SYNERGISTIC INHIBITORY EFFECT OF ENVIROXIME AND DISOXARIL ON POLIOVIRUS TYPE 1 REPLICATION

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Summary. – The effects of enviroxime, disoxaril and ribavirin in pair combinations on poliovirus type 1 (Mahoney) replication in FL cells were tested. Beforehand, the fifty percent inhibitory concentration (IC_{50}) was determined for each compound alone: enviroxime – 0.2 μmol/l, disoxaril – 0.3 μmol/l, ribavirin – 3 μmol/l. Combining enviroxime with disoxaril resulted in synergistic interaction, while combinations with ribavirin were markedly antagonistic. Enviroxime- and disoxaril-resistant poliovirus mutants appeared following 10 and 2 consecutive passages in FL cells, respectively. No cross-resistance was observed between these mutants towards disoxaril and enviroxime, respectively.

Key words: poliovirus; antivirals; enviroxime; disoxaril; ribavirin; combination effects; resistant mutants

Introduction

The role of enteroviruses as aetiological agents of severe diseases of the heart, liver, eyes and pancreas, as well as of well known acute infections of the central nervous system, has recently roused great interest in antiviral chepotherapy of enterovirus infections. Although a number of picornavirus replication inhibitors has been described, the therapy of enterovirus diseases remains elusive. The main reason for that is the fast development of resistant and even dependant mutants (Loddo, 1980), as well as the comparatively narrow antiviral range of the so far studied inhibitors.

To overcome these diffilculties, the use of synergistic combinations of antivirals could be one of the possible efficient approaches. Thus, the same effect could be potentially achieved with lower concentrations of drugs than with those required for the drugs used alone. In this way a higher selectivity ratio could be realized. Furthermore, discovery of synergistic combinations lacking cross-resistance would be especially favourable, because this would restrain the resistance phenomenon.

We made a preliminary study of the combined effects of a large number of picornavirus replication inhibitors. A special attention was paid to enviroxime, disoxaril and ribavirin, compounds with a known to a great extent mechanism of action. Enviroxime is thought to interfere with virus replication by affecting the formation of the RNA polymerase complex (Ninomiya *et al.*, 1985). Disoxaril inserts itself into the hydrophobic canyon within the poliovirus VP1 capsid protein, thus preventing uncoating (Fox *et al.*, 1986; Zeichhardt *et al.*, 1987). Ribavirin is a broad-spectrum antiviral agent exerting various effects on cellular metabolism, generally based on reduction of intracellular GTP pool and inhibition of 5'-cap formation on mRNAs. Its virus-specific action is complex and can be mainly attributed to inhibition of the formation of viral mRNAs and/or of the function of virus-coded RNA polymerase necessary to prime (initiate) and elongate viral mRNAs (Canonico, 1983; Sidwell *et al.*, 1985; Gilbert and Knight, 1986).

In the present paper we describe the effects of pair combinations of these three viral inhibitors on poliovirus 1 replication. A synergistic effect was observed when enviroxime with disoxaril were used. Pair combinations of these substances with ribavirin revealed a marked antagonism.

Abbreviations: DMSO = dimethylsulfoxide; MEM = minimal essential medium

Materials and Methods

Virus. Poliovirus type 1 (Mahoney) was grown in FL cells in Eagle's Minimal Essential Medium (MEM, Flow) with 5% heated calf serum and antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml). The stock virus titered to 3.2 x 10^8 PFU/ml.

Cells. All experiments were carried out in monolayer FL cells. Growth medium consisted of a mixture of equal parts of medium 199 (Difco) and Hanks' saline, supplemented with 10% heated calf serum and antibiotics.

Antivirals. Enviroxime (anti-6-[(hydroxyimino)-phenylmethyl]-1-[(1-methylethyl) sulfonylimidazol-2-amine, LY 122722) was supplied by the Lilly Laboratories of Eli Lilly & Co. (Indianapolis, USA). A stock solution of 30 mmol/l enviroxime in dimethylsulfoxide (DMSO) was prepared and then *ex tempore* diluted in Eagle's MEM to the required concentrations.

Disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methyl-isoxazole, WIN 51 711), supplied by Sterling Research Group of Sterling Drug, Inc. (Rensselaer, USA) was prepared as a 30 mmol/l stock solution in DMSO and then diluted in Eagle's MEM.

