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Broad-Spectrum Synergistic Antiviral Activity of Selenazofurin and Ribavirin

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The antiviral effects of selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide, selenazole), ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), and 3-deazaguanosine (6-amino-1-β-D-ribofuranosylimidazo-[4.5-C]pyridin-4(5H)-one) were investigated separately and in various combinations in an in vitro study. The combination interactions were evaluated at seven drug concentrations, graphically (isobolograms) or by using fractional inhibitory concentration indices against mumps, measles, parainfluenza virus type 3, vaccinia and herpes simplex virus type 2 viruses in Vero and HeLa cells. Selenazofurin in combination with ribavirin produced the greatest synergistic antiviral activity. However, the degree of synergy depended on the virus and cell line used. In contrast, selenazofurin combined with 3-deazaguanosine consistently yielded an indifferent or an antagonistic response, or both, whereas the ribavirin–3-deazaguanosine interaction was additive against the same viruses. Single-drug cytotoxicity was minimal for the cytostatic agents selenazofurin and ribavirin but was markedly higher for cytocidal 3-deazaguanosine, as determined by relative plating efficiency after drug exposure. The drug combinations did not significantly increase cytotoxicity (they were only additive) when used on uninfected cells. Therefore, the enhanced antiviral activities of the drug combinations (shown to be synergistic) were due to specific effects against viral replication. These results indicated that in Vero and HeLa cells (i) the combination of selenazofurin and ribavirin produced an enhanced antiviral effect, thus requiring smaller amounts of drug to cause the same antiviral effect relative to a single compound; (ii) selenazofurin when compared with ribavirin and 3-deazaguanosine appeared to have a somewhat different mode of antiviral action; (iii) 3-deazaguanosine combined with selenazofurin was an unsuitable antiviral combination; and (iv) the antiviral activity of 3-deazaguanosine appeared to be due largely to its general overall cytotoxic effect.

A new broad-spectrum antiviral agent, selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide, selenazole) (Fig. 1B), has recently been synthesized in our laboratory (48). Selenazofurin exhibited significant antiviral activity against numerous viruses and was significantly more potent in these cell culture experiments than was ribavirin (Fig. 1A) (27). Even though selenazofurin and ribavirin are structurally similar azole carboxamide nucleosides, their antiviral spectrum and cytotoxicity were somewhat different.

Other investigators have recently shown that the activity of ribavirin against herpes simplex virus type 1 (HSV-1), and HSV-2 (2), and various strains of influenza A (7, 8, 17, 20) and B (21, 54) viruses is enhanced when used in combination with arabinofuranosylhypoxanthine, amantadine (rimantadine), or interferon.

Combination studies seemed warranted to determine whether the antiviral potency of selenazofurin or ribavirin or both could be significantly enhanced, while their cytotoxicity would be simultaneously minimized. It was also hoped that through studies on such combinations insight might be gained as to the possible biochemical mode of action of selenazofurin; if the mechanisms of action of ribavirin and selenazofurin are found to be the same, then their use in combination should result in an additive response.

3-Deazaguanosine (Fig. 1C) was included in this study because reports have indicated that it also possesses a biochemical mode of action (11, 40, 49) and a breadth of antiviral spectrum against DNA and RNA viruses similar to those of ribavirin (1, 10).

In this report, we present further in vitro comparative studies of the antiviral action of selenazofurin in combination with ribavirin and 3-deazaguanosine and present evidence which indicates that (i) combinations of selenazofurin with ribavirin are more effective than either compound alone; (ii) selenazofurin appears to have a different mode of action than do ribavirin and 3-deazaguanosine; (iii) selenazofurin and 3-deazaguanosine are an ineffective combination; and (iv) in contrast to the activities of selenazofurin and ribavirin, the activity of 3-deazaguanosine can be explained by its general cytotoxic effect.

MATERIALS AND METHODS

Cells. Two types of continuous cell lines were used in this study: African green monkey kidney cells (Vero; ATCC CCL 1; American Type Culture Collection Cell Repository, Rockville, Md.) and human epithelioid cervical carcinoma cells (HeLa; Flow Laboratories, Inglewood, Calif.). Cells were grown in antibiotic-free Eagle minimum essential medium (EMEM) with Earle salts supplemented with 10% newborn bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Cells were passaged in 75-cm^3 plastic culture flasks (Corning Glass Works, Corning, N.Y.) at 37°C under humidified 5% CO_2–95% air. Experiments reported in this paper were conducted with cells passaged 5 to 30 times in our laboratory. All cultures were periodically tested and found to be mycoplasma free by direct culturing on agar plates (Flow Laboratories) under anaerobic conditions and

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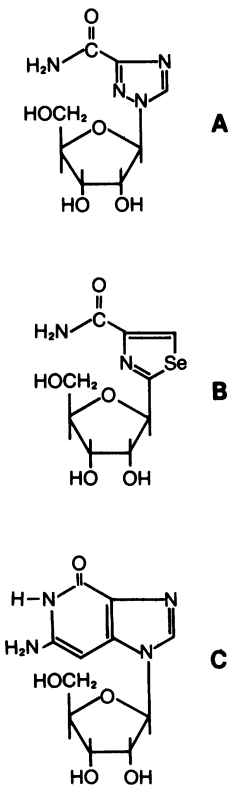

FIG. 1. Chemical structures of ribavirin (A), selenazofurin (selenazole) (B), and 3-deazaguanosine (C), the three related nucleosides used in this study.

by the absence of extranuclear fluorescence when stained with Hoechst stain 33258 (9).

Viruses. Two DNA and three RNA viruses known to be sensitive to ribavirin and selenazofurin (27) were used in this study. They included uncloned pools of HSV-2 strain 333, donated by F. B. Johnson, Brigham Young University, Provo, Utah, vaccinia virus (VV) strain Elstree, mumps virus strain Enders, measles virus strain Edmonston obtained from the American Type Culture Collection, and parainfluenza virus type 3 (Para-3) strain C243, donated by R. W. Sidwell, Utah State University, Logan.

