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Effects of double combinations of enterovirus replication inhibitors against Coxsackie B viruses

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Summary. – The effects of double combinations of enterovirus (EV) replication inhibitors against Coxsackieviruses B1 (neurotropic Connecticut-5 strain) and B3 (cardiotropic Woodruff and neurotropic Nancy strains) were tested in cell culture experiments. Compounds with different mechanisms of action were studied: pleconaril, guanidine.HCl, MDL-860 and oxoglaucine. A three-dimensional method was applied for determining the character of the combined effect. The study determined several synergistic double combinations: guanidine.HCL + pleconaril or MDL-860 against Coxsackievirus B1; MDL-860 + each of the other EV replication inhibitors and guanidine.HCl + pleconaril against the cardiotropic Woodruff strain of Coxsackievirus B3; MDL-860 + oxoglaucine against the neurotropic Nancy strain of Coxsackievirus B3. No increased cytotoxicity was manifested in any of the tested double combinations.

Keywords: antivirals; combination activity; Coxsackieviruses

Introduction

The total absence of success in the establishment of chemotherapy against enterovirus (EV) infections is due to EV's high mutation rate (10^{-3}) and the resueting formation of viral progeny consisting of quasispecies (Domingo *et al.*, 2008). This is the main reason for a quick development of drug resistance to almost all inhibitors of EV replication, which has been established in *in vitro* experiments. As a rational approach to the design of effective chemotherapy of EV infections, treatment with combinations of antivirals was introduced. In an attempt to overcome the drug-resistance barrier related to the application of monotherapeutic courses, combinations of antivirals were tested, beginning with the *in vitro* activity of double combinations of the most efficient enterovirus replication inhibitors. Application of Prichard and Shipman's (1990) methodology resulted in several markedby synergistic combinations (Nikolaeva and Galabov, 1999; Nikolaeva-Glomb and Galabov, 2004; Galabov *et al.*, 2012).

Coxsackie B viruses were preferable in these studies because they play a significant role in EV-induced human pathology: they are causative agents of a very wide range of EV infections, including myocarditis, dilated cardiomyopathy, pericarditis, endocarditis and juvenile diabetes mellitus, to name just a few (Pallansch and Roos, 2005). Table 1 summarizes data from previous *in vitro* studies of the effects of EV replication inhibitors combinations on Coxsackie B viruses.

We selected specific enterovirus replication inhibitors – pleconaril, guanidine hydrochloride, MDL-860 and oxoglaucine – and tested the effects of double combinations of these compounds on Coxsackieviruses B1 and B3. Viruses and compounds for this study were chosen with the concept of their use in the in vivo testing, as the selected viruses are very well adapted in mice. The selected compounds use different mechanisms of action. Pleconaril, a WIN compound, is a blocker of the VP1 protein in EVs (Pevear *et al.*, 1999), and guanidine.HCl targets the 2C protein (Pincus *et al.*, 1986; Cho and Ehrenfeld, 1991; Barton and Flanegan, 1997). The nitrobenzene derivative MDL-860 (Arita *et al.*, 2017) and oxoglaucine (Arita *et al.*, 2015) both

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Abbreviations: CC_{50} = 50% cytotoxic concentration; CPE = cytopathic effect; EV = enterovirus/es; IC_{50} = 50% inhibitory concentration; SI = selectivity index

Table 1. Combined effects of EV replication inhibitors against Coxsackie B viruses

Compound	Compound partner	Virus	Combination effect	References
HBB	Disoxaril	CV–B1	Synergistic	Nikolaeva-Glomb and Galabov (2004)
	Enviroxim	CV–B1	Synergistic	Nikolaeva-Glomb and Galabov (2004)
	S–7	CV–B1	Synergistic	Nikolaeva-Glomb and Galabov (2004)
Ribavirin	Oxoglaucine	CV–B1 CV–B3	Antagonistic	Nikolaeva-Glomb *et al.* (2011)
Disoxaril	Enviroxime	CV–B1	Synergistic	Nikolaeva-Glomb, Galabov (2004); Nikolaeva and Galabov (2000)
	MDL-860	CV–B3	Additive	Nikolaeva-Glomb *et al.* (2011)
Enviroxime	Guanidine	CV–B1	Synergistic	Nikolaeva-Glomb and Galabov (2004)
	PTU–23	CV–B1	Synergistic	Nikolaeva-Glomb and Galabov (2004)
Arildon	HBB	CV–B1	Synergistic	Nikolaeva-Glomb and Galabov (2004)
Oxoglaucine	Disoxaril	CV–B1	Additive	Nikolaeva-Glomb *et al.* (2007)
	Guanidine	CV–B1 CV–B3	Additive	Nikolaeva-Glomb *et al.* (2011)
	HBB	CV–B1 CV–B3	Additive	Nikolaeva-Glomb *et al.* (2011)

