ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1984, p. 476–480
0066-4804/84/100476-05$02.00/0

Vol. 26, No. 4

Synergistic Antiviral Effects of Ribavirin and the C-Nucleoside Analogs Tiazofurin and Selenazofurin Against Togaviruses, Bunyaviruses, and Arenaviruses

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Received 11 April 1984/Accepted 18 July 1984

Binary combinations of the N-nucleoside ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and the C-nucleoside analog selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide) or tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) were tested *in vitro* for activity against Venezuelan equine encephalomyelitis, Japanese encephalitis, yellow fever, Rift Valley fever, Korean hemorrhagic fever, and Pichinde viruses. The 50% effective dose for each compound alone or in a series of combinations was determined with a plaque reduction assay. Combinations of ribavirin and selenazofurin were synergistic against Venezuelan equine encephalomyelitis, Japanese encephalitis, yellow fever, and Pichinde viruses, with fractional inhibitory concentrations of 0.1, 0.2, 0.4, 0.4, respectively, but showed additive effects against Korean hemorrhagic fever and Rift Valley fever viruses. Combinations of ribavirin and tiazofurin were synergistic against yellow fever and Japanese encephalitis (fractional inhibitory concentrations, 0.41 and 0.48, respectively) but showed additive effects against Korean hemorrhagic fever virus. Combinations of selenazofurin and tiazofurin had additive effects against Japanese encephalitis, yellow fever, and Korean hemorrhagic fever viruses. The effect of combinations on cell toxicity was additive, both in monolayers of nondividing cells incubated under agar for the same period as the plaque assay and for rapidly dividing cells given short-term exposure (4 h), followed by determination of the proportion of surviving cells with a colony forming assay.

In the search for more effective antiviral compounds, we have screened numerous N-glycosyl and C-glycosyl nucleosides against selected members of the toga-, bunya-, and arenavirus families. The carboxamide nucleoside ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (8) was one of the most effective broad-spectrum agents tested. Limited structural modification of ribavirin did not improve its antiviral activity significantly; however, several analogs have shown comparable antiviral activity. Included were the novel selenazole carboxamide nucleoside selenazofurin (2-β-D-ribofuranosyl-selenazole-4-carboxamide) (19), with a broad spectrum (13) and the closely related thiazole carboxamide nucleoside tiazofurin (2-β-D-ribofuranosly-thiazole-4-carboxamide) (15, 18), with a narrower spectrum but exceptionally active in vitro against Japanese encephalitis (JE), yellow fever (YF), and Hantaan virus, the causative agent of Korean hemorrhagic fever (KHF) (13).

The structural similarities between ribavirin, selenazofurin, and tiazofurin and their known ability to inhibit IMP dehydrogenase prompted the in vitro evaluation of combinations to determine whether there would be potential benefits of such combinations over the use of a single compound. Binary combinations were evaluated in vitro with a plaque reduction assay to determine the most effective ratios and possible antagonistic combinations and to gain insight as to whether these related compounds have the same or different biochemical modes of antiviral action.

MATERIALS AND METHODS

Performance. All studies were performed in facilities designed for high-level microbiological containment (class 3) at the U.S. Army Medical Research Institute of Infectious Diseases in compliance with guidelines (4, 21).

Antiviral compounds. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), and selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide, selenazole) were synthesized as previously described (18, 19, 22).

Cells. All experiments were conducted with cells passaged 5 to 15 times in our laboratory. African green monkey kidney cells (Vero-76; ATCC CRL 1587) and a cloned line Vero E6 (Vero C1008; ATCC CRL-1586) were grown in Eagles minimum essential medium with Earle salts and 1× nonessential amino acids supplemented with 10% fetal bovine serum (KC Biologicals, Inc., Lenexa, Kans.). Rhesus monkey kidney cells (LLC-MK_2; ATCC CCL 7) were grown as described previously (13). All cell cultures were tested weekly and found to be free of bacterial and mycoplasmal contamination (6).