Virus pools were prepared by infecting confluent monolayers of Vero or HeLa cells. Infected monolayers were incubated for 2 to 6 days at 37°C in 5% CO_2 and harvested when cultures exhibited 90% cytopathic effect (CPE). Viruses were passaged at least twice in each cell line before the virus pool was used. The cell line used in any given experiment was infected with virus produced only in that same cell line. Infected cells and fluids were stored at −70°C. Intracellular virus was released by disrupting infected cells by freeze-thawing and vigorous trituration against the flask wall. Viral stock cultures were divided into 0.5-ml samples, frozen, and stored at −70°C until used. Samples of each virus were assayed for infectious virus by plaque formation assay (PFU per milliliter) and by determination of the 50% tissue culture infective dose by the method of Reed and Muench (37).

Antiviral compounds. Ribavirin, selenazofurin, and 3-deazaguanosine (Fig. 1) were synthesized and prepared in our laboratory as previously described (11, 48, 55). A 10^{-2} M stock solution was prepared in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.35). The stock solution was sterilized by passage through a 0.22-μm membrane filter (Gelman Sciences, Inc., Ann Arbor, Mich.).

Drug cytotoxicity evaluations. Ribavirin, selenazofurin, and 3-deazaguanosine were examined singly and in combination for their cytotoxic effect on Vero and HeLa cells. The cell monolayers and drug exposure times used in cytotoxicity determinations were identical to those used for the antiviral experiments.

The cells were grown overnight in 96-well plates (Corning) until near confluency. The growth medium was then replaced with fresh growth medium containing the test compounds at 1, 10, 100 and 1,000 μg/ml. Quadruplicate wells were used for each drug concentration, along with an equal number of control (nondrug) wells. Cells were exposed to the drug for 72 h, after which cells were harvested by trypsinization, triturated, and serially diluted, and the 1/10, 1/100, 1/1,000 dilutions were plated onto 12-well tissue culture plates (Costar, Data Packaging, Cambridge, Mass.). After 5 days of incubation in drug-free growth medium, the cells were washed with phosphate-buffered saline, fixed with 10% Formalin, and stained with 1% crystal violet. Cell colonies, as representatives of single, viable, original cells, were counted under a dissecting microscope. Relative plating efficiency, as percent survival, was calculated as the average number of colonies grown from drug-treated cells divided by the average number of colonies grown from control (nondrug) wells. In this study, the concentration of test compound that resulted in a relative plating efficiency of 50% was considered to be the maximum tolerated concentration (MTC) of the compound.

Evaluations of the cytotoxicity of ribavirin and selenazofurin when used in combination were performed by testing eight concentrations of each test compound close to the MTC of that compound. These concentrations, expressed as a percentage of the MTC, were 0, 10, 25, 50, 75, 100, 250, and 1,000%. Each concentration of ribavirin was tested in combination with each concentration of selenazofurin. In the combination experiments, the cell monolayer conditions, drug exposure times, and determinations of plating efficiencies were identical to those described for single compounds.

The concentrations of ribavirin and selenazofurin in combination that resulted in a relative plating efficiency of 50% were determined from computer-generated isobolograms as

TABLE 1. MTCs of ribavirin, selenzofurin, and 3-deazaguanosine used individually and in combination in uninfected cells

Treatment	MTC[a] (μg/ml) in:	
	Vero cells	HeLa cells
Single drug		
Ribavirin	1,000	1,000
Selenazofurin	1,000	500
3-Deazaguanosine	3.3	6.5
Combination[b]		
Ribavirin + selenazofurin	500 + 500	470 + 165
Ribavirin + 3-deazaguanosine	280 + 1.5	
Selenazofurin + 3-deazaguanosine	600 + 2.0	

[a] MTC is the concentration of compound that produces 50% inhibition of plating efficiency of cells after 72 h of exposure to the compound.

[b] The combination MTCs were obtained from computer-generated cytotoxicity isobolograms as described in the text.

described below. These concentrations were considered to be the MTC of ribavirin and selenazofurin in combination. The same procedure was followed for the evaluation of the cytotoxicity of the 3-deazaguanosine-ribavirin and the 3-deazaguanosine-selenazofurin combinations.

Determination of antiviral ED_{50} for single compounds. The antiviral activity of each individual drug was determined in each cell-virus system before its use in combinations. Vero or HeLa cells were inoculated into 96-well tissue culture plates (Corning) at a concentration of 4×10^4 cells per 0.2 ml per well and cultured to confluency for 24 h at 37°C under 5% CO_2. These monolayers were infected with a predetermined number of 50% tissue culture infective dose units of virus (0.1 ml per well) that resulted in the complete destruction of the monolayer within 72 h.

After 30 min of adsorption at 37°C and 5% CO_2, test compounds were added (0.1 ml per well) in seven 0.5 $\log_{10}$ dilutions ranging from 5×10^{-4} M (~100 μg/ml) to 5×10^{-7} M (~0.1 μg/ml). At each dilution level, duplicate wells were used for the evaluation of antiviral activity. Experimental controls included uninfected cells and virus-infected non-drug-treated cells.

The degree of inhibition of viral-induced CPE and drug cytotoxicity was observed microscopically after 72 h of incubation as described previously (27, 46). Briefly, CPE was scored numerically from 0 (normal control cells) to 4 (100% cell destruction as in virus controls). Scores for CPE were verified after the plates were fixed in 10% Formalin and stained with 1% crystal violet.

At each drug concentration, CPE scores were averaged and expressed as a percentage of the virus control scores. Least-squares fit dose-response curves were generated by plotting percent virus control values versus drug concentrations (47). The concentration of drug resulting in 50% inhibition of viral CPE was determined, and this concentration was considered to be the 50% effective dose (ED_{50}). The ED_{50} values reported are averages from at least two separate determinations.