Ribavirin (1-ß-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) (ICN, Irvine, USA) was kindly supplied by Prof. R.W. Sidwell, Logan, USA. It was dissolved straight to the necessary concentrations in Eagle's MEM.

Plaque inhibition test. Monolayer FL cell cultures in 20 ml scintillation glass vials (diameter 2.5 cm) were inoculated with 50 – 60 PFU of virus per vial and left for 1 hr at room temperature. Then 1 ml per vial of an agar overlay (1% purified Difco agar in Eagle's MEM supplemented with 10% heated calf serum, 2.25 mg/ml sodium bicarbonate and antibiotics) was laid over the cells. The tested compounds, either alone of in pair combinations, were included in the agar overlay. Following a 48 hrs incubation at 37 °C a second agar overlay (1.5% agar with 0.02% neutral red (Gibco) in physiological saline) was added and vials were kept at room temperature. The percentage of PFU inhibition was evaluated in comparison to the control (no compound in the agar overlay).

Estimation of the combination effects. The combination effect character was estimated by use of the three-dimensional model for analyzing drug-drug interactions of Prichard and Shipman (1990). The additivity assumption equations for both the single and different site inhibitors were used. The difference between the observed combination effects and those expected if effects were additive, was calculated. Positive values were indicative for synergism, while negative ones for antagonism. DELTA GRAPH computer programme, kindly supplied by Prof. W. Shannon (Birmingham, Alabama, USA) was applied for graphic representation of the dose-response surface.

Development of drug-resistant mutants. Consecutive passages of poliovirus type 1 (Mahoney) in FL cell monolayers in test tubes were carried out in the presence of 40 µmol/l enviroxime or disoxaril in Eagle's MEM supplemented with 5% heated calf serum. Virus yields were assayed (cultures were three times frozen and thawed) after CPE spread over the whole of the cell monolayer. The sensitivity test was monitored in a multi-cycle virus growth experiment using a multiplicity of infection of 0.01 PFU per cell, and a dose-response scheme for each compound. The infectious virus yields (in PFU/ml) were recorded after 24 hrs incubation at 37 °C and the percentage of inhibition was evaluated in comparison to the controls.

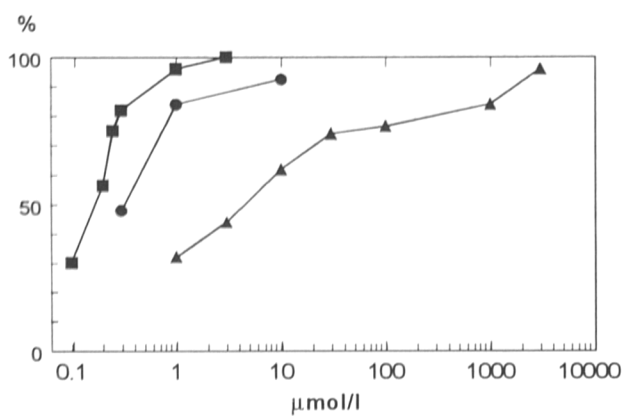

Fig. 1
Effect of enviroxime, disoxaril and ribavirin on poliovirus type 1 replication in FL cells (dose response curves)
Enviroxime (■), disoxaril (●), ribavirin (▲)

Results

Effects of enviroxime, disoxaril and ribavirin alone or in pair combinations on poliovirus replication

As a first step the IC_{50} values of each compound alone were determined. They were as follows: 0.2 µmol/l for enviroxime, 0.3 µmol/l for disoxaril and 3 µmol/l for ribavirin (Fig. 1).

Table 1 presents experimental data on the antiviral effect of the combination of enviroxime plus disoxaril. Fig. 2

Table 1. Effect of the combination of enviroxime with disoxaril on poliovirus type 1 replication in FL cells

Disoxaril IC_{50} (µmol/l)	Enviroxime IC_{50} (µmol/l)					
	0	1/8 (0.025)	1/4 (0.05)	1/2 (0.1)	1 (0.2)	2 (0.4)
2 (0.6)	22.5[a]	4	15.5	0.5	0	0.5
	55.5%[b]	92.1%	69.3%	99.0%	100%	99.0%
1 (0.3)	25	8.5	18	0	0	0
	50.5%	83.2%	64.4%	100%	100%	100%
1/2 (0.15)	23.5	12	20.5	0	0.5	1
	53.5%	76.2%	59.4%	100%	99.0%	98.0%
1/4 (0.075)	27	26	19.5	2	2.5	1
	46.5%	48.5%	61.4%	96.0%	95.1%	98.0%
1/8 (0.0375)	29	33.5	20	5	7	0
	42.6%	33.4%	60.4%	90.1%	86.1%	100%
0	50.5[c]	37.5	32.5	23	25.5	17
	0	25.7%	35.6%	54.5%	49.5%	66.3%

[a]Average number of plaques per vial.
[b]Inhibition.
[c]Virus control.

