CYTOTOXICITY OF THE SYNERGISTIC ANTIENTEROVIRAL COMBINATION OF ENVIROXIME AND DISOXARIL

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Summary. – When combined, enviroxime and disoxaril, two selective picornavirus inhibitors, exert a marked synergistic inhibitory effect on poliovirus type 1 replication in FL cells. The cytotoxicity of the compounds applied individually and in combination to the same cells was examined. The quantitative assay of the cytotoxic effect was made by determination of the growth curve of uninfected FL cells in the presence of increasing concentrations of the compounds applied alone and in combination. The obtained results indicate lack of a synergic cytotoxic effect of the combination of enviroxime and disoxaril. The previously established synergistic antiviral effect, and the lack of cross-resistance and synergic cytotoxic effect classify the combination of enviroxime and disoxaril as a very promising chemotherapeutic.

Key words: enviroxime; disoxaril; enterovirus; cytotoxicity; antivirals; additive effects; synergism

Introduction

Human and animal enteroviruses are of great medical and economic importance, being the causative agents of a variety of diseases and syndromes. Although a number of picornavirus replication inhibitors have been described, the therapy of enterovirus diseases still remains elusive. With high incidence of enteroviral infections and high rate of emergence of resistant and even dependent mutants (Loddo, 1980) there is a critical need for a new approach to antienteroviral chemotherapy. Combining enviroxime and disoxaril, antipicornavirus agents with a known mechanism of action (Heinz and Vance, 1995; Zeichhardt *et al.*, 1987), has resulted in a synergistic inhibitory effect on the replication of poliovirus type 1 in cell culture (Nikolaeva and Galabov, 1995). Lack of cross-resistance between the selected enviroxime- and disoxaril-resistant mutants has been established, too (Nikolaeva and Galabov, 1995).

In the present paper, the cytotoxicity of enviroxime and disoxaril applied individually and in combination is described as a part of the efforts to evaluate this potent antienteroviral combination.

Materials and Methods

Cells. FL cells cultures of Fr cells were used in all experiments, the cells being routinely subcultured weekly. The growth medium consisted of equal parts of Medium 199 with Hanks' salts (Gibco BRL) and Hanks' solution, supplemented with 10% of heated calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Antiviral compounds. Enviroxime (anti-6-[hydroxyimino-phenylmethyl]-1-[(1-methylethyl)sulfonylimidazol-2-amine; LY 122722), supplied by the Lilly Laboratories of Eli Lilly & Co., Indianapolis, IN, USA, and disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methyl-isoxazole; WIN51711), supplied by Sanofi Winthrop, Inc., Malvern, PA, USA, were first dissolved in dimethylsulfoxide (DMSO) and then diluted in the growth medium to the required concentrations.

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Abbreviations: $CGIC_{50}$ = 50% cell growth inhibitory concentration; DMSO = dimethylsulfoxide; IC_{50} = 50% virus inhibitory concentration; SI = selectivity index