Determination of combination antiviral ED_{50}. Eight concentrations of each drug were used in the antiviral drug combination experiments. These concentrations, expressed as a percentage of the single-drug antiviral ED_{50}, were 0, 10, 25, 50, 75, 100, 250, and 1,000%. Duplicate wells were used to test ribavirin in combination with each concentration of selenazofurin. Virus adsorption and incubation times as well as scoring for viral CPE were performed as described for individual drug ED_{50} determinations. Least-squares fit dose-response curves for ribavirin, used individually and in the presence of each concentration of selenazofurin, were generated (47). At each concentration of selenazofurin, the concentration of ribavirin needed to produce 50% inhibition of viral CPE (ribavirin ED_{50} in combination) was determined. The process was repeated for selenazofurin at each concentration of ribavirin (selenazofurin ED_{50} in combination). The same procedure was followed for the 3-deazaguanosine–ribavirin and 3-deazaguanosine–selenazofurin combinations.

Determination of FIC. Each ED_{50} concentration of ribavirin in combination was reexpressed as a fractional inhibitory concentration (FIC), that is, as a fraction of the ED_{50} of ribavirin used alone (ribavirin FIC = ribavirin ED_{50} in combination/ribavirin ED_{50} alone). The resulting ribavirin FIC was paired with the FIC of selenazofurin that was present in that combination (selenazofurin FIC = concentration of selenazofurin in combination/selenazofurin ED_{50} alone). The FIC values for selenazofurin, in the presence of each concentration of ribavirin, were determined (selenazofurin FIC = selenazofurin ED_{50} in combination/selenazofurin ED_{50} alone) and paired with the FIC values of ribavirin that were present in each combination (ribavirin FIC = concentration of ribavirin in combination/ribavirin ED_{50} alone).

The FIC values for the ribavirin–3-deazaguanosine combination and the selenazofurin–3-deazaguanosine combination were calculated in the same manner.

Interpretation of drug interactions in combinations. The evaluation of drug combination interactions and FIC index calculations were performed as previously described (2, 15, 22, 38, 53) from the graphic interpretation of isobolograms. Curves were generated by a least-squares fit of the data points to a polynomial equation with an Apple IIe polyfit program (47).

Calculation of AI. Antiviral indices (AIs) were used to evaluate the specific antiviral activity of individual compounds or their combinations, taking into account the cytotoxicity of the compound or combination. The AI of an individual compound was defined as the MTC of the compound divided by the antiviral ED_{50}. The AI of the ribavirin-selenazofurin combination, at the point at which the FIC index is a minimum on the computer-fitted isobologram, was calculated as follows: combination AI = ribavirin MTC in combination/ribavirin ED_{50} in combination + selenazofurin MTC in combination/selenazofurin ED_{50} in combination.

RESULTS

Cytotoxicity evaluations of individual compounds and their combinations. The MTCs of ribavirin, selenazofurin, and 3-deazaguanosine, used alone and in combination, in Vero and HeLa cells are summarized in Table 1. The MTC of ribavirin, as determined by relative plating efficiency of cells after 72 h of exposure to the drug, was 1,000 μg/ml in both Vero and HeLa cells. The MTC of selenazofurin was 1,000 μg/ml in Vero cells and 500 μg/ml in HeLa cells. 3-Deazaguanosine markedly reduced the plating efficiency of both HeLa and Vero cells after the 72-h exposure. The MTC of 3-deazaguanosine was 3.3 μg/ml in Vero cells and 6.5 μg/ml in HeLa cells. These data strongly suggest that 3-deazaguanosine acts in a cytocidal manner, whereas ribavirin and selenazofurin are cytostatic for these cells.

Computer-generated isobolograms and FIC indices showing the cytotoxicity of the ribavirin-selenazofurin combination on uninfected Vero and HeLa cells are shown in Fig. 2. Use of this drug combination in Vero cells resulted in an additive interaction, as indicated by the isobologram and by FIC indices of ca. 1.0 (Fig. 2A). The MTCs of the compounds when used in combination in Vero cells were 500 μg of ribavirin per ml plus 500 μg of selenazofurin per ml (Table 1).

The ribavirin-selenazofurin combination in HeLa cells exhibited a slightly synergistic interaction, as indicated by the isobologram and by the FIC indices of slightly less than 1.0 (Fig. 2B). The MTCs of ribavirin and selenazofurin used in combination in HeLa cells were those concentrations present in the combination at the point at which the FIC index reached a minimum value on statistically fitted isobolograms. This minimum FIC index for the ribavirin-selenazofurin combination in HeLa cells was 0.81. This value represents 470 μg of ribavirin per ml (FIC, 0.47) and 165 μg/ml of selenazofurin (FIC, 0.34). Isobolograms and FIC indices representing the cytotoxicity of 3-deazaguanosine in combi-

