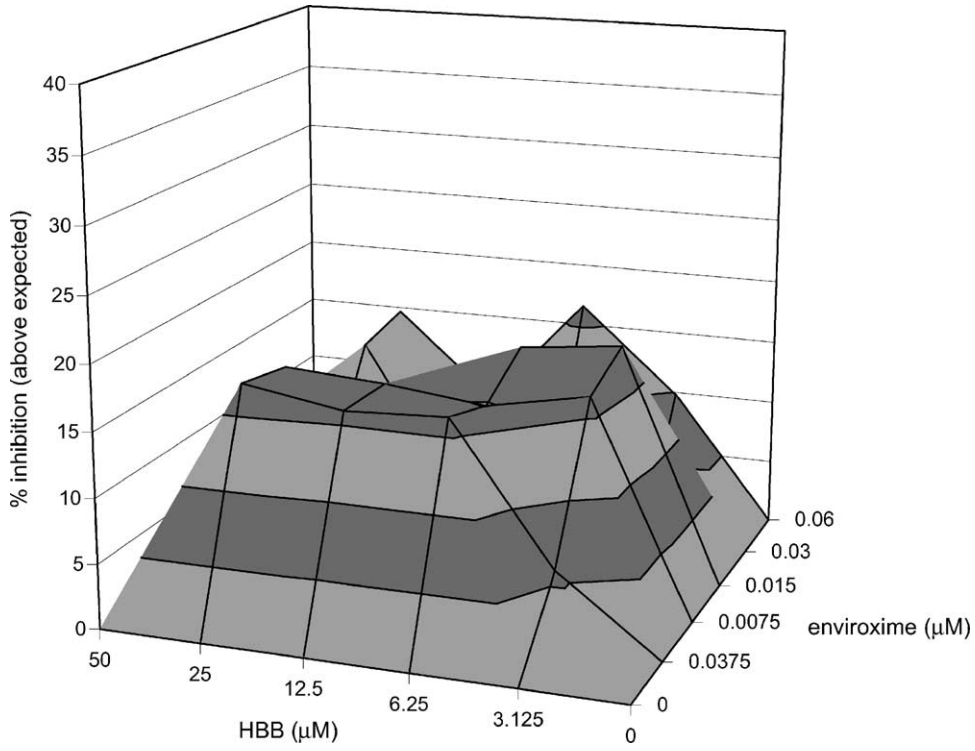

Fig. 3. Combined effects of enviroxime and HBB on the replication of CBV-1 in FL cells (dose–response surface).