Viruses. Rift Valley fever virus (RVF) Zagazig 501 strain, Venezuelan equine encephalomyelitis virus (VEE) Trinidad donkey strain, Pichinde virus (PIC) CoAn 3739 strain, YF Asibi strain, JE Nakayama strain, and Hantaan virus, the etiological agent of KHF, HBL7990 strain, are described elsewhere (13).

In vitro determination of compound ED_{50} by a plaque reduction assay. The 50% effective dose (ED_{50}) for each compound was determined in triplicate in 12-well plates (4.5 cm^2 per well; Linbro; Flow Laboratories, McLean, Va.) with a plaque reduction assay on monolayers of Vero-76 (VEE, JE, RVF, and PIC), Vero E6 (KHF), or LLC-MK_2 (YF) as described previously (13). Plaques were enumerated after staining with neutral red for visualization on the day that plaques were first fully developed in non-compound-

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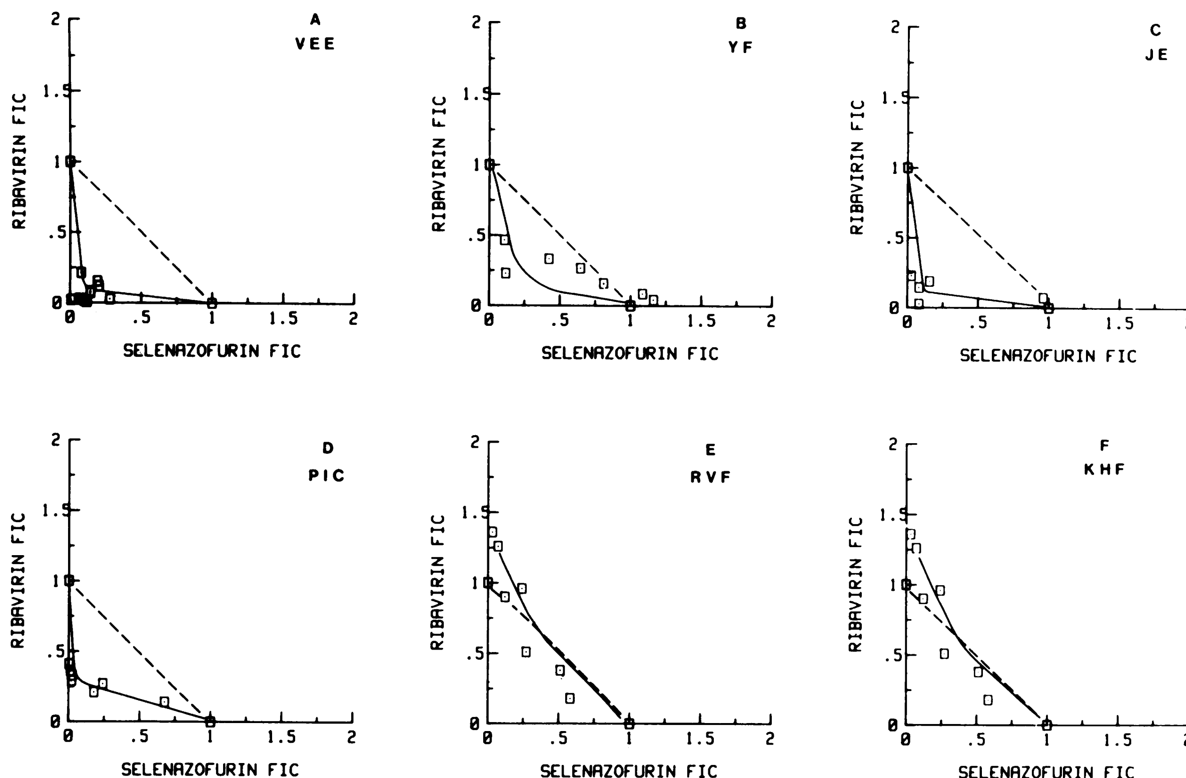

FIG. 1. Isobologram for combinations of ribavirin and selenazofurin alone and at various fixed ratios. Details are given in the text. Viruses were assayed in Vero-76 (A, B, D, and E), LLC-MK$_2$ (C), and Vero E6 (F) cells.

treated plates. The dose-response curves were sigmoid in shape, and the ED_{50} was obtained by using an appropriately weighted four-parameter logit curve-fitting computer program (16) which included criteria for goodness of fit.