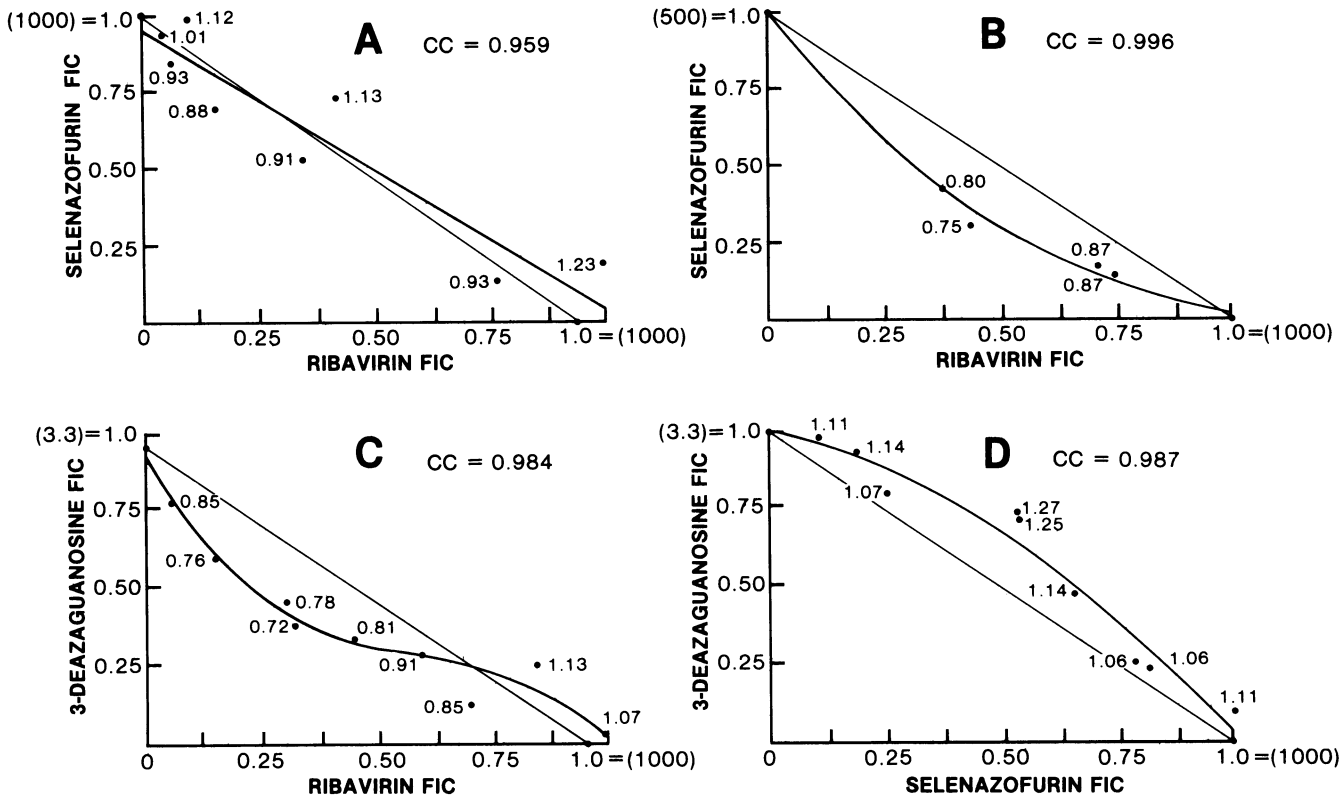

FIG. 2. Isobolograms of uninfected cells demonstrating the cytotoxic effects of the selenazofurin and ribavirin combinations in Vero (A) and HeLa cells (B), the ribavirin and 3-deazaguanosine combination in Vero cells (C), and the selenazofurin and 3-deazaguanosine combination in Vero cells (D). The number next to each data point is the FIC index for that point (see the text). The number within parentheses, next to the FIC of 1.0, is the MTC (micrograms per milliliter) of the single compound. CC, Correlation coefficient for the curve.

nation with ribavirin and with selenazofurin in Vero cells are shown in Fig. 2C and 2D. The ribavirin–3-deazaguanosine combination exhibited a slightly synergistic interaction, whereas the selenazofurin–3-deazaguanosine combination revealed an additive, if not slightly indifferent, interaction. The minimum FIC index for ribavirin–3-deazaguanosine was 0.74. This value represents 280 μg of ribavirin per ml (FIC, 0.28) and 1.5 μg of 3-deazaguanosine per ml (FIC, 0.46). The maximum FIC index for the selenazofurin–3-deazaguanosine combination was 1.20. This value represents 600 μg of selenazofurin per ml (FIC, 0.60) and 2.0 μg of 3-deazaguanosine per ml (FIC, 0.60).

Antiviral ED_{50} and AI of single compounds. The ED_{50} and AI values of single compounds for selected viruses replicating in Vero and HeLa cells are summarized in Table 2. Antiviral ED_{50} values for selenazofurin were ca. 10-fold lower than those for ribavirin in both cell lines. 3-Deazaguanosine was effective at concentrations ca. 10-fold lower than those values found for selenazofurin in Vero cells and was as active at approximately the same concentrations as those found for selenazofurin in HeLa cells.

When the cytotoxicity of the compounds was taken into account in the determination of the AI, selenazofurin was ca. 10 times more active than ribavirin in Vero cells. In HeLa cells, where selenazofurin was more toxic than ribavirin, the specific antiviral activity of selenazofurin was somewhat reduced but was still greater than the activity of ribavirin.

3-Deazaguanosine had a low specific antiviral activity as a result of its cytotoxicity for both HeLa and Vero cells, which was indicated by the low AI values (Table 2). Therefore, the

TABLE 2. Antiviral ED_{50} and AI for single compounds[a]

Virus	Vero cells						HeLa cells					
	Ribavirin		Selenazofurin		3-Deazaguanosine		Ribavirin		Selenazofurin		3-Deazaguanosine	
	ED_{50}	AI	ED_{50}	AI	ED_{50}	AI	ED_{50}	AI	ED_{50}	AI	ED_{50}	AI
Measles	21.0	47.6	3.7	270.3	1.60	2.1	7.0	142.8	1.5	333.3	1.3	5.0
Para-3	34.0	29.4	1.3	769.2	0.28	11.8	23.0	43.5	3.7	135.1	6.9	0.9
Mumps	32.0	31.2	8.0	125.0	ND	ND	23.0	43.5	7.5	66.7	ND	ND
VV	19.0	52.6	3.4	294.1	0.24	13.8	36.0	27.8	2.7	185.2	1.9	3.4
HSV-2	40.0	25.0	4.3	232.6	0.43	7.7	NA	NA	NA	NA	NA	NA

[a] ED_{50} values (micrograms per milliliter) were determined as described in the text. Values shown are averages of two to four separate determinations. AI = MTC/ED_{50}. NA, Not applicable; ND, not determined.