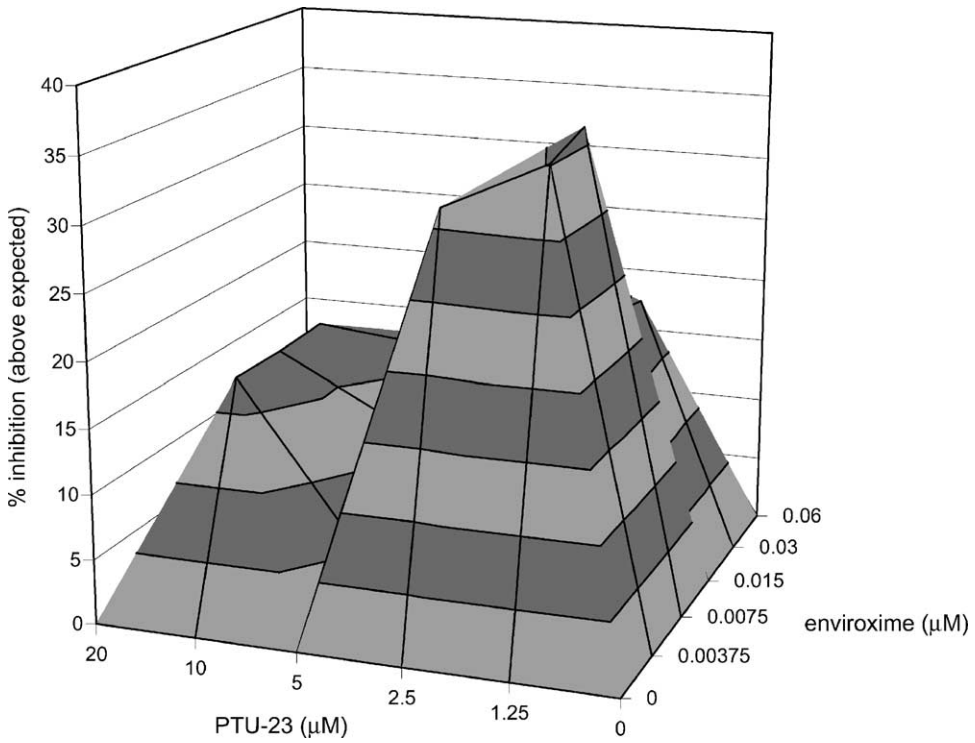

Fig. 4. Combined effects of enviroxime and PTU-23 on the replication of CBV-1 in FL cells (dose–response surface).

Ninomiya et al., 1985; Otto et al., 1985; Andries et al., 1990; Shimizu et al., 2000). The susceptibility of CBV-1 to the individual effect of the replication inhibitors has been compared to that of poliovirus type 1 (Mahoney) established previously in this laboratory by the same experimental set up (Nikolaeva and Galabov, 1999). CBV-1 is relatively more sensitive to the individual inhibitory effects of enviroxime, S-7, PTU-23 and HBB and relatively less sensitive

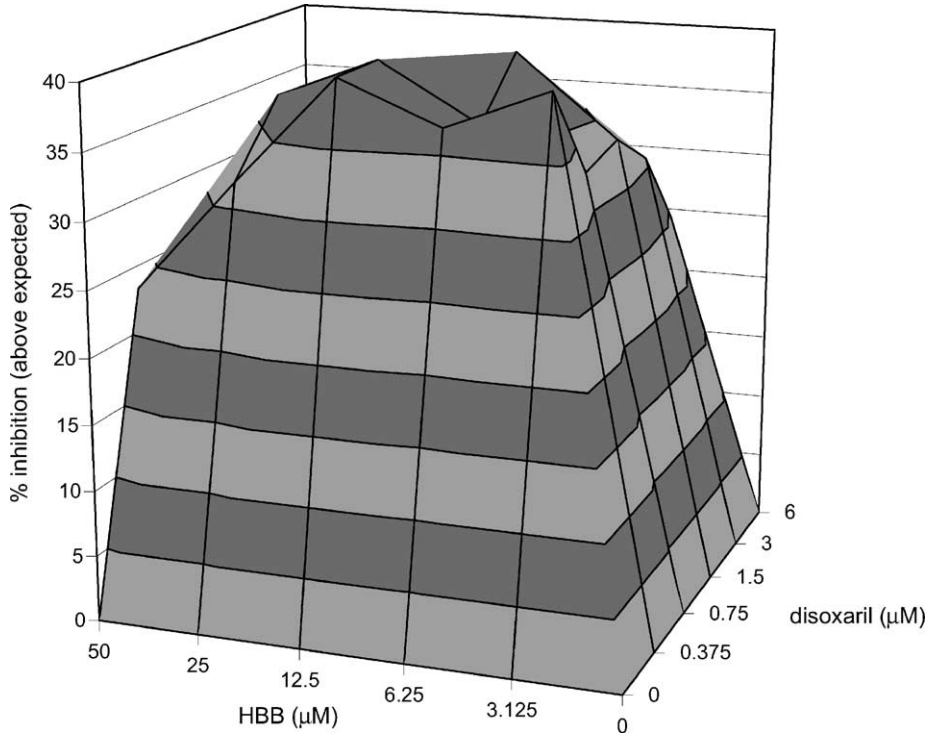

Fig. 5. Combined effects of disoxaril and HBB on the replication of CBV-1 in FL cells (dose–response surface).