Experimental design for evaluation of antiviral activity of compound combinations. Ribavirin, selenazofurin, or tiazofurin were tested alone and in binary combinations at seven fixed ratios by a modification of our previously published drug assay based on an in vitro plaque reduction test (13). Combination studies were designed, using the approach of Berenbaum (3), which is based on the dose of each compound causing a 50% response. To determine synergy, dose-response assays of compound alone (to verify the ED_{50}) and combinations of compounds at fixed ratios (typically, ratio of ED_{50} of compound A to ED_{50} of compound B, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10) were performed. The molar concentration of each compound in a combination was calculated as follows. Beginning with the null hypothesis that the effects of the compounds in combination would be additive, an isobologram response surface was constructed based on the ED_{50}s of each compound alone. Since virus inhibition is related among compounds by their ED_{50}, the ratios for testing were selected based on the ratios of the ED_{50}s by modeling in such a manner that each combination would contain four ED_{50}s of activity. The stock solution and seven threefold serial dilutions were tested in triplicate to determine the dose-response curve for plaque reduction and were designed to yield from 0 to 100% response. The antiviral activity of combinations was evaluated by two methods. First, isobolograms (1, 2, 7, 12, 17) were constructed to convert the family of curves associated with each experiment into a two-dimensional plot, in which the concentration of each compound alone or in combination that produced the same response (a 50% plaque reduction) was graphed. Such plots yield an isobologram of constant responses.

Second, the fractional inhibitory concentration (FIC) (2, 3, 19) was calculated by using the following formula: FIC =

TABLE 1. Maximal response of antiviral compounds[a] used alone or in combination

Virus	ED_{50} (μg/ml)			FIC[b]		
	Sel	Riba	Tia	Sel + Tia	Tia + Riba	Sel + Tia
Togaviridae						
Alphavirus VEE[c]	0.5	25	NA	0.1	ND	ND
Flavivirus						
JEV[c]	3.0	65	73.0	0.2	0.4	1.0
YF[d]	0.08	8	0.08	0.4	0.5	1.1
Bunyaviridae						
RVF[c]	2.8	80	1.0	0.8	0.8	1.0
KHF[e]	1.3	16	NA	1.0	ND	ND
Arenaviridae PIC[c]	4.0	60	NA	0.4	ND	ND

[a] Sel, Selenazofurin; Riba, ribavirin; Tia, tiazofurin. NA, Compound had no activity against the virus. ND, Not done because one compound had no activity against the virus.

[b] The interpretation of the FIC index as described by Allen et al. (1) is as follows. FIC indices: <0.5, significant synergism; 0.5 to 0.9, suggestive of synergism; 1, additive; 1.1 to 1.9, indifference or partial antagonism.

[c] Assayed in Vero-76 cells.

[d] Assayed in LLC-MK$_2$ cells.

[e] Assayed in Vero E6 cells.

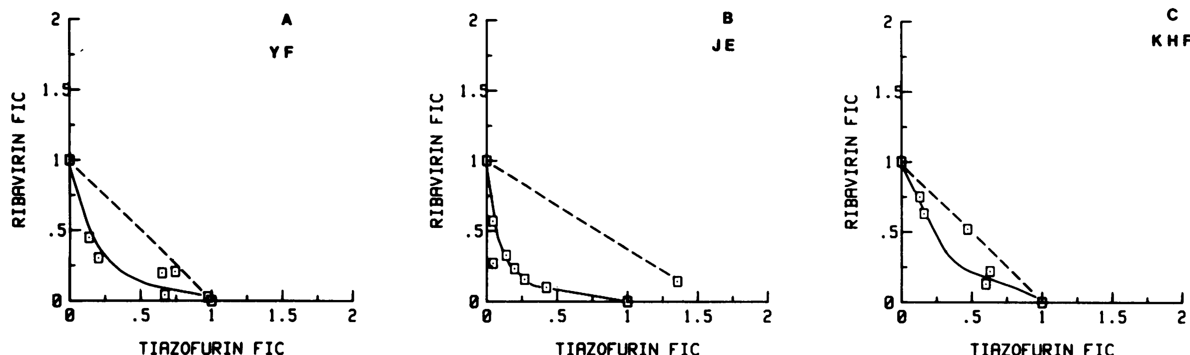

FIG. 2. Isobologram for combinations of ribavirin and tiazofurin alone and at various fixed ratios. Details are given in the text.

