Acta virol. **31** : 126—137, 1987

RAPID METHOD FOR THE DETECTION OF SYNERGISM IN COMBINATIONS OF ANTIVIRAL SUBSTANCES

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Received May 10, 1986

Summary. — The plaque inhibition method was modified in order to evaluate the effectiveness of various combinations of antiviral substances. One substance (A) diffuses from the centre of cell culture, the other (B) is incorporated into the agar overlay at subinhibitory concentration. The inhibitory effect of the combination (A + B) is demonstrated by the increase in size of the inhibitory zone in comparison with the control inhibitory zone produced by the substance A alone. The ratio of the diameter of the inhibitory zone with substance combination (A + B) to the diameter of single drug control zone (substance A) serves as index DI (degree of interaction). Quantitative evaluation of the degree of potentiation using isobolograms showed that DI > 1.5 indicate a synergistic effect of the respective combinations. This inexpensive method can serve for rapid selection of suitable combinations out of number of substances. Model experiments were performed with combinations of selected inhibitors of virus replication.

Key words: antiviral substances in combination; synergism of antiviral substances; method for detection of synergism

Introduction

If there are basic differences in the biochemical mechanism of action of two antiviral substances, combined therapy with these two active agents might be more effective than treatment with either drug alone. In antitumour chemotherapy drug combinations are widely used with good results. However, surprisingly low interest was devoted to this subject in virology. After the first report on the synergic action of isatin thiosemicarbazone and certain phenoxypyrimidines in vaccinia infected mice (Bauer, 1955) not many studies dealing with this subject were published. Most of them were carried out in cell cultures (Fiala *et al.*, 1974; Pancheva-Golowinska, 1975; Shannon *et al.*, 1977; Galabov, 1978; Wirjawan and Wigand, 1978; Ayisi *et al.*, 1980; Hayden *et al.*, 1980; Surjono and Wigand, 1981; Allen *et al.*, 1982; Janz and Wigand, 1982; Smith *et al.*, 1982; Ayisi *et al.*, 1985; Spector *et al.*, 1985), some were performed in mice (Bauer, 1955; Eggers, 1976;

Pushkarskaya *et al.*, 1977; Eggers, 1982; Herrmann *et al.*, 1982). This low interest in the study of antiviral substances in combination might be due to new methodological problems (Allen *et al.*, 1982) and to the amount of work involved. To evaluate the degree of potentiation by isobolograms is laborious; a demand for a rapid, inexpensive method of evaluating the activity of antiviral substance combinations seems desirable.

We have developed a new arrangement of the plaque inhibition method which permits quantitative evaluation of the potentiation of two inhibitory substances used in combination.

Materials and Methods

Viruses. Fowl plaque virus (FPV) strain Dobson was obtained from the collection of the Institute of Virology, Bratislava. Ten-day-old chick embryos were inoculated into the allantoic sacs. After 28 hr incubation, the allantoic fluid was harvested, divided into tubes and stored at −60 °C. Vaccinia virus strain WR was obtained from Dr. N. P. Salzman, National Institute of Allergy and Infectious Diseases, Bethesda, U.S.A. The stock prepared in HeLa cells was stored at −60 °C.

Agar-diffusion plaque-inhibition test. The test used was described (Rada and Závada, 1962). Chick embryo cell monolayers in 10 cm Petri dishes (in the case of several substance combinations 15 cm Petri dishes were used) were infected with a virus dose producing semiconfluent plaques. After adsorption of the virus inoculum (1 hr for FPV, 2 hr for vaccinia virus) the monolayers were overlaid with medium containing agar. When the agar overlay has solidified, glass cylinders were put into centre of agar overlay and filled with 50 µl of substance solutions.