attack the same target – the cellular phosphatidylinositol 4-kinase III ß (PI4KIIIß), which has an important role in the picornavirus replicative complex, but they probably bind to different regions of that target.

Materials and Methods

Compounds tested. Pleconaril, 3-{3,5-dimetyl-4-[3-(3-metyl-1,2-isoxazole-5-il)propoxi]phenyl}-5-(trifluoromethyl)-1,2,4-oxadiazole (WIN-63843, Picovir©), synthesized by Dr. Vadim Makarov, A. H. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow. For tests, the compound was diluted in polyethylene glycol 400 (PEG 400). MDL-860 [2-(3,4-dichlorophenoxi)-5-nitrobenzonitril, DNB], kindly provided by Prof. Gerhard Pürstinger, Faculty of Chemistry and Pharmacy, University of Innsbruck, Innsbruck, diluted in PEG 400. Oxoglaucine [1,2,9,10-tetramethoxy-7h-dibenzo(de,g) quinolone 7-on], synthesized by Assoc. Prof. Stefan Philipov, Institute of Organic Chemistry with Centre for Phytochemistry, Bulgarian Academy of Sciences, dissolved in DMSO (AppliChem GmbH, Darmstadt, Germany) and physiological saline 1/9 v/v. Guanidine.HCl (Eastman Organic Chemicals, New York), dissolved in PBS.

Cells. Monolayer cultures of human epithelial cells type 2 (HEp-2) from the collection of the Stephan Angeloff Institute of Microbiology, BAS, Sofia, were used. Cell cultures were grown in Dulbecco's minimal essential medium (Gibco BRL, Paisley, Scotland, UK) containing 10% fetal bovine serum (Gibco BRL, Paisley, Scotland, UK), 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml, fungizone; Gibco, Invitrogen, Paisley, Scotland, UK). Maintenance solution was Dulbecco's MEM containing 0.5% fetal bovine serum. Monolayer cell cultures in 96-well CELLSTAR plates (Greiner Bio-One GmbH, Frickenhousen, Germany) and at a cellular density 3-3.5 x 10^5 cells/ml were used to determine individual and combined effects of the compounds on the replication of Coxsackie B viruses, as well as their cytotoxicity.

Viruses. Coxsackievirus B1 neurotropic strain Connecticut-5 and Coxsackievirus B3 (neurotropic Nancy strain and cardiotropic Woodruff strain) were used. They were obtained by Dr. Andreas Henke (Friedrich Schiller University of Jena, Germany).

Virus assay, antiviral effect and cytotoxicity determination. HEp-2 monolayer cell cultures in 96-well plates were infected with 0.1 ml of 10-fold serial virus dilutions, *via* 60 min adsorption at 37°C followed by incubation for 48 h at 37°C with 5% CO_2. Infectious titer was determined by the end-point dilution method (Reed and Muench, 1938) based on CPE evaluation *via* light microscopy observation and the colorimetric method with neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Barnard *et al.*, 1997; Zhang *et al.*, 2003). Optical density was determined using a spectrophotometer ELISA Teknika Reader (Orgtanon, Germany) at the wavelength 540 nm. Virus titers were as follows: Coxsackievirus B1 – $10^{6.5}$ CCID50/ml; Coxsackievirus B3 Woodruff – $10^{6.3}$ CCID50/ml; Coxsackievirus B3 Nancy – $10^{5.1}$ CCID50/ml.