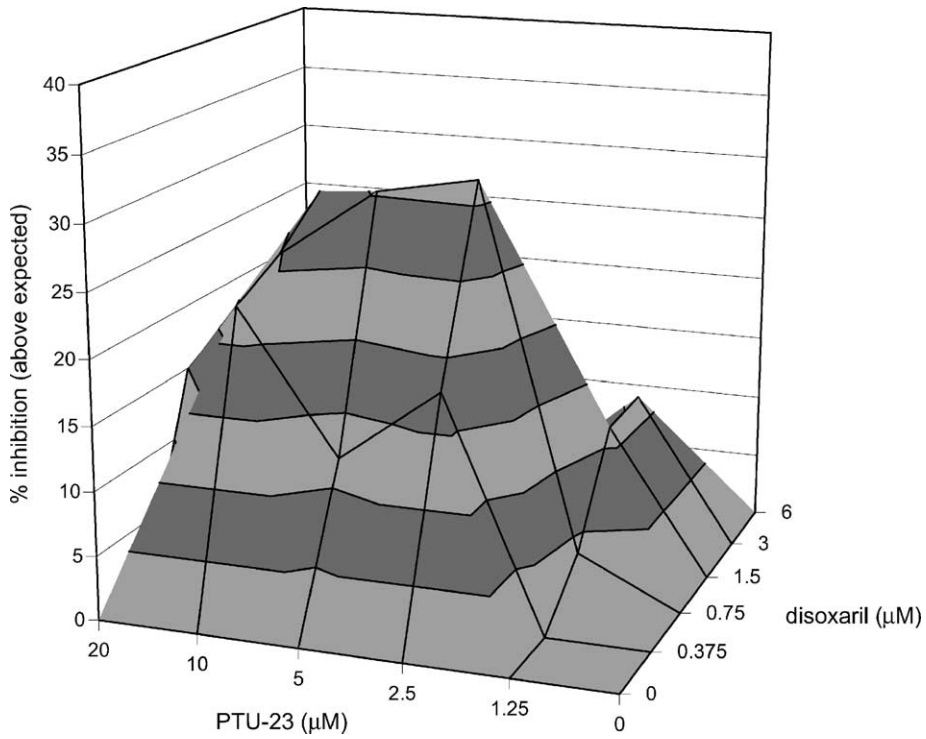

Fig. 6. Combined effects of disoxaril and PTU-23 on the replication of CBV-1 in FL cells (dose–response surface).

to the effect of uncoating "blockers" disoxaril and arildone.

All dual drug combinations tested provided synergistic antiviral effects with the exception of enviroxime in combination with arildone. The latter combination showed minor but significant value of antagonism against the replication of CBV-1. Mild antagonism between enviroxime and arildone has also been observed in our previous study on the