Dose response curves. One-step growth experiments were performed in suspension cultures of chick embryo cells (CEC). Secondary CEC obtained by careful trypsinization of primary monolayer cultures grown in Roux bottles were used. Medium 199 with 10% calf serum was employed for both the growth of primary monolayers and suspension cultures. For infection, cells were concentrated to 1×10^7 cells per ml and FPV was added at a multiplicity of 5 plaque-forming units (PFU) per cell. After adsorption at 37 °C for 1 hr the cells were centrifuged, washed three times to remove unadsorbed virus, and resuspended at a density of 2×10^6 cells per ml. The respective substance was added and the culture was incubated for 24 hr at 37 °C. The cells in suspension cultures were subjected to three cycles of freezing and thawing prior to virus titration. Virus titres were determined by plaque assay on chick embryo cell monolayers.

Chemicals. 6-Azauridine, 5-iodo-2′-deoxyuridine, rifampicin and DL-p-fluorophenylalanine were obtained from Calbiochem, U.S.A.; adenine arabinoside was obtained from Serva, F.R.G.; cycloheximide was obtained from Sigma Chemicals, U.S.A.; 9-(2-hydroxyethoxymethyl)guanine (acyclovir, acycloguanosine) was kindly provided by Dr. G. B. Elion (Burroughs Wellcome Co., Research Triangle Park, N.C., U.S.A.); 9-(S)-(2, 3-dihydroxypropyl)adenine was kindly provided by Dr. A. Holý (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

Results and Discussion

Experimental arrangement

One substance (A) diffuses from the centre of the cell culture, the other (B) is incorporated into the agar overlay at subinhibitory concentration. The appropriate amount of the concentrated solution of substance B is added to double concentrated Eagle's medium before mixing with double concentrated agar solution. The respective monolayer cultures are overlaid with this medium containing agar supplemented with the studied substance

Table 1. Concentrations of combinations of rimantadine and cycloheximide causing 90 per cent inhibition of FPV replication

Rimantadine		Cycloheximide	
ng/ml	Fractional inhibitory concentration	ng/ml	Fractional inhibitory concentration
18.0	1	0	0
7.7	0.43	50	0.09
5.4	0.3	100	0.19
3.8	0.21	250	0.41
0	0	530	1

(substance B). After solidification of the agar overlay glass cylinders are mounted in the centre of dish cultures. Solution of substance A is given into the cylinders. After incubation of three days (in the case of FPV or vaccinia virus) the cultures are stained with neutral red.

The experimental arrangement is shown in Figs. 1—6. In Fig. 2 ribavirin diffuses from the centre and exerts an inhibitory zone of 66 mm in diameter. Fig. 3 shows another drug control: with rimantadine incorporated into the agar overlay. Fig. 4 shows the combination when ribavirin diffuses from the centre and rimantadine is incorporated into the agar overlay. Positive result was indicated by the increase in size of the inhibitory zone to 92 mm in diameter. Another example: cycloheximide gave an inhibitory zone of 48 mm in diameter (Fig. 5). The combination of cycloheximide plus rimantadine increased the inhibitory zone to 74 mm (Fig. 6).

Table 2. Antiviral activity and DI indexes of selected substances in combination against fowl plague virus

Substance diffusing from the centre of the dish culture	Single substance	In combination with rimantadine (1 μg/ml) incorporated in the agar overlay	
Cycloheximide (0.5 mg/ml)	48*	74*	1.5**
Ribavirin (100 mg/ml)	66	92	1.4
6-Azauridine (500 mg/ml)	31	54	1.7
(S)-9-(2,3-Dihydroxypropyl)-adenine(50 mg/ml)	0	0	—
9-β-D-Arabinofuranosyladenine (0.3 mg/ml)	0	0	—