Analogical methodology was used to determine the antiviral effect and cytotoxicity of each individual compound. IC_{50} was evaluated using formula described by Zhang *et al.* (2003). Cell survival was evaluated as a percentage against the control (arbitrarily set as 100%). CC_{50} values were determined based

Table 2. Antiviral activity of EV antivirals against Coxsackie B viruses quantitated by CC50, μM, IC50, μM and SI values

Compound	CV-B1 Connecticut-5 strain			CV-B3			
				Woodruff strain		Nancy strain	
	CC50	IC50	SI	IC50	SI	IC50	SI
Pleconaril	1345.7	0.2	6408.1	1.9	727.4	-	-
MDL-860	490.7	1.7	285.3	1.5	329.3	2.3	218.1
Oxoglaucine	110.8	1.5	76.4	0.6	181.6	0.6	194.4
Guanidine.HCl	>10000.0	182.4	54.8	168.2	59.5	392.9	25.5

on the constructed concentration-survival curves (Mosmann, 1983). Selectivity index values were determined based on CC_{50}/IC_{50} ratios.

Determination of the antiviral effects of the double combinations of compounds. This determination was based on the requirements of Prichard and Shipman's (1990) three-dimensional model. For this aim, 24-hour monolayer cultures of HEp-2 cells in 96-well plates were inoculated with 100 $CCID_{50}$. After 60 min adsorption, non-adsorbed virus was removed and 0.1 ml of maintenance solution was added, modified with each of the substances tested. The following compound concentrations were applied: $2IC_{50}$, IC_{50}, $IC_{50}/2$, $IC_{50}/4$, $IC_{50}/8$ and $IC_{50}/16$. Both in the virus and the cell controls, 0.2 ml per well DMEM containing 0.5% fetal bovine serum were added. Treated cell cultures were incubated at 37°C, 5% CO_2 and high humidity for 48h. CPE was recorded by light microscopy, and cultures were stained as described. The results were analyzed using the program MacSynergy™ II (Prichard *et al.*, 1993a, b), with evaluations requested for the application of Prichard and Shipman's three-dimensional model. The principle of this model is as follows: a formula, applied according to whether the partners in the combination have similar or different mechanisms of action, determines the values of the theoretically additive "dose-response" surface. The next step is evaluating the difference between the actually observed combined effect and the theoretically evaluated additive effect. Positive values suggest synergism, while negative values suggest antagonism. When the difference is zero, the combination is with additive effect. Through such obtained values, a dose-response surface is constructed. The surface expresses the deviation from the weighted theoretically additive combined effect, which has a horizontal plane of 0% (when the combination effect is purely additive). Elevation over this plane is a sign for synergism, decline under it is a sign of antagonism. The height and depth are proportional to the degree of synergism and antagonism, respectively.

The program MacSynergy™ II also permits a statistical treatment of the results in several confidence intervals. The figures in this study present dose-response surfaces for each of studied combinations at a 95% confidence interval. MacSynergy™ II determines the total value of synergism/antagonism in the entire dose range investigated. The results obtained could be interpreted as follows: (a) values of synergism/antagonism up to 25 $\mu M^2\%$ are considered as statistically insignificant; (b) values between 25 and 50 $\mu M^2\%$ are indicators of weak but statistically significant synergism/antagonism; (c) values between 50 and 100 $\mu M^2\%$ indicate a moderate synergism/antagonism; (d) values over 100 $\mu M^2\%$ are a sign of strong synergism/antagonism; (e) values over 1000 $\mu M^2\%$ are considered a result of experimental mistake or occasional phenomenon and are statistically insignificant.

Table 3. Effect of double combinations of EV antivirals on the replication of Coxsackievirus B1 (Connecticut strain)

Synergy plot (95%) *Bonferroni Adj.*	Synergy	Antagonism
Pleconaril + MDL-860	22.5 $\mu M^2\%$	-0.8 $\mu M^2\%$
Pleconaril + Guanidine.HCl	58.4 $\mu M^2\%$	-7.4 $\mu M^2\%$
Pleconaril + Oxoglaucine	27.0 $\mu M^2\%$	-8.9 $\mu M^2\%$
MDL-860 + Oxoglaucine	7.8 $\mu M^2\%$	-3.1 $\mu M^2\%$
MDL-860 + Guanidine.HCl	47.5 $\mu M^2\%$	-8.5 $\mu M^2\%$

Results

Before testing the antiviral activities of the combinations of the selected compounds against Coxsackie B virus strains, the individual effects of these compounds were determined, as shown in Table 2. Pleconaril was not tested against the Nancy strain of Coxsackievirus B3, because the inactivity of the substance against this strain had already been proven by Schmidtke *et al.* (2005).