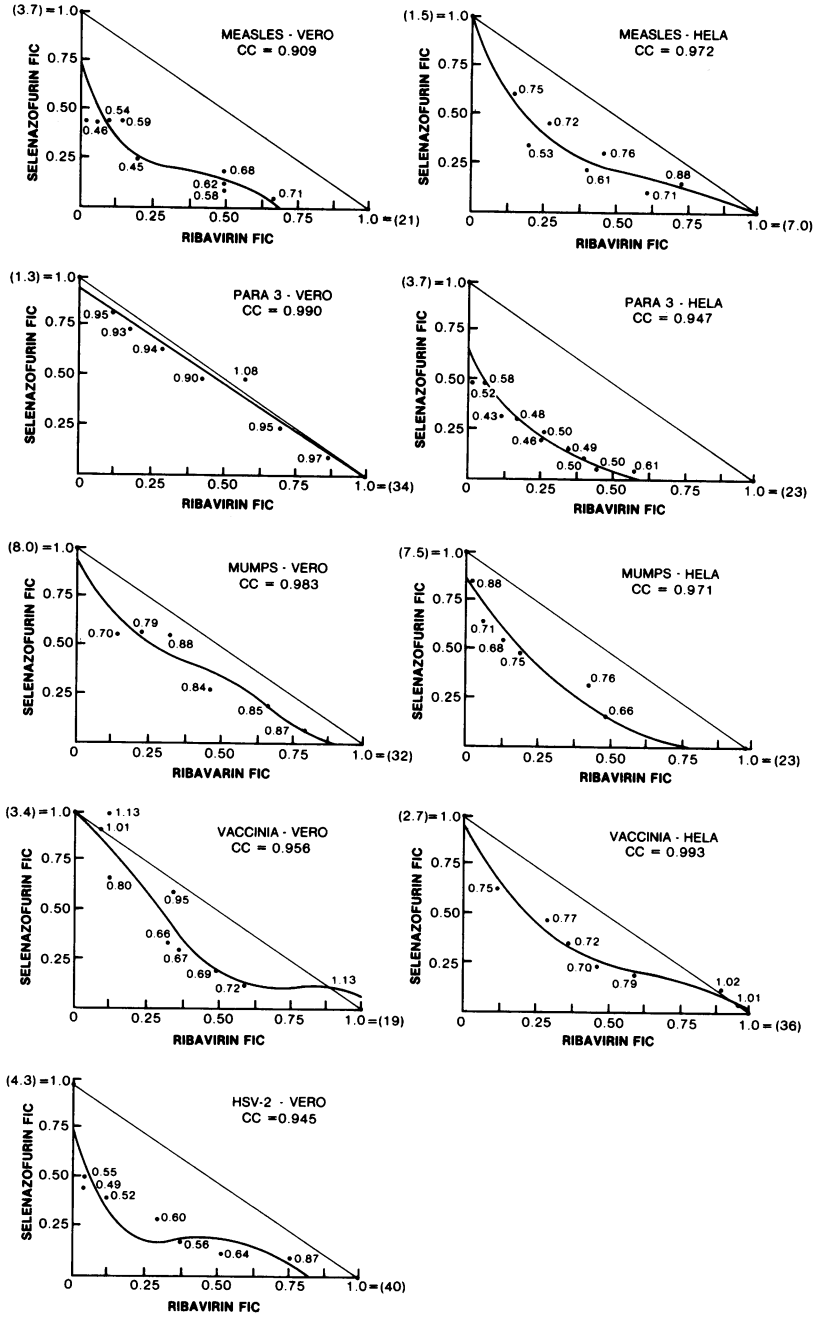

FIG. 3. Isobolograms representing the antiviral effects of the selenazofurin-ribavirin combination in Vero and HeLa cells infected with the indicated viruses. The number next to each data point is the FIC index for that point, as described in the text. The number within parentheses next to the FIC of 1.0 for each compound is the antiviral ED_{50} (micrograms per milliliter) for that compound. CC, Correlation coefficient for the curve.

antiviral activity of this compound might be explained largely as a result of this cytotoxicity.

Antiviral effectiveness of ribavirin and selenazofurin in combination. The antiviral effectiveness of the ribavirin-selenazofurin combination is expressed graphically in statistically fitted isobolograms (Fig. 3). These isobolograms are used to give a qualitative indication of the type of interaction that these compounds have on virus replication. The FIC indices shown next to each data point in Fig. 3 (and in Table 3) give a quantitative indication of the degree of synergism. The FIC index values of less than 1.0 indicate a synergistic interaction.

The greatest degree of synergism by this drug combination in Vero cells was against measles virus and HSV-2. A milder degree of synergism was observed against VV and mumps virus, whereas an additive interaction was observed against Para-3. In HeLa cells, the same drug combination was synergistic against all viruses tested. The greatest degree of

TABLE 3. Antiviral effectiveness of ribavirin and selenazofurin used in combination

Virus	Drug combination[a]	Vero cells					HeLa cells[b]				
		Minimum FIC index[c]	FIC[d]	Amt of drug required (μg/ml)		Decrease in ED_{50} (%)[f]	Minimum FIC index[c]	FIC[d]	Amt of drug required (μg/ml)		Decrease in ED_{50} (%)[f]
				ED_{50} combination[e]	ED_{50} alone				ED_{50} combination[e]	ED_{50} alone	
Measles	Riba + Selena	0.46	0.20	4.20	21.0	80	0.63	0.29	2.8	7.0	60
			0.26	0.96	3.7	74		0.34	0.5	1.5	67
Para-3	Riba + Selena	0.96	0.48	16.30	34.0	52	0.42	0.19	4.4	23.0	81
			0.48	0.62	1.3	52		0.23	0.8	3.7	78
Mumps	Riba + Selena	0.76	0.23	7.40	32.0	77	0.66	0.44	10.1	23.0	56
			0.53	4.20	8.0	48		0.22	3.1	14.0	78
VV	Riba + Selena	0.64	0.43	8.20	19.0	57	0.70	0.34	12.2	36.0	66
			0.21	0.71	3.4	79		0.36	1.0	2.7	63
HSV-2	Riba + Selena	0.35	0.21	8.40	40.0	79	NA	NA	NA	NA	NA
			0.14	0.60	4.3	86		NA	NA	NA	NA