* Diameter of the zone of inhibition in mm
** DI Index

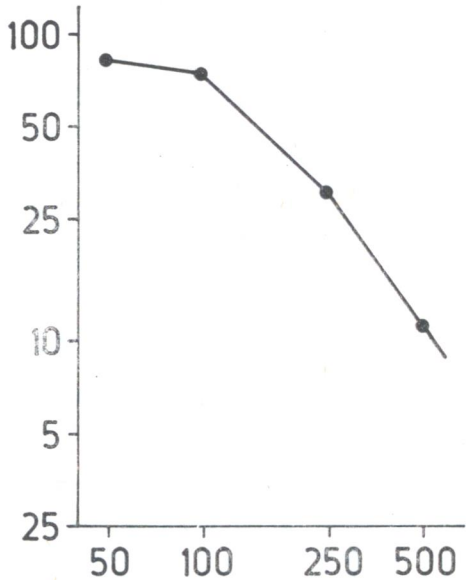

Fig. 7.
Inhibition of fowl plaque virus replication by cycloheximide Suspension of CEC was infected with FPV (multiplicity 5). After virus adsorption, cycloheximide was added. The virus yields were determined by plaque assay at 24 hr post-infection. Abscissa: cycloheximide (ng/ml); ordinate: virus yield (per cent of control)

Concentration of substances

The concentration of substance (A) diffusing from the centre was chosen neither to elicit a toxic zone nor to form a very large inhibition zone, thus leaving enough space for a potential increase of the inhibitory zone exerted by combination of both substances. In our experiments, inhibitory zones of substances A (in the absence of substance B) were about 50—70 mm or smaller with less effective substances (Table 2, Table 3). Usually this concentration (of the substance A) for diffusion was about two logs higher than the subinhibitory concentration when the same substance was present in the agar overlay. Cycloheximide requiring a 4 logs higher concentration for diffusion in agar overlay was an exception.

The substance (B) which was incorporated into the agar overlay was used at concentration causing reduction of the plaque size (subinhibitory concentration). This concentration was determined in preceding experiment; with different viruses it was different. There was no reason to use higher concentration of substance B, since if the plaques disappeared or if their number was highly reduced, the estimation of the inhibitory zone was impossible. If a lower concentration was used, the desired positive effect of drug combination could disappeare.

To estimate the positive effect of the combination it is always necessary to include control cultures in which substance A was diffusing in a normal agar overlay not containing the substance B (Figs. 2, 5).

Table 3. Antiviral activity of selected substances in combination against vaccinia virus

Substance (A) diffusing from the centre of the dish culture	Single substance*	In combination with the substance (B) incorporated in the agar overlay* IdUrd (0.1 μg)**	DHPA (100 μg)	RFP (50 μg)	CYH (0.05 μg)	araA (1.5 μg)	FPA (100 μg)	ACV (30 μg)
6-Azauridine (500 mg)**	69	73	140	77	96	98	93	95
5-Iodo-2′-deoxyuridine (0.5 mg)	67	—	140	64	65	71	60	76
(S)-9-(2,3-Dihydroxy-propyl)adenine (50 mg)	64		—	71	80	68	54	81
Rifampicin (3 mg)	17			—	21	22	26	15
Cycloheximide (0.5 mg)	65				—	82	72	71
9-β-D-Arabinofuranosyl-adenine (0.3 mg)	55					—	64	57
p-Fluorophenylalanine (5 mg)	14						—	14

*: Diameter of zone of inhibition in mm
** Concentration of the substance per ml
ACV = acycloguanosine

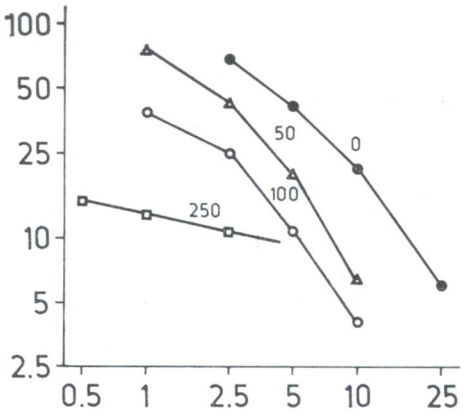

Fig. 8.
Inhibiton of fowl plague virus replication by rimantadine and by the combination of rimantadine plus cycloheximide
Concentrations of cycloheximide: (●——●) — none; (△——△) — 50 ng/ml; (○——○) — 100 ng/ml; (□——□) — — 250 ng/ml.
Abscissa: rimantadine (ng/ml); ordinate: virus yield (per cent of control)

