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Synergism between anti-rhinovirus antivirals: various human interferons and a number of synthetic compounds

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Summary

DCF (dichloroflavan), enviroxime, chalcone Ro-09-0410 and HuIFN (Human interferon)-α2, HuIFN-β, HuIFN-β × 401 and HuIFN-γ, showed antiviral activity in vitro against RV2 (rhinovirus type 2) and RV9.

Binary combinations of these drugs showed synergistic activity of which the combinations of HuIFN-γ or HuIFN-α and enviroxime were of most interest. They were studied in more detail in tissue culture by virus yield experiments and in organ culture of human embryonic nasal epithelium and human embryonic tracheal culture in which there was a potent antiviral synergy. These results indicate that such combinations of drugs may be worthy of clinical study.

rhinovirus; antivirals; interferon; synergy; dichloroflavan; enviroxime

Introduction

Rhinoviruses have long been considered a suitable goal for antiviral prophylaxis and therapy. Four active compounds were evaluated in vitro and in vivo in this Unit (see Reed et al. [20]) but were not effective against rhinovirus challenge in man, possibly because they were not sufficiently potent as inhibitors of rhinoviruses. Since then more active compounds have been synthesised and although enviroxime has been shown to have some effect in human volunteers [15,16,26], dichloroflavan (DCF) and the chalcone Ro-09-0410 have not [17,18,26].

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On the other hand, since interferon was discovered by Isaacs and Lindenmann in 1957, it has been thought that it might be an ideal antiviral agent [11], as it is active against essentially all viruses and is relatively non-toxic. In volunteers 2×10^6 units (about 10 μg) per day given in three divided doses as an intranasal spray prevents rhinovirus colds, but much larger doses fail to improve colds if given a day or more after the virus [13,21]. However, prolonged intranasal administration gives rise to local inflammation and this will limit the use of interferons in the prophylaxis of common colds.

It has been known for many years that the synergy exhibited by certain combinations of antibacterial drugs in vitro can also be demonstrated in animals and patients. However, there has been little work of this sort with antivirals. Eggers found in 1976 that HBB and guanidine act synergistically and protect mice infected with echo 9 virus [6]; recently a number of workers have reported synergistic activity between certain antiviral agents, mainly those against herpes viruses. Often the effect has been small and there is little evidence that such synergism is useful in clinical practice [2,3,7–9,23].

We have now tested for synergy between a number of substances against both rhinoviruses types 2 and 9 and also checked the toxicity of the combined drugs with the object of selecting a potent non-toxic combination for further study in man.

Materials and Methods

Cells

WISH cells and MRC-5 were grown at 37°C in MEM (Gibco) supplemented with 10% foetal bovine serum (FBS), 1% glutamine, penicillin, 100 unit/ml and streptomycin, 0.1 mg/ml.

Ohio HeLa cells were grown at 37°C in BME (Gibco) supplemented with 10% newborn calf serum (NCS), penicillin 100 U/ml and streptomycin 0.1 mg/ml. Maintenance medium (BME) was supplemented with 5% NCS and antibiotics.

Overlay for the plaque assay was prepared by combining 1 volume of BME containing twice the required concentration of antiviral agent with 1 volume of 0.4% agarose in PBS.

The following components were also added to the overlay at the final concentration indicated in parentheses: FBS (2%), $NaHCO_3$ (0.08%), $MgCl_2$ (1 mg/ml), EAE-dextran (1 mg/ml) and tryptose phosphate broth (2.6 mg/ml).

Cytopathic effects were read by microscopy of unstained cell monolayers or by eye after staining.

Viruses

Laboratory passaged strains of RV2 and RV9 were grown in Ohio HeLa cell monolayers maintained in BME supplemented with 2% FCS. Cultures were harvested at full cytopathic effect (CPE), frozen and thawed, clarified by centrifugation and the supernatant was stored at −70°C.

Semliki Forest Virus (SFV) 210 was obtained from CAMR, Porton Down, Salisbury, UK.

Antivirals

Enviroxime was prepared at the Lilly Research Laboratory, Indianapolis, Ind., as an equal mixture of *syn-* and *anti-*isomers. Dichloroflavan (DCF) (6830 77) was supplied by Wellcome Research Laboratories, Kent, UK. Chalcone Ro-09-0410 and Ro-09-0415 were supplied by Nippon Roche Research Center, Kamakura, Japan. All were prepared as stock solutions of 10 μg/ml in dimethyl sulphoxide and stored at 4°C.

Interferons

Recombinant human IFN-γ (*E. coli*-derived) D0002 with a titre of 6.8×10^7 IU/ml was a gift from Dr. G.R. Adolf, Ernst-Boehringer-Institute, Vienna, Austria. Hybrid HuIFN-β × 401 (this is normal HuIFN-β in which some of the N-terminal sequence has been removed and replaced with HuIFN-α_2 sequence), 90% pure, contained 5×10^6 IU/ml and was a gift from Drs. M. Parker and O. Neill, G.D. Searle, Wickham, Berks., U.K.