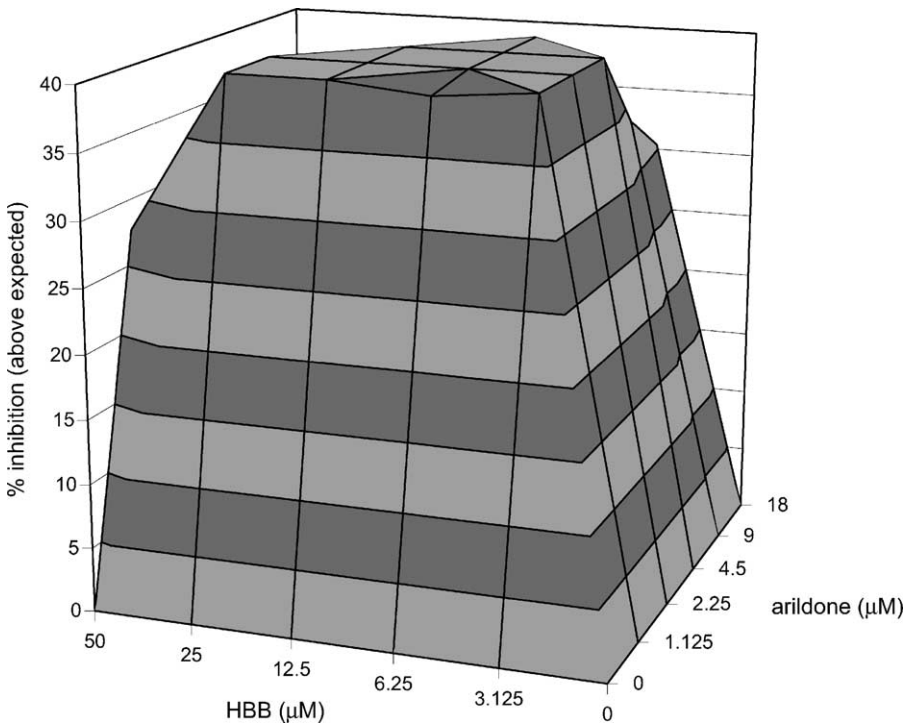

Fig. 7. Combined effects of arildone and HBB on the replication of CBV-1 in FL cells (dose–response surface).

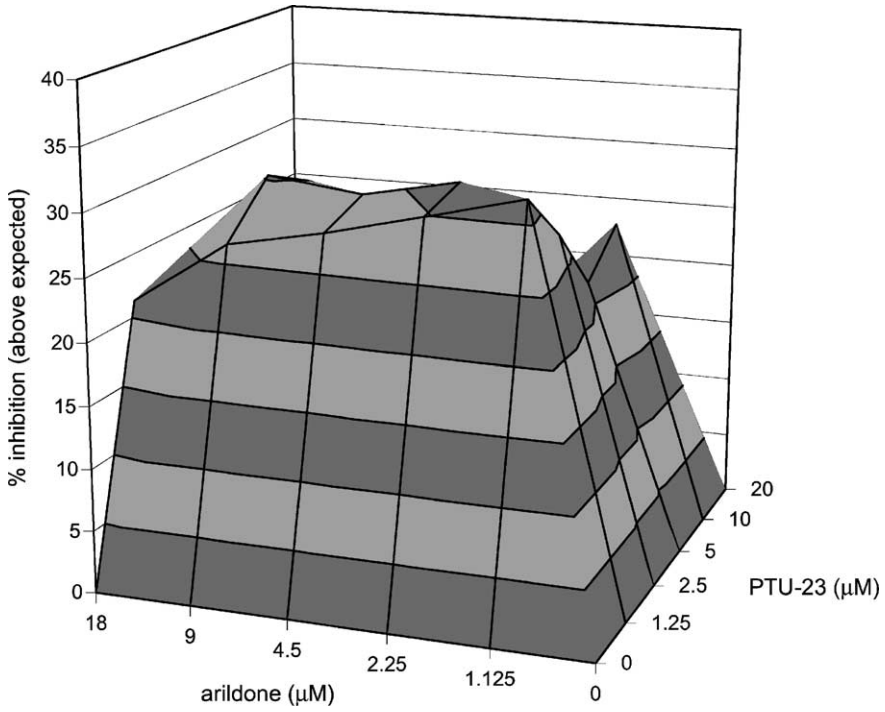

Fig. 8. Combined effects of arildone and PTU-23 on the replication of CBV-1 in FL cells (dose–response surface).

combined effects of those inhibitors against the replication of PV-1 (Nikolaeva and Galabov, 1999).

As seen from Table 1, the highest volume of synergy along with a smooth and homogenous shape of the dose–response surface was represented for the combinations of the benzimidazole derivative HBB with arildone (Fig. 7) and disoxaril (Fig. 5). In contrast, combination with enviroxime, another benzimidazole derivative, revealed a significantly lower synergy (Table 1, Figs. 11and 12). In the case of the combination of enviroxime with arildone (Fig. 12), the