Quantitative evaluation of the antiviral effect of substance combinations

The question arises how to distinguish synergism from a simple additive effect in this arrangement. For this purpose we performed a quantitative evaluation of the antiviral effect of combinations of cycloheximide plus rimantadine and ribavirin plus rimantadine. We used one-step growth experiments — FPV replicating in CEC suspension cultures. Dose response curves were performed with either of these substances alone. Fig. 7 shows the dose response curve for cycloheximide. The dose response curve for rimantadine alone and the dose response curves for the combination of both substances are shown in Fig. 8. A concentration of 90 % inhibition of virus replication was chosen as comparative value. These concentrations are given in Table 1, which also shows the "fractional inhibitory concentration" of

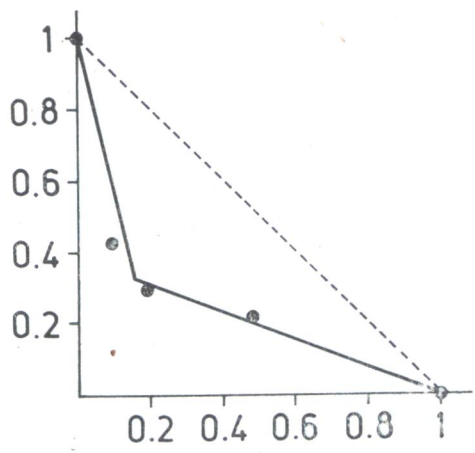

Fig. 9.
Effects of combinations of cycloheximide and rimantadine on the replication of fowl plague virus in terms of fractional inhibitory concentrations
Abscissa: cycloheximide; ordinate: rimantadine

each substance. This fractional inhibitory concentration was calculated by dividing the concentration of the substance present in combination by concentration of the substance which would be required to give the same degree of inhibition by itself.

The fractional inhibitory concentrations of each pair of inhibitors were then plotted as shown in Fig. 9. This curve — termed isobologram — was introduced for the study of combinations of substances (originally in bacteria) by Elion *et al.*, (1954). The isobologram indicates the mode of interaction: additive effect, synergism or antagonism. When the effect of two substances is additive, the points fall on a straight line, connecting unity of the ordinate with unity on the abscissa. Deviations to the right of this theoretical line represent interference or antagonism between the substances, deviations to the left indicate synergism. The higher the degree of synergism, the nearer the curve is to the axes. Thus, the curve in Fig. 9 indicates a synergism of a medium degree.

Index DI — numerical expression of the interaction

Cycloheximide exerts an inhibitory zone of 48 mm in diameter, the combination of cycloheximide and rimantadine exerts an increase in size of the inhibitory zone to 74 mm (Figs. 5, 6, Table 2).

We introduce a simple index permitting numerical expression of this interaction. This index termed degree of interaction (DI) is the ratio of the diameter of the inhibitory zone with the combination of substances (A + B) to the diameter of the zone of the substance A alone in control culture.

$$DI = \frac{\text{Diameter of the inhibitory zone exerted by the combination (A + B)}}{\text{Diameter of the inhibitory zone exerted by the control substance (A) alone}}$$

For combination of cycloheximide plus rimantadine:

$$DI = \frac{\text{Diameter of the inhibitory zone of cycloheximide plus rimantadine} = 74 \text{ mm}}{\text{Diameter of the inhibitory zone of cycloheximide} = 48 \text{ mm}} = 1.5$$

Quantitative evaluation of the degree of potentiation using the presented isobologram (Fig. 9) shows that DI = 1.5 indicates synergism of a medium degree.