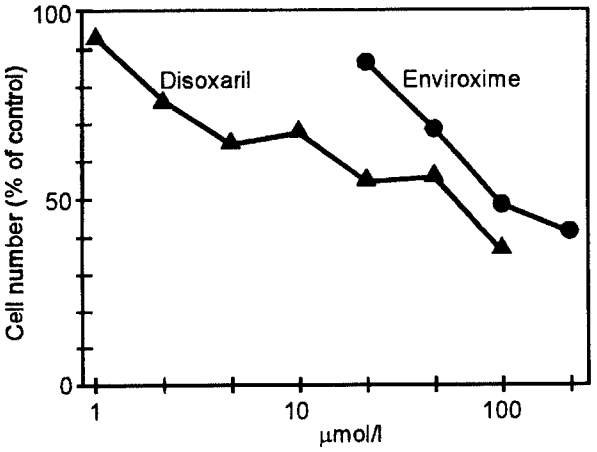

Fig. 1
Effects of enviroxime and disoxaril on the growth of FL cells

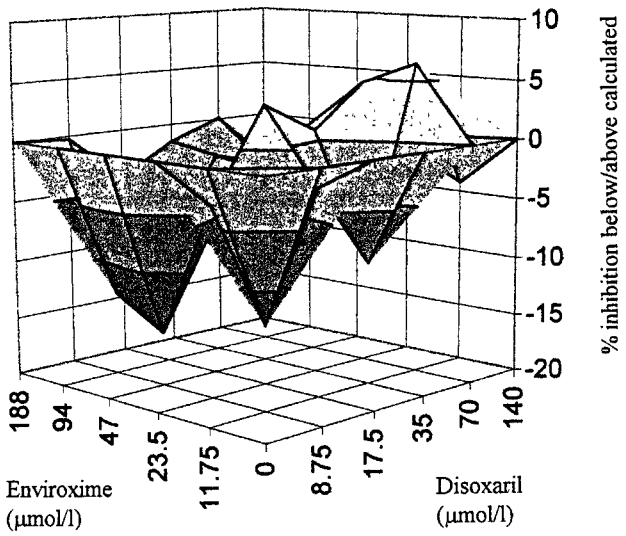

Fig. 2
3D picture of the observed cytotoxic effect of the combination of enviroxime and disoxaril
Negative/positive values represent a lower/higher cytotoxicity than the expected additive one.

Table 1. Observed cytotoxic effects of enviroxime and disoxaril applied alone and in combination

	Enviroxime					
Disoxaril	0	$CGIC_{50}/8$	$CGIC_{50}/4$	$CGIC_{50}/2$	$1xCGIC_{50}$	$2xCGIC_{50}$
$2xCGIC_{50}$	27.70	26.43	23.02	23.11	18.65	16.86
$1xCGIC_{50}$	45.55	29.97	30.60	28.94	25.19	20.41
$CGIC_{50}/2$	50.06	49.63	37.76	35.66	25.98	24.10
$CGIC_{50}/4$	59.04	49.95	42.03	45.27	37.37	30.82
$CGIC_{50}/8$	68.50	67.73	56.94	59.89	45.14	31.11
0	100[a]	79.91	77.10	64.81	57.13	44.96

Results are % values of the control.
[a]Cell control.

Table 2. Theoretical additive cytotoxic effect of the combination of enviroxime and disoxaril

	Enviroxime				
Disoxaril	$CGIC_{50}/8$	$CGIC_{50}/4$	$CGIC_{50}/2$	$1xCGIC_{50}$	$2xCGIC_{50}$
$2xCGIC_{50}$	77.86	78.64	82.05	86.94	87.55
$1xCGIC_{50}$	63.60	64.88	70.48	78.53	79.52
$CGIC_{50}/2$	60.00	61.40	67.56	76.41	77.49
$CGIC_{50}/4$	52.82	54.48	61.74	72.17	73.46
$CGIC_{50}/8$	45.26	47.19	55.61	67.72	69.20

Table 3. Observed cytotoxic effect of the combination of enviroxime and disoxaril after subtraction of theoretical additive cytotoxic effect of the combination

	Enviroxime				
Disoxaril	$CGIC_{50}/8$	$CGIC_{50}/4$	$CGIC_{50}/2$	$1xCGIC_{50}$	$2xCGIC_{50}$
$2xCGIC_{50}$	-4.29	-1.66	-5.16	-5.59	-4.41
$1xCGIC_{50}$	6.43	4.52	0.58	-3.72	0.07
$CGIC_{50}/2$	-9.63	0.84	-3.22	-2.39	-1.59
$CGIC_{50}/4$	-2.77	3.49	-7.01	-9.54	-4.28
$CGIC_{50}/8$	-12.99	-4.13	-15.5	-12.86	-0.31

Cytotoxicity assay. Approximately 1 x 10^5 cells were seeded in each well of 24-well microtiter plates. The compounds tested, either alone or in combination, were added to the growth medium immediately before seeding. The plates were incubated for 48 hrs at 37°C to allow the cells to reach the stationary phase. The medium was then removed and viable cells were counted. The cell growth curves in the presence of the compounds, applied alone and in combination, were determined, and the 50% cell growth inhibitory concentration ($CGIC_{50}$) values were calculated in relation to the untreated control (no compound in the growth medium). To characterize the cytotoxic effect of the combination, the three-dimensional model of Prichard and Shipman (1990), was used. Briefly, theoretical additive effects were calculated from the individual dose-response curve of each compound according to the dissimilar site additivity equation. The theoretical additive effects, which represented a predicted cytotoxicity, were then subtracted from the observed experimental effects. Positive differences were indicative for a synergistic cytotoxic effect, while negative ones for antagonism.