[a] Riba, Ribavirin; Selena, selenazofurin.
[b] NA, Not applicable.
[c] Minimum FIC index, taken from computer-fitted isobologram, represents the drug combination in which the lowest concentrations of ribavirin and selenazofurin are needed to inhibit the viral CPE by 50%.
[d] FIC, FIC of ribavirin and selenazofurin present at the minimum FIC index point.
[e] ED_{50} combination is the concentration of ribavirin and selenazofurin present at the minimum FIC index point.
[f] The percent reduction in viral ED_{50} for the drug used in combination as compared with that for the drug used alone.

synergism was observed against Para-3, whereas less pronounced synergism was seen against measles virus, VV, and mumps virus (Fig. 3).

The minimum FIC indices for each drug combination, chosen from the statistically fitted isobolograms are shown in Table 3. The minimum FIC index represents the lowest concentrations of ribavirin and selenazofurin which were needed to inhibit viral CPE by 50%. The relative amounts of ribavirin and selenazofurin present in the drug combination which comprised the minimum FIC index are shown as FIC values in Table 3, column 2. The actual drug concentrations (expressed in micrograms per milliliter) that the FIC numbers represent are expressed as ED_{50} combination values (Table 3, column 3). The combination ED_{50} values were contrasted with the ED_{50} values of the single compounds by calculating the percent decrease in their ED_{50} values (column 5). In all combinations that resulted in a synergistic response, the amount of each drug required for the inhibition of viral CPE was considerably reduced (maximum, 86%) as compared with the use of either drug alone.

Based on the minimum FIC index values in Table 3, the degree of synergism of the ribavirin-selenazofurin combination in Vero cells was HSV-2 > measles virus > VV > mumps virus > Para-3.

AIs for ribavirin and selenazofurin, used individually and in combination, are summarized in Table 4. The reason for using AIs in this type of study is that these values take into account the cytotoxicity of the compounds. In Vero cells, the sum of the AI (ΣAI) values for the drug combination was increased over the ΣAI values of individual drugs by 247% for HSV-2, 121% for VV, 101% for measles virus, 19% for mumps virus, and 5% for Para-3. In HeLa cells, the percent increase in ΣAI values for the combination over that of the individual compounds was 76% for Para-3, 26% for mumps virus, 19% for measles virus, and −4% for VV. The degree of improvement in the antiviral activity seemed to be a reflection of the type of drug interaction occurring in the combination.

Antiviral effectiveness of 3-deazaguanosine in combination with ribavirin and selenazofurin. The isobolograms and FIC indices which resulted from the combination of 3-deazaguanosine with ribavirin or selenazofurin in Vero cells are shown in Fig. 4. The antiviral effect of the 3-deazaguanosine–ribavirin combination was consistently additive against all viruses tested. When 3-deazaguanosine was combined with selenazofurin, the interaction was consistently one of indifferences; no additivity or synergy was observed with these cell-virus assay systems. The most pronounced deviation from an additive effect was seen in Para-3, followed by measles virus, VV, and HSV-2.

DISCUSSION

It is relatively well established that antiviral agents used in combination should possess one or more of the following advantages to be considered therapeutically useful: (i) they should interact to produce at least an additive, or better yet, a synergistic effect; (ii) the combination should minimize cytotoxicity, so that there is no increase in the toxicity relative to that of the agents used alone; (iii) the combination should prevent the appearance of mutants resistant to either drug.

In this study, the specific enhanced antiviral activity of the selenazofurin-ribavirin combination was clearly evident in Fig. 3. The synergistic interaction observed in both cell lines (Vero and HeLa) could not be attributed solely to the cytotoxicity of the drugs. If cytotoxicity alone were the cause of the synergistic effect, then the combination ought to show equal cytotoxic and antiviral effects. However, the isobolograms are fundamentally different (Fig. 2 and 3). Cytotoxicity was additive, whereas the overall antiviral action was synergistic against the same viruses tested in both cell lines (Fig. 3).

Quantitative evidence for specific antiviral synergism was obtained by calculating the indices of the antiviral combinations (Tables 3 and 4), in which the cytotoxic effects of the drugs were taken into account. Although the usual antiviral

TABLE 4. AIs for ribavirin and selenazofurin used alone or in combination

Virus	Treatment[a]	Vero cells				HeLa cells			
		AI[b]	ΣAI[c]	% Increase[d]	Drug interaction	AI[b]	ΣAI[c]	% Increase[d]	Drug interaction
Measles	Riba	47.6	317.9			142.8	476.1		
	Selena	270.3				333.3			
	Riba + Selena	119.0	639.8	101	Synergistic	235.0	565.0	19	Slightly synergistic
		520.8				330.0			
Para-3	Riba	29.4	798.6			43.5	178.6		
	Selena	769.2				135.1			
	Riba + Selena	30.7	837.1	5	Additive	107.8	314.0	76	Synergistic
		806.4				206.2			
Mumps	Riba	31.2	156.2			43.5	79.2		
	Selena	125.0				35.7			
	Riba + Selena	67.6	186.6	19	Slightly synergistic	46.5	99.7	26	Slightly synergistic
		119.0				53.2			
VV	Riba	52.6	346.7			27.8	213.0		
	Selena	294.1				185.2			
	Riba + Selena	61.0	765.2	121	Synergistic	38.5	203.5	−4	Additive
		704.2				165.0			
HSV-2	Riba	25.0	257.6						
	Selena	232.6							
	Riba + Selena	60.0	893.3	247	Synergistic				
		833.3							

[a] Riba, Ribavirin; Selena, selenazofurin.