When combinations of ribavirin and rimantadine were tested, the potentiation was found to be similar (Fig. 10). With combination of ribavirin and rimantadine the index DI = 1.4 was found (Table 2). Previously, Pushkarskaya *et al.* (1977) reported an increased antiviral effect with rimantadine and ribavirin in in vitro experiments with FPV virus and in mouse protection experiments with influenza A2/Frunze virus. The enhanced antiviral effect of ribavirin and rimantadine combination was confirmed with several

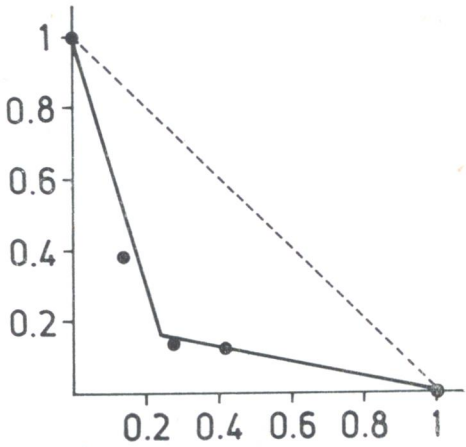

Fig. 10.
Effects of combinations of ribavirin and rimantadine on the replication of fowl plague virus in terms of fractional inhibitory concentrations
Abscissa: ribavirin; ordinate: rimantadine.

influenza virus strains. An assay of Madin-Darby canine kidney cell proliferation in the presence of drugs showed that the enhanced inhibitory effect of drug combinations was not due to their increased cytotoxicity (Hayden *et al.*, 1980).

We have noticed a difference in sensitivity of FPV against rimantadine when studied by two different tests: in single cycle experiment (dose response curve) and in the partial inhibition of plaque formation when rimantadine was present in the agar overly. A similar difference — high sensitivity of FPV in single cycle experiment and resistance in plaque reduction and inhibition test against amantadine — was observed previously by Scholtissek and Faulkner (1979).

Model experiments with vaccinia virus

For experiments with vaccinia virus there is a greater choice of inhibitors. We have chosen eight inhibitors with distinct sites of action and studied the effect of their mutual combinations, altogether 28 combinations (Table 3). Several of these substances are inhibitors of DNA synthesis (5-iododeoxyuridine, adenine arabinoside, acycloguanosine); 6-azauridine and (S)-dihydroxypropyladenine inhibit RNA synthesis; p-fluorophenylalanine and cycloheximide are inhibitors of protein synthesis. Rifampicin acts on several sites.

Table 3 shows diameters of the inhibitory zones of the studied combinations. Several positive results could be observed. The DI indexes showed (Table 4) that combination of rifampicin and p-fluorophenylalanine resulted in medium synergism; the potentiation of 6-azauridine with either cycloheximide, adenine arabinoside or acycloguanosine was close to this degree of synergism. Strong synergism was observed with combination of (S)-dihydroxypropyladenine and either 6-azauridine or 5-iododexyuridine.

Table 4. DI indexes of selected substances in combination against vaccinia virus

Substance (A) diffusing from the centre of the dish culture	In combination with the substance (B) incorporated in the agar overlay						
	IdUrd (0.1 μg)	DHPA (100 μg)	RFP (50 μg)	CYH (0.05 μg)	araA (1.5 μg)	FPA (100 μg)	ACV (35 μg)
6-Azauridine (500 mg)	1.1	2.0	1.1	1.4	1.4	1.3	1.4
5-Iodo-2′-deoxyuridine (0.5 mg)	—	2.1	1.0	1.0	1.1	0.9	1.1
(S)-9-(2,3-Dihydroxypropyl)adenine (50 mg)		—	1.1	1.3	1.1	0.8	1.3
Rifampicin (3 mg)			—	1.2	1.3	1.5	0.9
Cycloheximide (0.5 mg)				—	1.3	1.1	1.1
9-β-D-Arabinofuranosyladenine (0.3 mg)					—	1.2	1.1
p-Fluorophenylalanine (5 mg)						—	1.0

In parentheses concentration of the substance per ml

The purpose of presented method is to serve in the search of combinations of antivirals resulting in synergism. It is clear that not each combination results in synergism. In efficient drug combinations (Table 4) such as 5-iododeoxyuridine plus (S)-dihydroxypropyladenine or 6-azauridine plus (S)-dihydroxypropyladenine — the three substances differ in their mode of action.