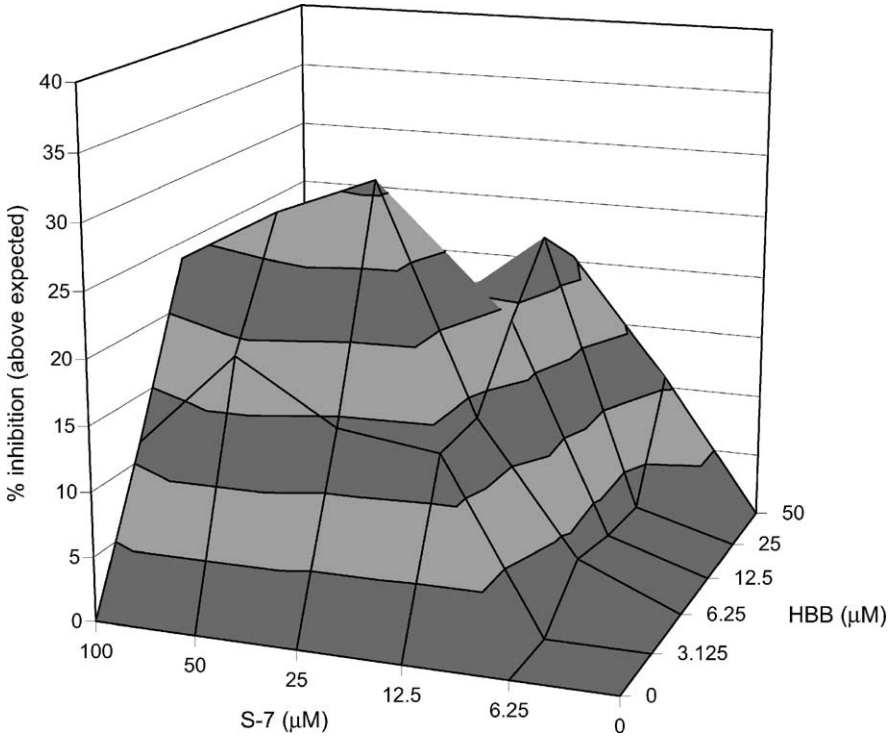

Fig. 9. Combined effects of S-7 and HBB on the replication of CBV-1 in FL cells (dose–response surface).

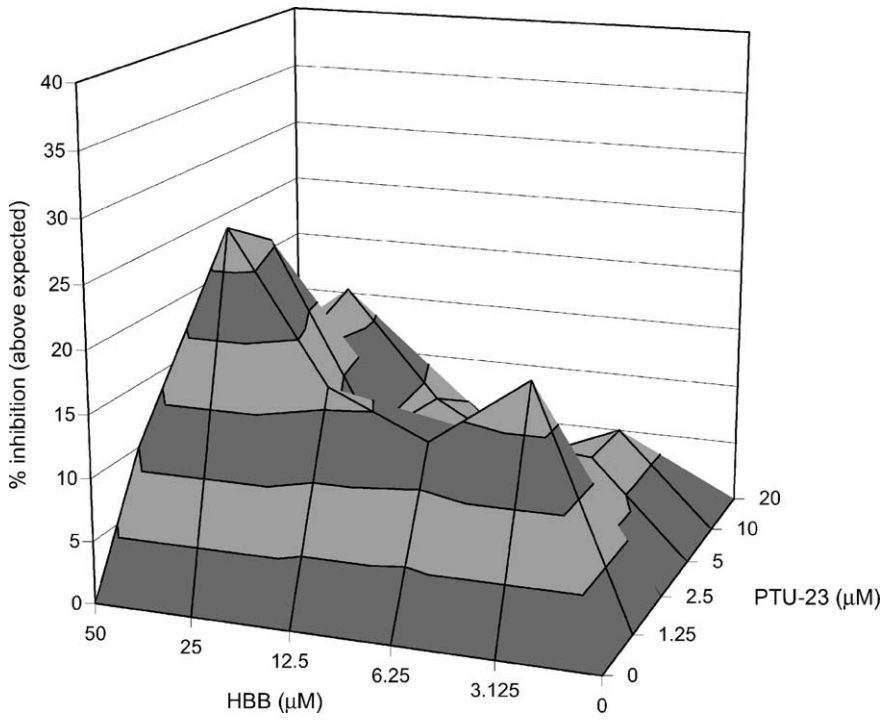

Fig. 10. Combined effects of PTU-23 and HBB on the replication of CBV-1 in FL cells (dose–response surface).

combined effect was additive with even minor but significant antagonism. The different character of the effects of the combinations of the two benzimidazole derivatives with the uncoating "blockers" arildone and disoxaril might serve as a proof for the different mechanism of action of HBB and enviroxime despite the chemical similarity. In favor of the idea for a different mechanism of action of the two benzimidazoles serves also the fact for significant synergy in the combination between both of them (Fig. 3). The third compound in our study that inhibited uncoating (besides dis-