Effect of the double combinations on replication of Coxsackievirus B1

The *in vitro* antiviral effect of pleconaril combined with MDL-860, oxoglaucine or guanidine.HCl against Coxsackievirus B1 is shown in Table 3 and Fig. 1a–c. The combination with MDL-860 is additive (Fig. 1a). The total volume of the observed synergism did not reach the sta-

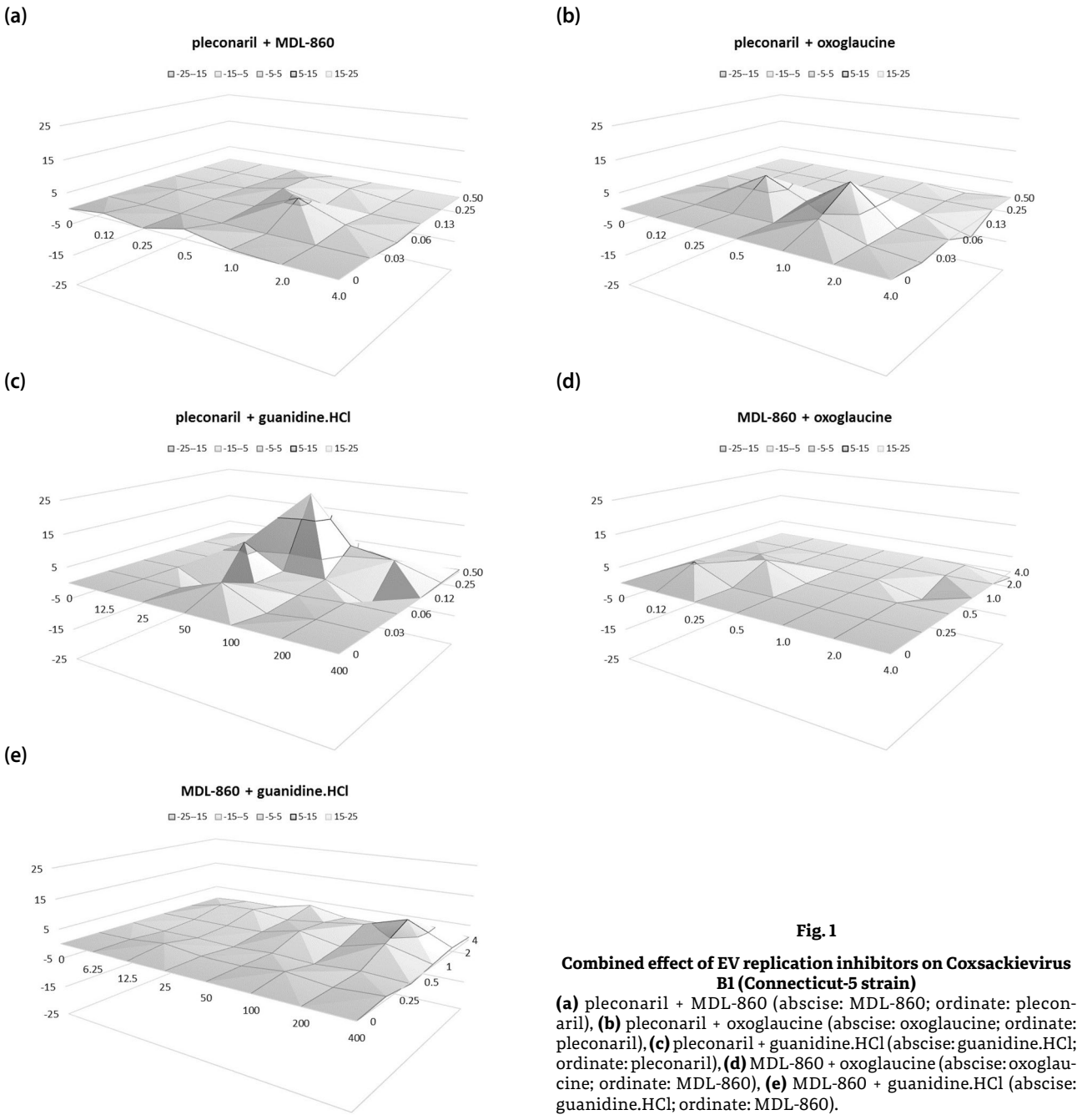

Fig. 1

Combined effect of EV replication inhibitors on Coxsackievirus B1 (Connecticut-5 strain)