The mode of action of 5-iododeoxyuridine was studied in details by Prusoff and Goz (1973; 1975). Thymidine kinase, thymidylate kinase and DNA polymerase are competitively inhibited by 5-iododeoxyuridine or one of its phosphorylated derivatives. However, these effects do not account for the antiviral activity of 5-iododeoxyuridine. Recently, a direct parallelism between the incorporation of 5-iododeoxyuridine into viral DNA and the inhibition of herpes simplex virus replication was demonstrated (Fischer *et al.*, 1980).

6-Azauridine in vivo is converted by uridine kinase to 6-azauridine-5'--phosphate which is an inhibitor of orotidylic acid decarboxylase (Pasternak and Handschumacher, 1959). In contrast to 5-halogeno substituted deoxyuridines, 6-azauridine is not incorporated into nucleic acids. In the replication cycle of vaccinia virus this analogue affects both the early (uncoating) as well as late events (synthesis of late m-RNA) in the latent period (Rada and Doskočil, 1984).

(S)-Dihydroxypropyladenine inhibits the hydrolysis of S-adenosyl-L-homocysteine (SAH) catalyzed by S-adenosyl-L-homocysteine hydrolase. Accumulation of SAH in vivo by this inhibition could be one of the causes of the antiviral effect of (S)-dihydroxypropyladenine (Votruba and Holý, 1980). Inhibition of RNA methylation seems to the site of action, since (S)-dihydroxypropyladenine exerts an effect upon both virus-infected or proliferating cells. Unlike most nucleoside analogues (S)-dihydroxypropyladenine was not found to be metabolized during the latent period of vaccinia virus replication cycle or in uninfected cell cultures (Rada *et al.*, 1980).

The present modest positive results do not permit any conclusion about the respective mode of action of substances to be combined for potentiation. For the combination of 6-azauridine plus (S)-dihydroxypropyladenine it would seem that their distinct loci of action are arranged along the same biochemical pathway, i.e. the biosynthesis of RNA, but for combination of 5-iododeoxyuridine and (S)-dihydroxypropyladenine this is not the case. Why these combinations and not others do result in synergism. This question opens a new interesting field of antiviral research — selection of combinations of antiviral substances giving synergism. This search will aim at understanding which different modes of action of substances are suitable for use in combination.

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Explanation to Figures (Plates XXIX–XXX):

Fig. 1. Control culture. Chick embryo cells infected with 5×10^3 PFU of fowl plaque virus incubated for three days at 37 °C. All cultures (Figs. 2–6) were infected with the same virus inoculum.

Fig. 2. Inhibition of FPV plaque formation by ribavirin. In the cylinder 50 µl of ribavirin solution, 100 mg/ml.

Fig. 3. Plaque-reduction by rimantadine. Monolayers of chick embryo cells infected with FPV covered with overlay containing rimantadine 1 µg/ml.

Fig. 4. Inhibitory effect of the combination ribavirin plus rimantadine. Ribavirin (100 mg/ml) diffuses from the centre, rimantadine is incorporated into the overlay.

Fig. 5. Inhibitory effect of cycloheximide. Cylinder contains cycloheximide solution, 500 µg/ml.

Fig. 6. Inhibitory effect of the combination cycloheximide plus rimantadine. Cycloheximide (500 µg/ml) diffuses from the centre, rimantadine (1 µg/ml) is incorporated into the overlay.