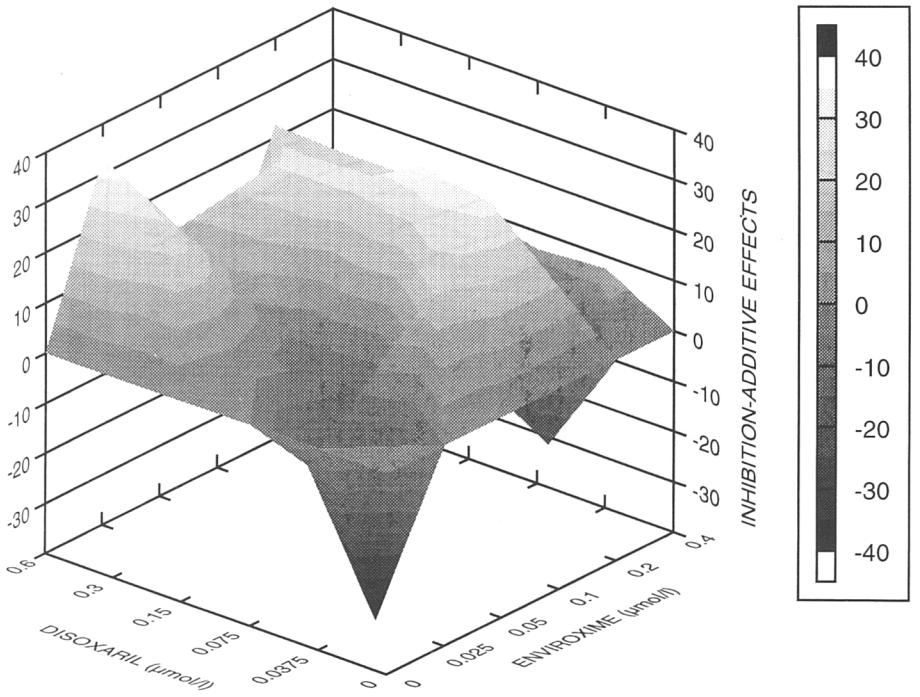

Fig. 2
Effect of the combination of enviroxime with disoxaril on poliovirus type 1 replication in FL cells
Dose-response surface, different site assumption equation. Peaks (positive values) and valleys (negative values) indicate synergy and antagonism, respectively.

shows that combining enviroxime with disoxaril led to a well manifested synergism. A well reproducible additive to antagonostic effect "valley" is demonstrated when enviroxime participates at a concentration of 0.05 μmol/l.

Combining enviroxime with ribavirin revealed a marked antagonism (Table 2). Fig. 3 and 4 illustrate the effect calculated according to the assumption of single site and different site inhibitors, respectively.

A strong antagonism was also observed when a combination of disoxaril plus ribavirin was used (Table 3, Fig. 5 and 6).

Cross-resistance studies

Just two virus passages in the presence of disoxaril were sufficient for selection of a disoxaril-resistant mutant. With enviroxime-resistant mutants the selection was slow. The resistance was found only after the tenth passage in the presence of this compound.

The examination of enviroxime-resistant and disoxaril-resistant mutants of poliovirus for cross-sensitivity revealed lack of cross-resistance, i.e. the enviroxime-resistant mutant preserved its sensitivity to disoxaril, and *vice versa*, the sensitivity of the disoxaril-resistant mutant towards enviroxime was preserved too (Table 4).