(a) pleconaril + MDL-860 (abscise: MDL-860; ordinate: pleconaril), **(b)** pleconaril + oxoglaucine (abscise: oxoglaucine; ordinate: pleconaril), **(c)** pleconaril + guanidine.HCl (abscise: guanidine.HCl; ordinate: pleconaril), **(d)** MDL-860 + oxoglaucine (abscise: oxoglaucine; ordinate: MDL-860), **(e)** MDL-860 + guanidine.HCl (abscise: guanidine.HCl; ordinate: MDL-860).

tistically significant values for a synergistic effect, and the volume for antagonism is not significant. The combination effect with oxoglaucine could also be characterized as additive (Fig. 1b).

The combination with guanidine.HCl was evaluated as moderately synergistic (Fig. 1c). The dose-response surface was for the most part uniform, and it covered approximately all tested dose combinations; the synergism was most markedly expressed in the combination of 0.25 μM pleconaril + 50 μM guanidine.HCl.

The effects of combinations of MDL-860 with oxoglaucine and guanidine.HCl are presented in Fig. 1d,e. The combination of MDL-860 + oxoglaucine was additive (Fig. 1d), while the combination MDL-860 + guanidine. HCl had a moderately synergistic character (Fig. 1e), which was distributed uniformly on the dose-response surface.