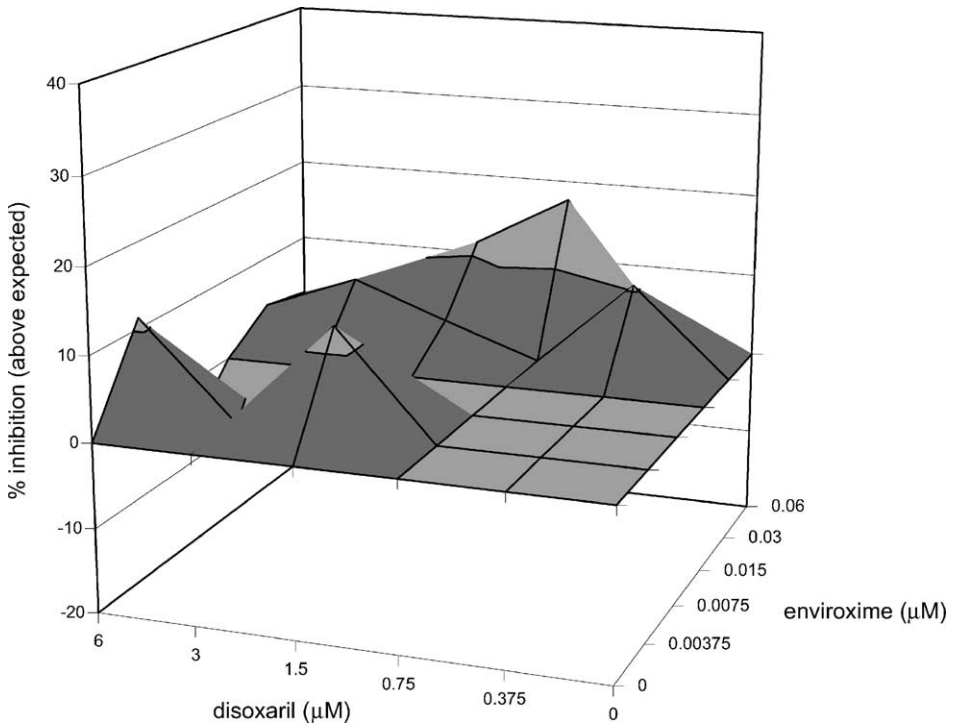

Fig. 11. Combined effects of enviroxime and disoxaril on the replication of CBV-1 in FL cells (dose–response surface).