Table. 2. Effect of the combination of enviroxime with ribavirin on poliovirus type 1 replication in FL cells

Ribavirin IC_{50} (μmol/l)	Enviroxime IC_{50} (μmol/l)					
	0	1/8 (0.025)	1/4 (0.05)	1/2 (0.1)	1 (0.2)	2 (0.4)
2	16.5[a]	18.5	14	11	10.5	12.5
(6)	64.1%[b]	59.8%	69.6%	76.1%	77.2%	72.8%
1	25	18	18.5	18	11	10
(3)	53.3%	60.9%	59.8%	60.9%	76.1%	78.3%
1/2	22.5	18.5	19	17.5	12	11.5
(1.5)	51.1%	59.8%	58.7%	62.0%	73.9%	75.0%
1/4	26	22.5	22	16.5	19	13
(0.75)	43.5%	51.1%	54.4%	64.1%	58.7%	71.7%
1/8	31	25.5	24	23	18.5	12.5
(0.375)	32.6%	43.6%	47.8%	50.0%	59.8%	72.8%
0	46[c]	35	28.5	27	20	16
	0	23.9%	38.0%	41.3%	56.5%	65.2%

[a]Average number of plaques per vial.
[b]Inhibition.
[c]Virus control.

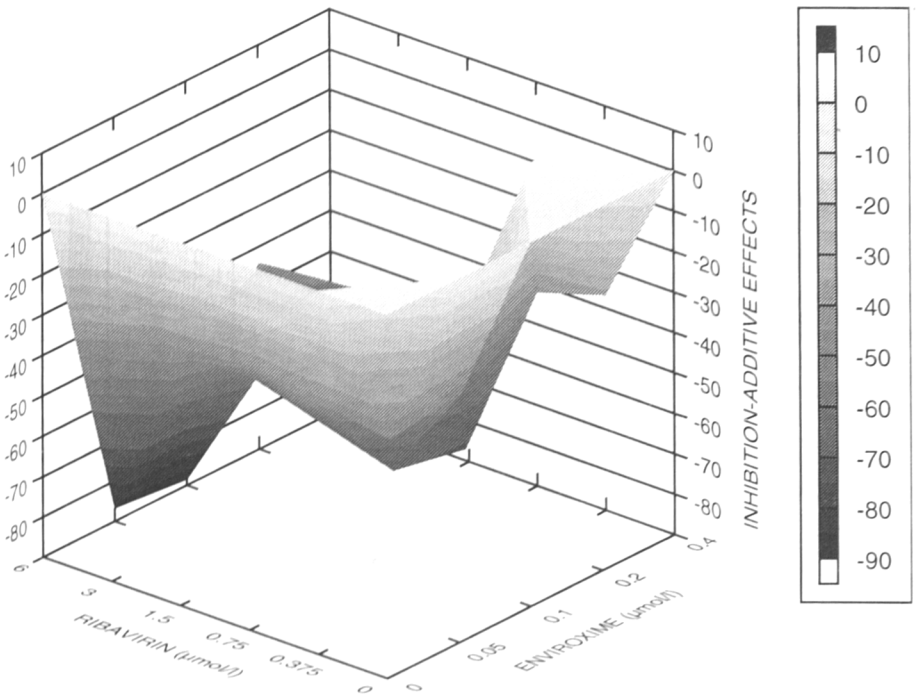

Fig. 3
Effect of the combination of enviroxime with ribavirin on poliovirus type 1 replication in FL cells
Dose-response surface, single site assumption equation. For the rest of legend see Fig. 2.