[(ED_{50} of drug A in combination)/(ED_{50} of drug A alone)] + [(ED_{50} of drug B in combination)/(ED_{50} of drug B alone)]. The calculations were done by using the ED_{50} for each individual compound obtained from the experiment. Experiments with all viruses were repeated at least twice with low-passage level cells and samples of virus from the same pool.

Toxicity. Toxicity determinations for stationary, nondividing monolayers were based on an assessment of cytopathic effects in treated cultures compared with those in control cells which were also maintained under agar overlay. Toxicity was determined in parallel with antiviral activity, using the same concentrations. Phase-contrast microscopy was used to evaluate morphological changes along with uptake of neutral red. Cultures were examined daily, including the day that plaques were read in the virus-challenged cultures. Acute toxicity in dividing cells was determined as follows: rapidly dividing subconfluent cells, subcultured 2 days previously, were removed with trypsin-EDTA, washed, and incubated with the compound (500 μg/ml) or combinations as above for 4 h at 37°C, and the number of viable cells remaining was determined with a colony-forming assay in the standard growth media.

RESULTS

In vitro activity of ribavirin and selenazofurin in combination. The combination studies yielded a family of sigmoidal dose-response curves corresponding to each compound or fixed ratio of combinations. The partial FIC of each compound was calculated from the resulting ED_{50} obtained from each curve. Construction of the isobologram allows for a graphical representation of the family of curves as a single constant response line.

Combinations of selenazofurin with ribavirin were tested against six viruses that serve as models for other members of the toga-, bunya-, and arenavirus families. A representative experiment for each virus is shown in Fig. 1. The three togaviruses tested possess significantly different virus properties within this family; however, all show similar responses to the antiviral compound tested. Against the alphavirus VEE and the flaviviruses YF and JE, ribavirin-selenazofurin combinations show significant deviation below the theoretical additive line (Fig. 1A, B, and C). VEE and JE were assayed in Vero-76 cells, and the FIC values for combinations (Table 1) show significant synergy at 0.1 and 0.21, respectively. The Asibi strain of YF does not form plaques well on Vero-76 cells, so assays were performed on LLC-MK_2 cells. The isobolograms show a significant deviation below the additive effect line, although less than for VEE or JE. YF was inhibited at much lower concentrations of selenazofurin or ribavirin than was VEE or JE (10-fold), and significant synergy is still seen at a FIC of 0.43. For the toga- and arenaviruses none of the compounds show antagonism at higher ratios of one compound compared with the other.

The isobologram for the arenavirus PIC assayed in Vero-76 cells (Fig. 1D) shows a deviation below the theoretically additive line (dashed), with a FIC of 0.4, indicating significant synergy (Table 1).

The bunyavirus RVF assayed in Vero-76 cells showed no significant deviation below the additivity line, whereas combination studies with the bunyavirus KHF showed either no effect or antagonism (antagonism was seen when ribavirin