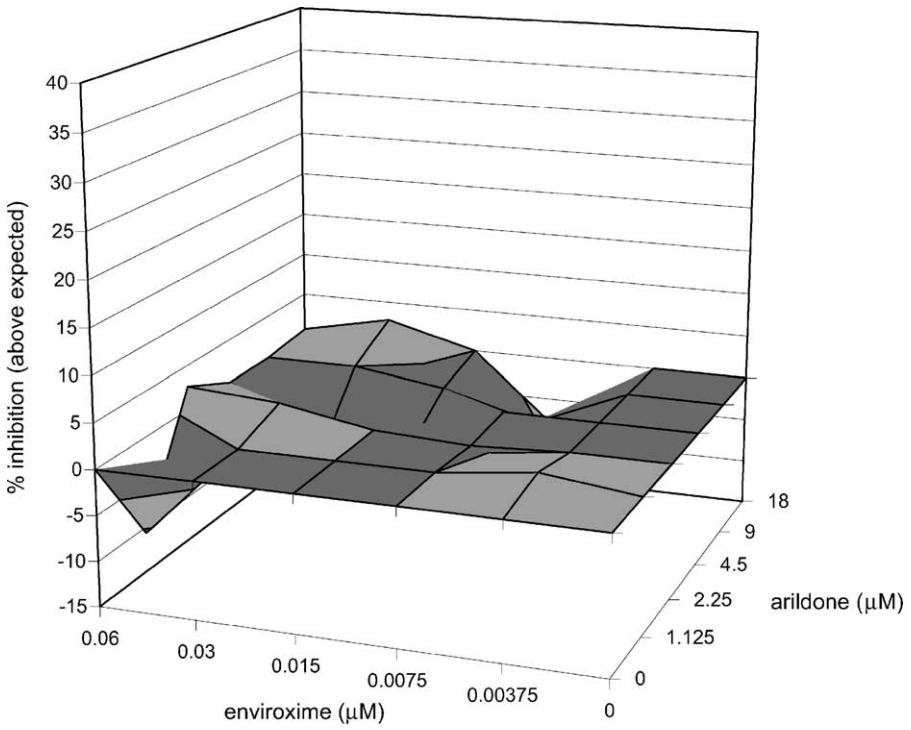

Fig. 12. Combined effects of enviroxime and arildone on the replication of CBV-1 in FL cells (dose–response surface).

oxaril and arildone), S-7, also resulted in a synergistic combination with the benzimidazoles (Figs. 2 and 9). Evidently, the synergy is based on the attack on different viral targets involved in different stages of the virus replication cycle.

Compounds that inhibit uncoating are promising partners in combinations with the benzimidazole derivatives resulting in significant synergy. What is more, combinations with HBB revealed greater synergy than the respective combinations with enviroxime (Table 1). The more favorable effect of HBB, in comparison to enviroxime, as a partner in combinations with the uncoating "blockers" arildone and disoxaril was observed also in the case of the replication of poliovirus 1 (Nikolaeva and Galabov, 1999).

Combinations with the original Bulgarian compound PTU-23 were all highly synergistic (Table 1), with characteristic twin-peaked dose–response surfaces (Figs. 4, 6, 8 and 10). PTU-23 might be considered as a partner for anti-enteroviral combinations. The combined effects of this compound deserve further investigations having also in mind its marked antiviral effect in vivo (Galabov and Velichkova, 1974; Galabov, 1979).

As far as the statistically insignificant antagonism mentioned in the combination of enviroxime and disoxaril is concerned, this antagonism was observed only in certain dose combinations of the inhibitors. A similar antagonistic "valley" at a certain concentration of enviroxime was seen in the case of the same combination tested against the replication of poliovirus 1 (Nikolaeva and Galabov, 1999). This could quite well illustrate the very important postulate in combined chemotherapy that there are no synergistic or antagonistic combinations in general but only certain combinations of doses that act synergistically, additively or antagonistically. In this respect, different dose combinations of the same agents may exhibit different types of interactions (Prichard and Shipman, 1990).

Generally, the synergistic combined effects against the replication of CBV-1 are stronger as compared to the effects of the same combinations against PV-1 replication. Simi-

Table 1
Picornavirus inhibitors interactions in their combined application against the replication of CBV-1 in FL cells

Compound	Mean volume of synergy (or antagonism) in $\mu M^2\%$					
	Enviroxime	Disoxaril	Arildone	PTU-23	HBB	S-7
Enviroxime	–	82 (−13)	9 (−39)	373	253	399
Disoxaril	82 (−13)	–	n.d.	314	689	n.d.
Arildone	9 (−39)	n.d.	–	519	852	n.d.
PTU-23	373	314	519	–	275 (−4)	n.d.
HBB	253	689	852	275 (−4)	–	368

n.d.: not done. The 95% confidence value is listed. The degree of antagonism is indicated by a negative value in parentheses.

lar synergistic combined effects could be expected in other enterovirus models as well. The results in this study indicate that anti-enterovirus agents with different mechanisms of antiviral action interact principally in an additive or synergistic manner. This would be easily explained by the fact that the partners in the combinations attack different targets, or different steps in the virus replication cycle.

Values of synergy observed in the tested combinations in most cases reveal strong synergy and indicate that those combinations would be probably worth testing in vivo. The combined administration of those antiviral agents should not only produce additive or synergistic effect but should certainly delay the development of drug resistance. The synergistic combinations described above deserve further investigation with regard to their cytotoxicity as well as the presence (or absence) of cross-resistance. In general, combinations of different picornavirus replication inhibitors would possibly contribute to a more successful experimental chemotherapy of picornavirus infections.

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