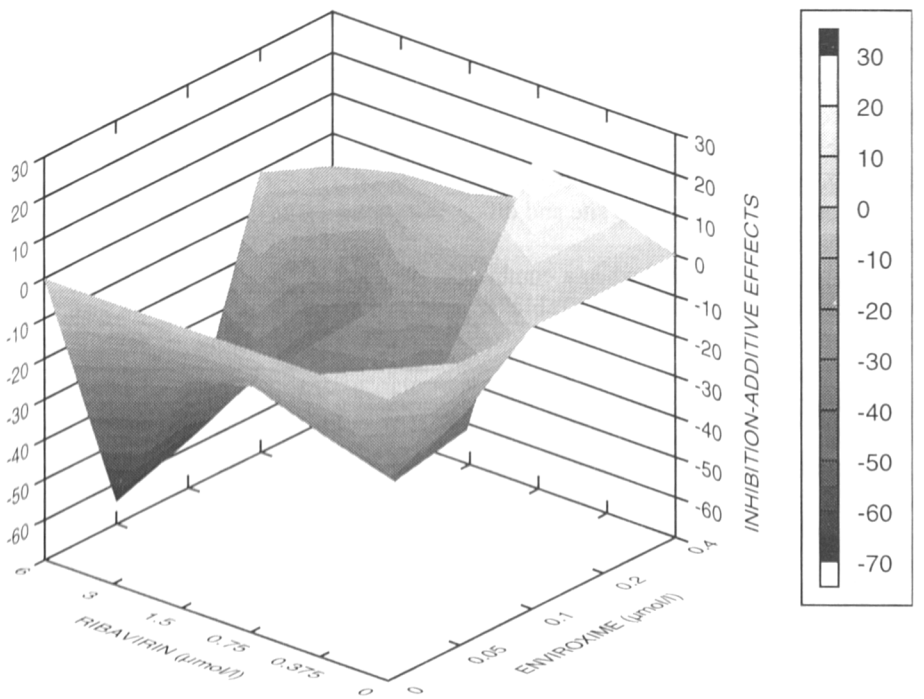

Fig. 4
Effect of the combination of enviroxime with ribavirin on poliovirus type 1 replication in FL cells
For the legend see Fig. 2.

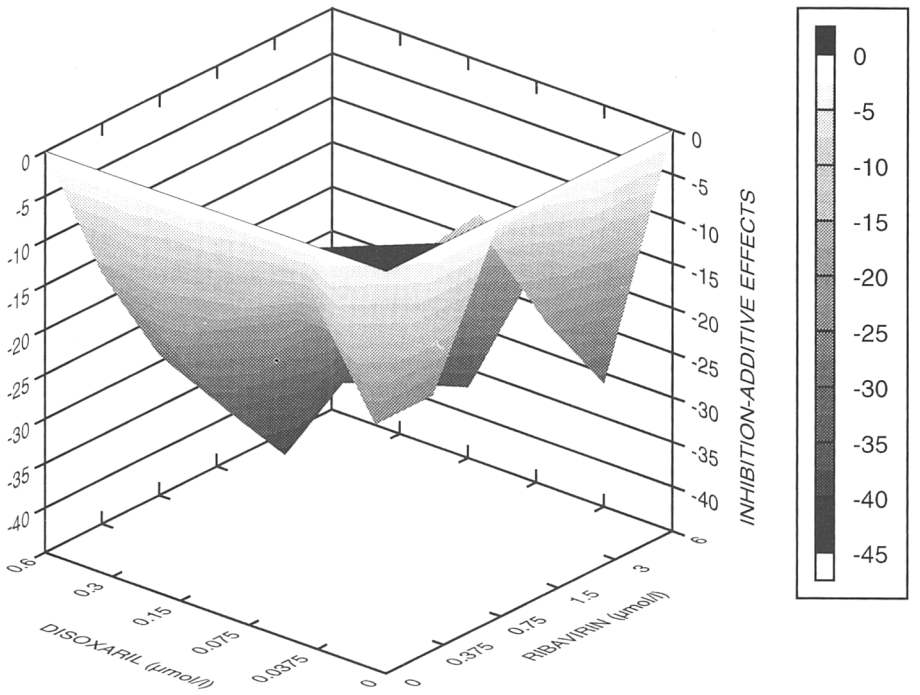

Fig. 5
Effect of the combination of disoxaril with ribavirin on poliovirus type 1 replication in FL cells
For the legend see Fig. 3.