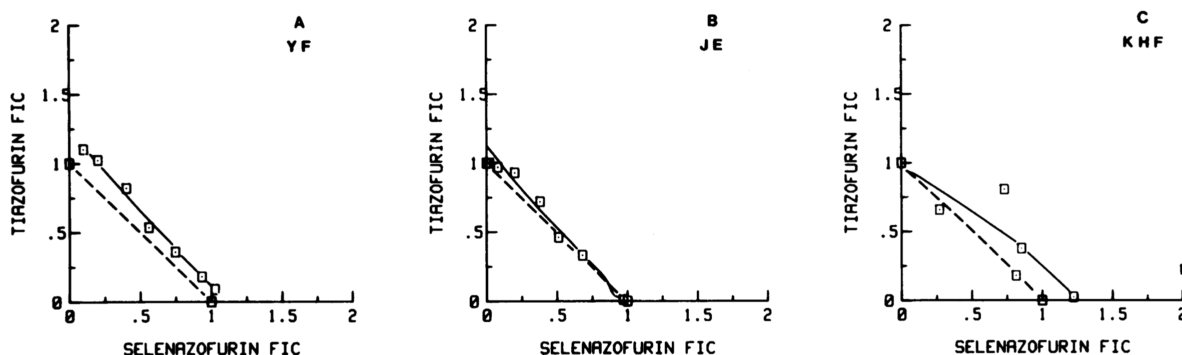

FIG. 3. Isolobologram for combinations of selenazofurin and tiazofurin alone and at various fixed ratios. Details are given in the text.

TABLE 2. Maximal noncytotoxic concentration of antiviral compounds tested

Cell line	Compound[a]					
	Alone[a]			In combination[c]		
	Riba	Sel	Tia	Sel + Riba	Tia + Riba	Sel + Tia
Vero-76	1,000	1,000	1,000	6 + 90 (1:20) 6 + 147	146 + 84 (1:0.6)	6 + 146 (1:29)
Vero E6	1,000	1,000	1,000	4 + 42 (1:13)	23 + 42 (1:1)	3 + 23 (1:9)
LLC-MK_2	1,000	1,000	1,000	0.6 + 42 (1:88)	23 + 100 (1:5)	0.6 + 23 (1:45)

[a] Cytotoxicity was assessed in stationary monolayers maintained under agar, both by cytopathic effect and by uptake of neutral red, as described in the text. Riba, Ribavirin; Sel, selenazofurin; Tia, tiazofurin.
[b] Micrograms per milliliter.
[c] Molar ratio of compounds in combination.

was present in greater amounts [left side of figure]). Both viruses were inhibited by either compound alone at low concentrations, and FIC values were 0.8 and 1.0 for RVF and KHF, respectively.

In vitro activity of ribavirin and tiazofurin in combination. Ribavirin-tiazofurin combinations were tested only against those viruses inhibited by tiazofurin (JE, YF, and KHF) (10). Isobolograms for YF (Fig. 2A) show a marked deviation below the additivity line and are very similar to the patterns seen with selenazofurin-ribavirin combinations. The FIC was 0.48, near the arbitrary division point in our system (Table 1). JE showed a profile that was essentially the same as that for selenazofurin-ribavirin (Fig. 2B). The isobologram for KHF (Fig. 2C) shows no significant deviation below the additivity line.

In vitro activity of selenazofurin and tiazofurin in combinations. Selenazofurin-tiazofurin combinations tested against YF, JE, and KHF show an additive effect as judged both by the isobolograms (Fig. 3) and FICs near 1 (Table 1).

In vitro toxicity of combinations. In vitro toxicity was determined by phase-contrast morphology and qualitative neutral red uptake by individual cells. No significant changes in morphology were observed in confluent monolayers of Vero-76, Vero E6, or LLC-MK_2 cells for any of the binary combinations at any of the ratios tested. In each case, combinations were tested at each ratio up to a maximum combined FIC of 4 (Table 2). Morphology was followed up to the day that plaques were read. Neutral red uptake and sequestration into vacuoles were not affected. Assessment in dividing cells of toxicity from a 4-h exposure to the compounds showed no significant reduction in cell viability, as judged by the ability of cells to form colonies after compound exposure, with a plating efficiency assay.

DISCUSSION

Combinations of the antiviral drug ribavirin, an N-glycosyl nucleoside, and the closely related C-glycosyl nucleoside analogs tiazofurin and selenazofurin were tested against a number of RNA viruses representative of virus classes which cause notable human disease. In vitro studies were performed to determine the interactions of the compounds at various ratios relative to their individual ED_{50}s. Combinations of ribavirin and selenazofurin were most effective against togaviruses VEE, YF, and JE, and the arenavirus PIC, with FIC values of 0.1 to 0.4 indicative of substantial synergy. Ribavirin-selenazofurin combinations were least effective against bunyaviruses RVF and KHF, with additive compound effects (FIC values of 0.8 and 1.0, respectively). Isobolograms of this combination show a virus-dependent variation in the response profile. The difference in responses among viruses indicates that the inhibition is not strictly a cell-dependent (cytotoxic) event. Three viruses, VEE, PIC, and RVF, were all assayed in Vero-76 under identical conditions, with synergy seen against VEE and PIC but not RVF. As with selenazofurin in combination, ribavirin-tiazofurin combinations show synergism against YF and JE but not KHF. This comparison also held for the shape of the isobolograms and the magnitude of the FICs. The combinations of selenazofurin-tiazofurin tested against JE, YF, and KHF consistently showed an additive effect.