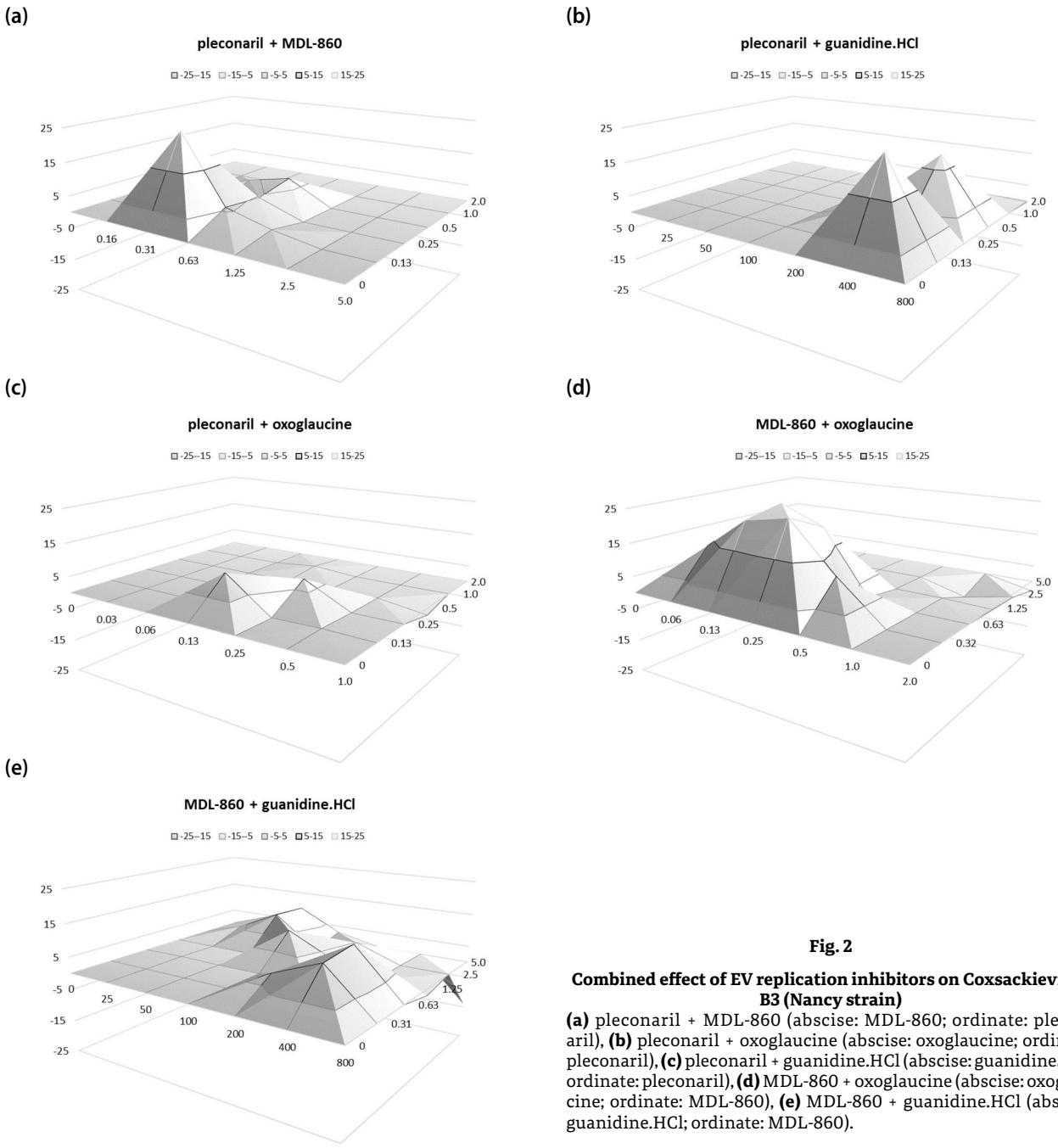

Fig. 2

Combined effect of EV replication inhibitors on Coxsackievirus B3 (Nancy strain)

(a) pleconaril + MDL-860 (abscise: MDL-860; ordinate: pleconaril), **(b)** pleconaril + oxoglaucine (abscise: oxoglaucine; ordinate: pleconaril), **(c)** pleconaril + guanidine.HCl (abscise: guanidine.HCl; ordinate: pleconaril), **(d)** MDL-860 + oxoglaucine (abscise: oxoglaucine; ordinate: MDL-860), **(e)** MDL-860 + guanidine.HCl (abscise: guanidine.HCl; ordinate: MDL-860).

Effect of the double combinations on replication of Coxsackievirus B3 Woodruff strain

Combinations of pleconaril with MDL-860 and guanidine.HCl demonstrated a synergistic character (Table 4, Fig. 2a,b), and that of pleconaril + oxoglaucine was additive (Table 4, Fig. 2c). The combined antiviral effect of MDL-860 + oxoglaucine and MDL-860 + guanidine.HCl against Coxsackievirus B3 Woodruff strain manifested a marked synergism (Table 4), which was distributed uniformly on most of the dose-response surface (Fig. 2d,e).

Effect of the double combinations on replication of Coxsackievirus B3 Nancy strain

A previous study on the Nancy strain established a pronounced decreased susceptibility to pleconaril (IC_{50}

Table 4. Effect of double combinations of EV antivirals on the replication of Coxsackievirus B3 (Woodruff strain)

Synergy plot (95%) ***Bonferroni Adj.***	**Synergy**	**Antagonism**
Pleconaril + MDL-860	61.1 μM²%	0.0 μM²%
Pleconaril + Guanidine.HCl	56.2 μM²%	-0.4 μM²%
Pleconaril + Oxoglaucine	18.6 μM²%	-2.1 μM²%
MDL-860 + Oxoglaucine	151.0 μM²%	-8.4 μM²%
MDL-860 + Guanidine.HCl	83.9 μM²%	-15.7 μM²%

Table 5. Effect of double combinations of EVantivirals on the replication of Coxsackievirus B3 (Nancy strain)

Synergy plot (95%) ***Bonferroni Adj.***	**Synergy**	**Antagonism**
MDL-860 + Guanidine.HCl	1.1 μM²%	-2.9 μM²%
MDL-860 + Oxoglaucine	220.6 μM²%	-1.6 μM²%

> 12.5 μM) (Pevear *et al*., 1999). Thus, combinations with pleconaril were not tested.

The combination MDL-860 + oxoglaucine manifested a strong synergism (Table 5 and Fig. 3a). In contrast to Coxsackievirus B3 Woodruff strain, the combined activity of MDL-860 + guanidine.HCl had an additive effect on the Nancy strain (Table 5 and Fig. 3b).

Determination of the combined cytotoxicity of double combinations in HEp-2 cells

A lack of increased cytotoxicity is an obligatory condition for a favorable combination. Figures 4a and 4b, which illustrate the combined cytotoxicity of double combinations including pleconaril in HEp-2 cells, demonstrate a complete lack of increased cytotoxicity. The flat character of the dose-response surfaces at point 0 of the ordinate proves this conclusion. The results obtained for combinations of MDL-860 with oxoglaucine and guanidine.HCl are similar – no increase in the cytotoxicity (Fig. 4c,d).