Results and Discussion

As a first step for estimating the cytotoxicity of the two compounds their individual effects on FL cells growth were determined. The following $CGIC_{50}$ values were found: 94 μmol/l for enviroxime and 70 μmol/l for disoxaril (Fig. 1). Having in mind previous data on their individual effects on the replication of poliovirus type 1 (Mahoney) in the same cell culture expressed by their 50% virus inhibitory concentrations (IC_{50}), i.e. 0.2 μmol/l for enviroxime and 0.3 μmol/l for disoxaril (Nikolaeva and Galabov, 1995), the selectivity index ($SI = IC_{50}/CGIC_{50}$) values of both

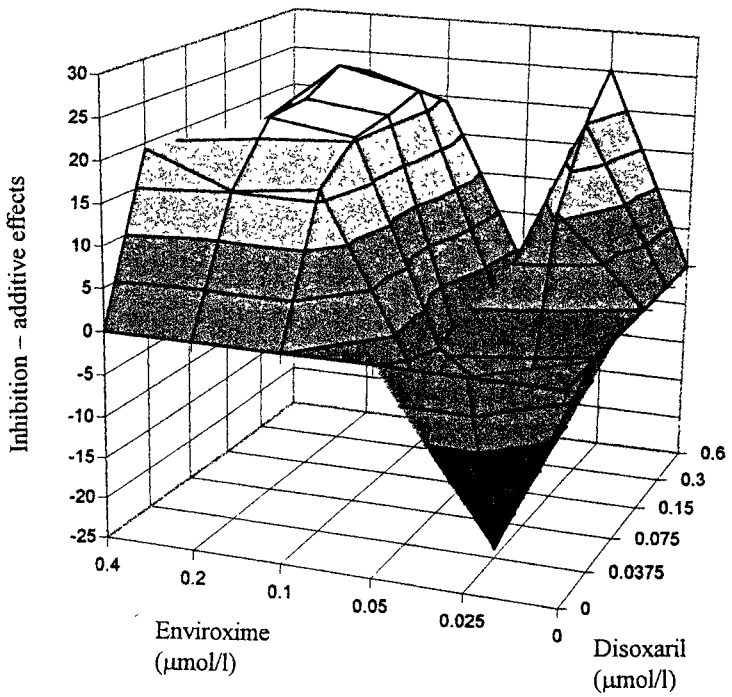

Fig. 3
3D picture of the observed inhibitory effect of enviroxime and disoxaril on poliovitus type 1 replication in FL cells
Data from Nikolaeva and Galabov (1995). Positive and negative values indicate synergism and antagonism, respectively.

compounds was found to be quite high: 470 for enviroxime and 233.3 for disoxaril.

Then a checkerboard design of the experiment for estimation of the cytotoxic effect of the combination was made and the following concentrations of each partner in the combination were used: $2xCGIC_{50}$, $1xCGIC_{50}$, $CGIC_{50}/2$, $CGIC_{50}/4$, and $CGIC_{50}/8$. The experimental data obtained are presented in Table 1 as mean values. These data were analyzed according to the three-dimensional model (Tables 2 and 3) and lack of synergism in the cytotoxicity of the combination was revealed (Fig. 2). A marked discrepancy is evident between the previously observed synergistic character of the antiviral effect of the combination (Fig. 3) (Nikolaeva and Galabov, 1995) and the additive (in some concentrations even sub-additive) character of the cytotoxic effect of the combination. This should be considered as an additional argument in favor of the virus-specific inhibitory effect of both compounds.

The previously established synergistic combined antiviral effect of enviroxime and disoxaril, the lack of cross-resistance (Nikolaeva and Galabov, 1995), and the presently demonstrated lack of synergistic cytotoxic effect classifies the combination of enviroxime and disoxaril as a very promising chemotherapeutic.

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