Synergistic interactions could be explained if the two compounds had different sites of action. The inhibition of several cellular enzymes such as IMP dehydrogenase has been demonstrated for all three compounds (9–11, 20), and this can be expected to alter nucleoside metabolism, with resulting changes in intracellular nucleotide pools. Ribavirin has been shown to accumulate in cells as the ribavirin triphosphate at concentrations approaching ATP levels (23). Selenazofurin and tiazofurin also form a NAD-like product which is a potent inhibitor of IMP dehydrogenase (5). These changes in cellular metabolism are believed to be important in the cytotoxicity of the compounds, but the differences in virus response to identical conditions argue that these changes are unlikely to explain the antiviral activity. Although the number of viruses tested was small, the synergy exhibited by combinations of ribavirin and selenazofurin or tiazofurin against members of the togavirus and arenavirus families suggests that each compound is acting at a different site. The studies with selenazofurin and tiazofurin against JE, YE, and KHF showed no evidence of synergy; thus we could not find any evidence that these close structural analogs worked at different sites. The known alteration in intracellular nucleotide pools caused by these compounds may not be a sufficient explanation for their synergistic activity, since each family of viruses responded differently in its growth properties to the same concentration of compound. The viruses tested have significant differences in their mechanism of viral replication which may account for differences in compound sensitivity. The use of drug combinations may prove useful in the study of the replication mechanisms of these viruses.

Ribavirin, selenazofurin, and tiazofurin are known to be cytostatic for rapidly dividing cells (12, 14). This inhibition appears to correlate with the inhibition of IMP dehydrogenase in resistant P388 cells (10). The inhibition is reversible in most cases after removal of the compound. Cells in stationary culture, however, fail to show any evidence of morphological changes, including cytopathic effect, and remain viable as assayed by neutral red uptake even when cultured continuously in the presence of high concentrations of compound for 14 days. Similarly, no toxicity, as judged by

the ability of cells to grow and form colonies (a test recognized as among the most sensitive), was found when dividing cells were incubated with the compounds for 4 h. Kirsi et al. in a companion study (12) also have shown that the toxicity for Vero cells of the three compounds in combination is additive, as judged by inhibition of subconfluent dividing cells. In our hands, Vero and Vero-76 cells responded identically to the inhibitory properties of these compounds. These toxicity findings are in opposition with those of studies based on cell division (unpublished data) in which all three compounds were found to inhibit cell replication in a dose-dependent manner. Published reports have shown that both tiazofurin and selenazofurin cause a reversible cell cycle arrest. Thus the toxicity appears to be classified as cytostatic and not cytocidal in nature. The use of such methods would thus measure a type of toxicity that may not be relevant to this study in which the virus is replicating in slowly dividing or stationary cells, and a cytostatic compound would have little effect. Definitive toxicity studies would probably be most appropriately done in vivo, along with in vitro studies with selected cell types more suitable for toxicity studies than for viral replication.

The demonstration of synergy between ribavirin and selenazofurin or tiazofurin is an important first step in the design of improved antiviral therapy. The patterns of interactions suggest starting combinations for in vivo studies and with certain viruses warn of the potential for antagonism at improper ratios of ribavirin-selenazofurin. The potential use of combinations to overcome single-compound shortcomings makes this an important approach to the treatment of serious human viral infections, and these studies provide the information required to design animal trials for these compounds.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Tony Spears and Jeff Stiefel and the valuable advice of Harold Lupton and Carol Linden in the preparation of this manuscript.

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