Discussion

The study of the combined effects of specific inhibitors of EV replication should be included as an important approach in the development of effective antivirals against EV infections occupying a significant spot in human infectious pathology. Prichard and Shipman's three-dimensional method (Prichard and Shipman, 1990; Prichard *et al*., 1993a,b) is the principal and highly precise tool for selecting synergistic double combinations and measuring their effects. Studies on such combination effects have contributed to clarifying the mechanisms of action of the substances included in the combinations. It is clear that the combination approach is usually based on choosing partner compounds that attack different targets in the viral replication cycle. In our study, however, we included two compounds, MDL-860 and oxoglaucine, that have the same target, the cellular protein PI4KIIIβ. This protein is an important component of the viral replication complex and thus it plays a significant role in the EV life cycle (Pincus *et al*., 1999; Cho and Ehrenfeld, 1991; Arita, 2014, 2016; Arita *et al*., 2008). Nevertheless, MDL-860 and oxoglaucine manifested a synergistic effect against Coxsackievirus B strains. Evidently, the partner compounds attack different points in the protein target. However, it is

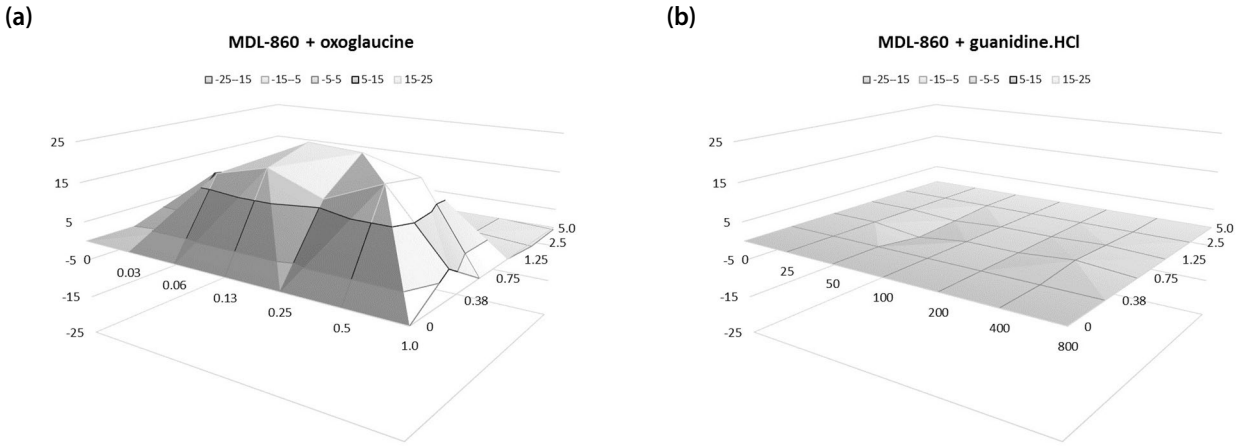

Fig. 3

Combined effect of EV replication inhibitors on Coxsackievirus B3 (Woodruff strain)
(a) MDL-860 + oxoglaucine (abscise: oxoglaucine; ordinate: MDL-860), **(b)** MDL-860 + guanidine.HCl (abscise: guanidine.HCl; ordinate: MDL-860).