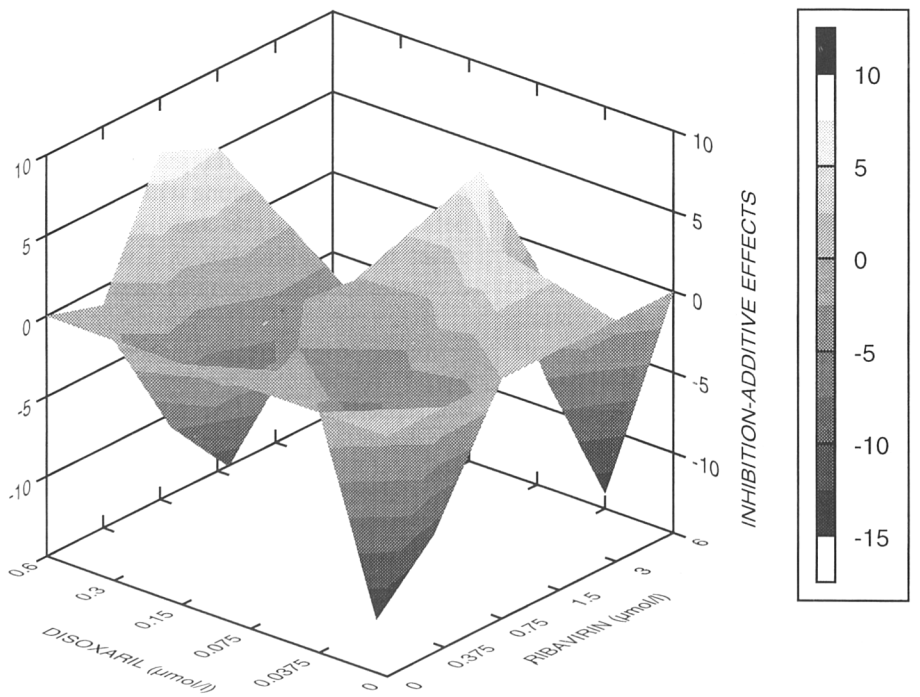

Fig. 6
Effect of the combination of disoxaril with ribavirin on poliovirus type 1 replication in FL cells
For the legend see Fig. 2.

Table 3. Effect of the combination of disoxaril with ribavirin on poliovirus type 1 replication in FL cells

Disoxaril IC_{50} (μmol/l)	Ribavirin IC_{50} (μmol/l)					
	0	1/8 (0.025)	1/4 (0.05)	1/2 (0.1)	1 (0.2)	2 (0.4)
2 (0.6)	24[a]	20.5	32.5	19	16	16.5
	58.3%[b]	64.4%	43.5%	67.0%	72.2%	71.3%
1 (0.3)	32.5	25.5	28	25.5	18.5	18.5
	43.5%	55.7%	51.3%	55.7%	67.8%	67.9%
1/2 (0.15)	34.5	28.5	29	21.5	18	20
	40.0%	50.4%	49.6%	62.6%	68.7%	65.2%
1/4 (0.075)	41.5	29	38	23	19	20.5
	27.8%	49.6%	33.9%	60.0%	67.0%	64.4%
1/8 (0.0375)	54	44.5	35.5	34	22.5	24
	6.1%	22.6%	38.3%	48.7%	60.9%	58.3%
0	56.5[c]	41	35	33	25.5	26
	0	28.7%	39.1%	42.6%	55.7%	54.8%

[a]Average number of plaques per vial.
[b]Inhibition.
[c]Virus control.

Table 4. Effects of disoxaril and enviroxime on replication of wild type poliovirus 1 and its enviroxime- and disoxaril-resistant mutants in FL cells

Drug (μmol/l)		Inhibition (%)		
		Wild type	Disoxaril-resistant mutant	Enviroxime-resistant mutant
Enviroxime	1	98.7	98.2	0
	3	98.3	98.0	0
	10	98.5	97.8	0
	30	98.8	97.6	0
Disoxaril	1	98.3	0	93.4
	3	98.0	1.5	92.9
	10	97.8	0	93.7
	30	98.2	0	94.0

Discussion

The synergistic effect of the combination of disoxaril plus enviroxime could be explained by the fact that these partners attack different targets at even different steps of poliovirus replication, namely, the uncoating for disoxaril (Fox *et al.*, 1986; Zeichhardt *et al.*, 1987) and the formation of viral RNA polymerase complex for enviroxime (Ninomiya *et al.*, 1985).

Our data about the antagonism between ribavirin and enviroxime correlate with those reported by Al-Nakib and Tyrell (1987) who have tested the same combination towards human rhinovirus 2.

Moreover, ribavirin antagonizes the effect of almost all known inhibitors of picornavirus replication: vs. rhinoviruses (Al-Nakib and Tyrell, 1987) and vs. enteroviruses (L. Nikolaeva and A.S. Galabov, unpublished data).

The explanation of the observed antagonistic effects in the combinations with ribavirin is considerably more sophisticated. In the case of the combination of enviroxime plus ribavirin a competitive inhibition of the processes of initiation and elongation of viral RNAs could be suspected. As far as the combination of disoxaril plus ribavirin is concerned, the explanation of the antagonism is completely missing. Having in mind the so far unclear participation or cellular components in the replication of picornaviruses (Dmitrieva *et al.*, 1979; Richards *et al.*, 1990), a possible involvement of a cell-dependent mechanism in the formation of the disoxaril target could be presumed.

The fast development of resistance to disoxaril (in two passages only) is quite similar to the development of resistance to arildone, a compound with analogous mechanism of action (McSharry *et al.*, 1979), observed by Eggers and Rosenworth (1988). Similar data exist also about guanidine hydrochloride (Caliguiri and Tamm, 1972).