[b] AIs for ribavirin and selenazofurin used alone (AI alone = MTC/ED_{50}) or in combination (ribavirin AI in combination = ribavirin MTC in combination/ribavirin ED_{50} in combination; selenazofurin AI in combination = selenazofurin MTC in combination/selenazofurin ED_{50} in combination).

[c] ΣAI alone = ribavirin AI alone + selenazofurin AI alone; ΣAI in combination = ribavirin AI in combination + selenazofurin AI in combination.

[d] The percent increase in the ΣAI values of the combination over ΣAI values of ribavirin and selenazofurin used alone. % Increase = ΣAI in combination − ΣAI alone/ΣAI alone.

effect was synergistic, the degree of antiviral synergism varied among the different viruses tested. The interaction with VV and Para-3 was cell line dependent but was still at least additive in its effect, not indifferent or antagonistic like the 3-deazaguanosine combinations (Fig. 4). This indicates improved antiviral usefulness of the selenazofurin-ribavirin combination over the individual use of the drugs. Although it may be possible that some antiviral activity could be ascribed to cytotoxicity, especially in HeLa cells, it would appear more likely that the observed synergistic antiviral activity was viral specific.

When a two-drug combination produces a synergistic effect it is generally accepted that the two agents have different sites or mechanisms, or both, of biochemical action (2, 4, 29). Conversely, an additive interaction between two drugs may indicate that they share the same mode of action (3, 19, 41). If this commonly held interpretation is correct, then the synergistic antiviral effect observed with the selenazofurin-ribavirin combination suggests that the two agents differ at least in certain aspects of their modes of antiviral action.

The interactions of 3-deazaguanosine also suggest that selenazofurin and ribavirin had different modes of action (Fig. 4). Ribavirin and 3-deazaguanosine combinations were consistently additive against the DNA and RNA viruses tested, whereas the interaction of selenazofurin with 3-deazaguanosine was indifferent or antagonistic in their action against the same viruses.

The observed additivity of the ribavirin–3-deazaguanosine interaction is consistent with other evidence that both 3-deazaguanosine and ribavirin are inhibitors of IMP dehydrogenase (46, 49, 52). Our data also indicate that the antiviral action of selenazofurin is different from that of either 3-deazaguanosine or ribavirin (Fig. 2, 3, and 4).

However, with our data we can only generalize and compare the known modes of action for ribavirin and 3-deazaguanosine with the (as yet unconfirmed) specific mode(s) of action of selenazofurin. Through an analysis of the data presented in this communication, the biochemical mechanism of the antiviral and cytotoxic actions of selenazofurin may be inferred from an interpretation of the isobolograms obtained from the studies on drug combinations (Fig. 2, 3, and 4).

Some specific modes of antiviral action for ribavirin have been proposed by several investigators. These include (i) selective inhibition of influenza RNA polymerase and mRNA synthesis by ribavirin-5′-triphosphate (16, 42); (ii) inhibition of capping guanylation and methylation of VV mRNA (18); (iii) inhibition of viral protein synthesis (5, 6, 26, 33, 39); and (iv) a general inhibition of tumor virus transformation (24, 36, 43, 44). All of these antiviral activities affect virus-dependent events and reflect the broad-spectrum antiviral actions characteristic of ribavirin.

The specific antiviral action for selenazofurin is not presently known, and further investigation is needed both to determine whether selenazofurin shares any common antiviral action sites with ribavirin and to identify the differences between their antiviral modes of action.

When trying to postulate the biochemical effects of these drugs, it is necessary to separate drug cytotoxic effects from the antiviral activity of the compounds. The results with uninfected control cells in this study (Fig. 2) indicate that the cytotoxicity produced by the selenazofurin-ribavirin combination was clearly additive in Vero cells and varied in HeLa