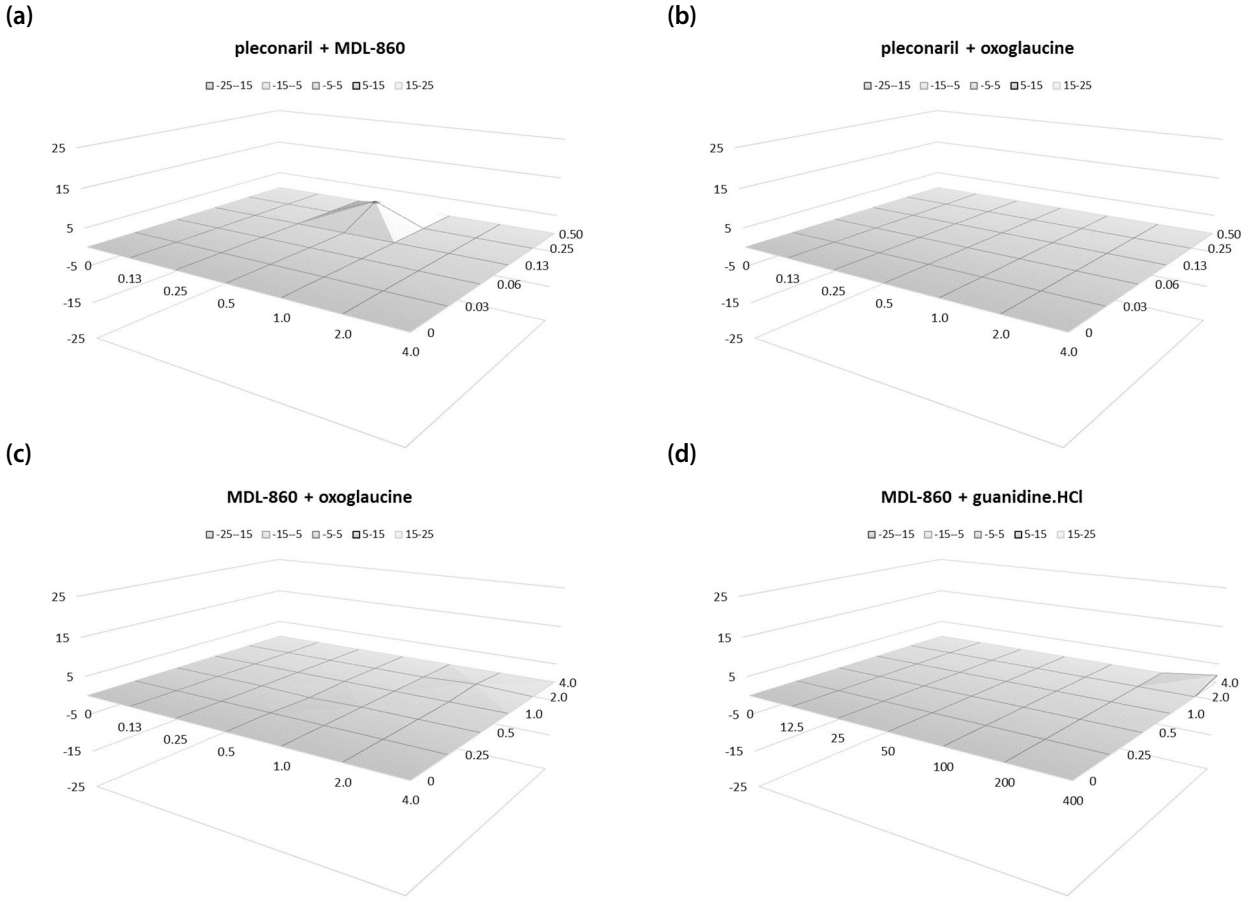

Fig. 4

Combined cytotoxicity of EV replication inhibitors on HEp-2 cells
(a) pleconaril + MDL-860 (abscise: MDL-860; ordinate: pleconaril), **(b)** pleconaril + oxoglaucine (abscise: oxoglaucine; ordinate: pleconaril), **(c)** MDL-860 + oxoglaucine (abscise: oxoglaucine; ordinate: MDL-860), **(d)** MDL-860 + guanidine.HCl (abscise: guanidine.HCl; ordinate: MDL-860).

necessary to keep in mind that with a combination of two EV replication inhibitors, resistance to the two partners in the combination can potentially develop.

Our study, carried out via cell culture experiments, revealed several synergistic double combinations: (a) guanidine.HCl + pleconaril or MDL-860 against Coxsackievirus B1 (Connecticut-5 strain); (b) the three combinations with MDL-860 (+ pleconaril, + guanidine.HCl, + oxoglaucine) and the combination of guanidine.HCl + pleconaril against the cardiotropic Woodruff strain of Coxsackievirus B3; and (c) MDL-860 + oxoglaucine against the neurotropic Nancy strain of Coxsackievirus B3.

The *in vitro* tests in our study could be considered an important guide when choosing EV replication inhibitors for in vivo tests involving experimental Coxsackie B virus infections in laboratory animals (mice). Our study thus contributes significantly to the development of chemotherapy for EV infections. This is especially important given that among the several hundred compounds registered as inhibitors in *in vitro* tests, only 20 have manifested individual activity in experimental EV infections in laboratory animals and no active antivirals have been confirmed in trials on humans (De Palma *et al.*, 2008; Fechner *et al.*, 2011; Galabov *et al.*, 2012; Tan *et al.*, 2014; Van der Linden *et al.*, 2015; Stoyanova, 2019).

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