There are examples of favourable combination effects, e.g. of 2-(α-hydroxybenzyl)benzimidazole (HBB) and guanidine – two classic enterovirus replication inhibitors (Tamm and Eggers, 1962; Tamm and Caliguiri, 1972). Unfortunately, a strikingly expressed cross-resistance has been found in that case.

The established synergism between enviroxime and disoxaril, especially along with the lack of cross-resistance between the enviroxime- and disoxaril-resistant mutants classifies this combination as a very promising one from a chemotherapeutic point of view.

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References

Al-Nakib W, Tyrell DAJ (1987): A 'new' generation of more potent synthetic antirhinovirus compounds: comparison of their MICs and their synergistic interactions. *Antivir. Res.* **8**, 179–188.

Caliguiri LA, Tamm I (1972): Guanidine. In Bauer DJ (Ed.): *International Encyclopedia of Pharmacology and Therapeutics* (Sect. 61), *Chemotherapy of Virus Diseases* (Vol. I). Pergamon Press, Oxford, pp. 181–230.

Canonico PG (1983): Ribavirin. A review of efficacy, toxicity and mechanisms of antiviral activity. In Hahn FE (Ed.): *Antibiotics* (Vol. 6), *Modes and Mechanisms of Microbial Growth Inhibitors.* Springer-Verlag Berlin, Heidelberg, pp. 109–134.

Dmitrieva TM, Shchoglova NV, Agol VI (1979): Inhibition of activity of encephalomyocarditis virus-induced RNA polymerase by antibodies against cellular components. *Virology* **92**, 271–277.

Eggers HJ, Rosenwirth B (1988): Isolation and characterization of an arildone-resistant poliovirus 2 mutant with an altered capsid protein VP1. *Antivir. Res.* **9**, 23–26.

Fox MP, Otto MJ, McKinley MA (1986): The prevention of poliovirus and rhinovirus uncoating by WIN 51 711: a new antiviral drug. *Antimicrob. Agents Chemother.* **30**, 110–116.

Gilbert BE, Knight V (1986): Biochemistry and clinical application of ribavirin. *Antimicrob. Agents Chemother.* **30**, 201–205.

Loddo B (1980): Development of drug resistance and dependence in viruses. *Pharm. Ther.* **10,** 431–460.

McSharry JJ, Caliguiri LA, Eggers HJ (1979): Inhibition of uncoating of poliovirus by arildone, a new antiviral drug. *Virology* **97**, 307–315.

Ninomiya Y, Aoyama M, Umoda Y, Suhara Y, Ishitsuka H (1985): Comparative studies on the mode of action of antirhinovirus agents Ro OS-0410, Ro 09-0179, RMI-15 731, DCF and enviroxime. *Antimicrob. Agents Chemother.* **27**, 595–599.

Prichard MN, Shipman CJr (1990): A three-dimensional model to analyze drug-drug interactions. *Antivir. Res.* **14**, 181–206.

Richards OC, Ehrenfeld E (1990): Poliovirus RNA replication. In Racaniello VR (Ed.): *Current Topics in Microbiology and Immunology.* Vol. 161, Springer-Verlag Berlin, Heidelberg, pp. 90–115.

Sidwell RW, Revankar GR, Robins RK (1985): Ribavirin: review of a broad-spectrum antiviral agent. In Shugar D (Ed.): *International Encyclopedia of Pharmacology and Therapeutics. Viral Chemotherapy.* Vol. 116/2, Pergamon Press, Oxford, pp. 49–107.

Tamm I, Caliguiri LA (1972): 2-(α-hydroxybenzyl)benzimidazole and related compounds. In Bauer DJ (Ed.): *International Encyclopedia of Pharmacology and Therapeutics* (Sect. 61), *Chemotherapy of Virus Diseases* (Vol. I). Pergamon Press, Oxford, pp. 115–180.

Tamm I, Eggers HJ (1962): Differences in the selective virus inhibitory action of 2-(α-hydroxybenzyl)benzimidazole and guanidine-HCl. *Virology* **18**, 439–447.

Zeichhardt H, Otto MJ, McKinlay MA, Willingmann P, Habermehl KO (1987): Inhibition of poliovirus uncoating by disoxaril (WIN 51 711). *Virology* **160**, 281–285.