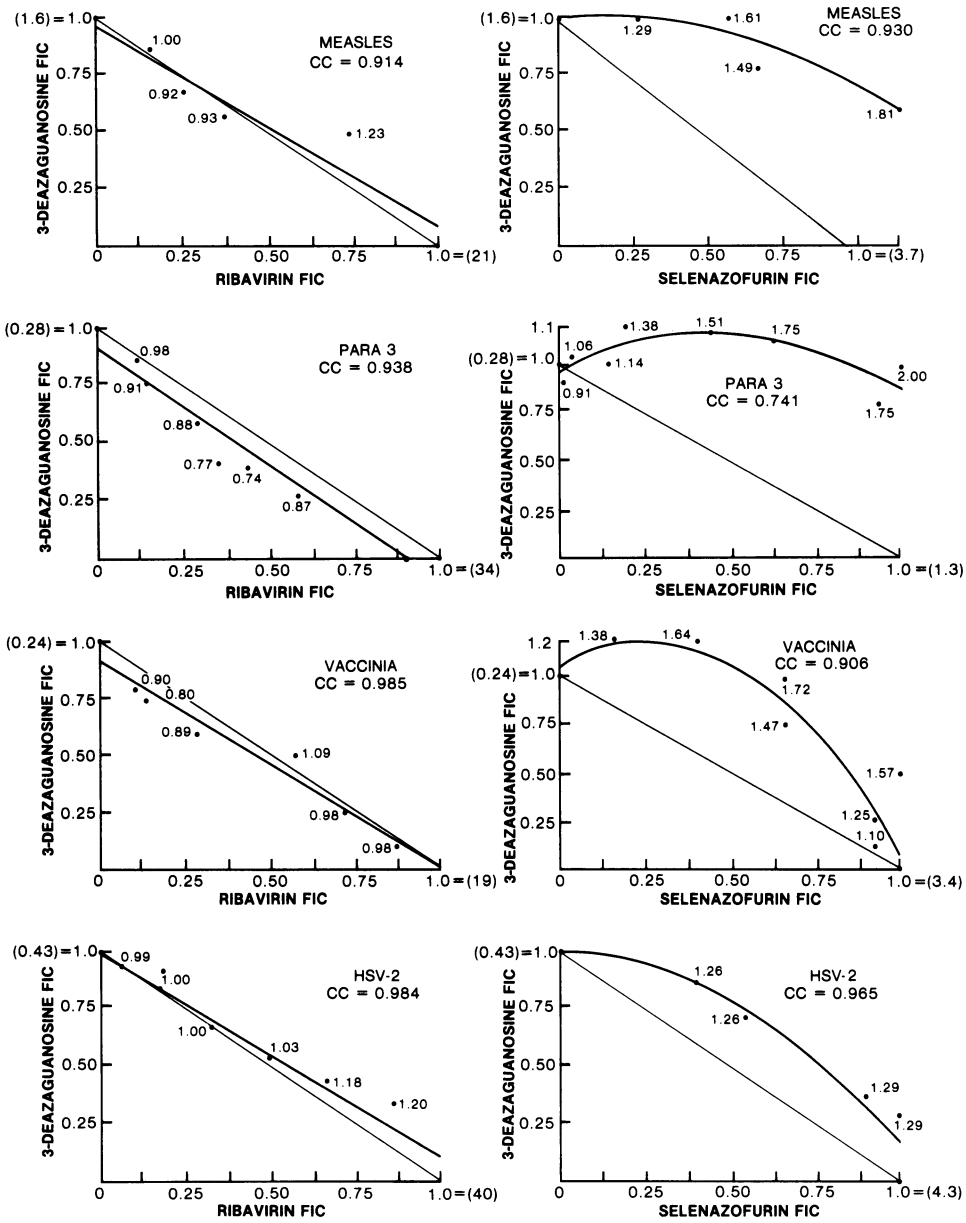

FIG. 4. Antiviral isobolograms and FIC indices for 3-deazaguanosine combined with selenazofurin or with ribavirin in Vero cells against the indicated viruses. The number within parentheses next to the FIC of 1.0 is the antiviral ED_{50} (micrograms per milliliter) for that compound. CC, Correlation coefficient for the curve.

cells from being additive to slightly synergistic. The synergistic response in HeLa cells seems to reflect the increased sensitivity of actively growing cells to these compounds (25, 34, 35).

The additive effect on cytotoxicity of the selenazofurin-ribavirin combination implies that these drugs exerted their cytotoxic activity by a similar mode(s) of action, possibly by competitive inhibition of cellular IMP dehydrogenase (45, 46, 49, 52). This enzyme is involved in the de novo synthesis of guanosine. Therefore, the inhibition of this enzyme by selenazofurin could also lead to a depletion of the intracellular guanine nucleotide pools, as observed by others in ribavirin-treated cells (30, 32, 45, 56).

Although ribavirin has been reported as a potent inhibitor of cellular DNA and RNA synthesis (13, 28, 32), this inhibition has been shown to be indirect and artifactual. Drach et al. (14) and Smith and Kirkpatrick (46) reported that the inhibition of nucleic acid synthesis, which had been reported in a previous paper of Drach and Shipman (13) and by others (12, 28, 32), is caused by an inhibition of [^{3}H]dTTP formation and an expansion of the dTTP pool size. These combined effects caused a marked decline in the specific activity of [^{3}H]dTTP and, consequently, a significant decrease in the labeling of DNA. The perturbations of the deoxy- and ribonucleotide pools had been observed previously by Lowe et al. (30) and Zimmerman et al. (56), respectively. Ribavirin does not have a direct inhibitory effect on several eucaryotic or procaryotic DNA and RNA polymerases (16, 32), nor is ribavirin an analog for DNA or RNA synthesis (14, 30, 50, 56). Therefore, the best descrip-

tion of the cytotoxic action for ribavirin and perhaps for selenazofurin seems to be cytostatic, especially for stationary, confluent, monolayer cells (28, 32).

The slight synergistic effect of the selenazofurin-ribavirin combination on HeLa cell growth may be due to multiple sites of action or altered enzymatic kinetics, or both, in tumor cells. The qualitative and quantitative differences in metabolism between normal and tumor cells could explain the observed increase of combination cytotoxicity in HeLa cells. Both compounds have shown increased cytotoxicity against actively dividing tumor cell lines (23, 31, 36, 51). How selenazofurin might exert cellular cytotoxicity affecting tumor cell polymerases or their substrate specificities is not presently well understood.

The mechanism of action of selenazofurin is currently under investigation in this and other laboratories (23, 31, 51). These studies suggest that selenazofurin and ribavirin have a similar cytotoxic effect via the inhibition of IMP dehydrogenase. Our results with uninfected Vero cells (Fig. 2) are in agreement with these observations, although when high drug concentrations were used in HeLa cells a mild synergism was noted. This implies a different or more potent cytotoxic effect for selenazofurin against these tumor cells.

In summary, the synergistic interaction between selenazofurin and ribavirin observed in this study is indicated by a substantial reduction of the dose of both drugs, when used in combination, while still maintaining the same degree of viral inhibition. The antiviral activity of the combination was much better than the activity of the individual drugs alone (Tables 3 and 4). This synergism also suggests that ribavirin and selenazofurin may vary in some aspects of their antiviral activity. Since no enhancement of the cytotoxicity was observed, but a synergistic antiviral response was commonly noted (isobolograms and AI indices), the antiviral inhibitory effects of the selenazofurin-ribavirin combinations seem to be specific against certain events necessary for viral replication. The antiviral effects of the selenazofurin–3-deazaguanosine combination varied from indifferent to antagonistic, implying that this combination would be therapeutically ineffective. Furthermore, the observed antiviral activity of the 3-deazaguanosine can be accounted for by its cytotoxicity alone, with a minimal direct antiviral effectiveness (Table 2).

These findings, demonstrating the marked enhancement of the antiviral activity of the selenazofurin-ribavirin combination, warrant further evaluation of its efficacy in other cell-virus assay systems as well as an investigation of its therapeutic usefulness in animal models. Further studies are in progress in our laboratory to elucidate the antiviral mechanism of selenazofurin against different viruses.

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