Recombinant HuIFN-β_{ser} was supplied by Triton Biosciences Inc., Alameda, California.

Recombinant HuIFN-α2 was supplied by Dr. Leon Gauci of Schering-Plough, Lyons, France.

Methods of detecting synergy

Enviroxime, DCF, chalcone Ro-09-0410 and Ro-09-0415, HuIFN-γ, HuIFN-α2, HuIFN-β and hybrid HuIFN-β × 401 were first tested individually to determine their MIC. Ro-09-0415 was inactive but the remainder were studied in binary combinations by chequerboard titration. Twofold serial dilutions of one drug starting at 2 MIC were made and added in unit volume to rows of wells or plates containing confluent monolayers of cells. Similar dilutions of a second drug were added to the columns of wells in order to produce all possible combinations within the chosen range of concentrations.

When IFNs were used the plates were incubated overnight, medium was removed and the second drug and virus were added.

To each well was added 100 $TCID_{50}$ of RV2 or RV9 in microtitre plates or 50 PFU in 5-cm petri dishes. The plates were incubated at 33°C. Ohio HeLa cells, MRC-5 or WISH cells were used according to the antiviral tested. The end-point was complete prevention of CPE in all wells. All experiments were run in triplicate and usually repeated 2 or 3 times. The results were usually identical or differed at most by 2-fold (i.e. one dilution). All tests included wells which received each drug and no virus.

Virus yield experiments: to confluent monolayers of cells in 5-cm petri dishes, undiluted virus (MOI 10) was allowed to absorb at room temperature for 90 min, then washed 3 times with PBS, and the maintenance medium with or without different concentrations of drug was added or removed at different time intervals and incubation was continued for 24 h at 33°C.

Certain drug combinations were tested in organ cultures of human embryonic nasal epithelium (HENE) and human embryonic tracheal culture (HETC) and the results were assessed by virus yield. The method for culture of HENE and HETC was basically that of Tyrrell and Blamire [25]; specimens were obtained from The Tissue

Bank, Institute of Cancer Research, The Royal Marsden Hospital, London, U.K. One piece (about 1 cm^2) of nasal epithelium with the underlying cartilage of septum or turbinate or one or two rings of human embryonic trachea were cultured in 1 ml maintenance medium MEM, supplemented with 0.2% BPA and 100 μ/ml penicillin and 0.1 mg/ml streptomycin.

All cultures were incubated in roller tubes at 33°C and the organs were examined for ciliary activity and only those showing good ciliary activity 24 h after preparation of the culture were used. HuIFN was added 24 h before virus. Enviroxime was added at the same time as virus. The medium was then harvested daily for up to 5 days and replaced by fresh medium containing the same concentration of enviroxime but no IFN. The organs were examined daily for ciliary activity and the harvested fluids titrated for virus in Ohio HeLa cells.

Results

Studies in tissue culture: toxicity

The toxicity of each compound was assessed in Ohio HeLa cells and/or MRC-5 and WISH cells, by inspection of monolayers maintained for 5 days in media with various concentrations of the compounds. In HeLa cells and MRC-5, 16 μg/ml of DCF and enviroxime and 8 μg/ml of chalcone Ro-09-0410 and Ro-09-0415 induced morphological changes or cell death, but HuIFN-α2, HuIFN-β, HuIFN-β × 401 and HuIFN-γ had no effect at 1000 U/ml, in MRC-5 or WISH cells. The toxicity of drugs in combination was no greater than that of the same drugs separately; indeed IFNs seemed to slightly reduce the toxic effects of some synthetic drugs.

Inhibition of CPE by single drugs

Serial 2-fold dilutions of compounds were made starting just below the toxic concentrations. These were added with virus to the wells of 96 well microtitre plates containing confluent monolayers of Ohio HeLa cells, MRC-5 or WISH cells. They were observed for CPE daily for 5 days. The minimal inhibitory concentration (MIC) of each drug was calculated according to Kärber as a 50% end-point. The MICs of compounds in such cells were 0.03 μg/ml for chalcone Ro-09-0410 (Ro-09-0415 was inactive), 0.03 μg/ml for DCF, 0.125 μg/ml for enviroxime (Table 1).

The IFNs were assayed in a similar way, but HuIFN dilutions were added the day before the virus. As expected all IFNs were inactive in HeLa cells. In the other combinations rhinoviruses were inhibited by a few IU of all interferons. In MRC-5 cells HuIFN-γ showed no activity (or against SFV; H. Wynne-Davies, personal communication).

In order to study antirhinovirus activity we used WISH cells as they are sensitive both to rhinoviruses and HuIFN-γ; although MDBK, MDCK and Hep-2 were sensitive to HuIFN-γ they were insensitive to rhinovirus type 2 and type 9.

Demonstration of antiviral synergy

Combinations of active drugs were next tested. As described in Materials and

TABLE 1

MICs of antirhinoviral compounds in different cell systems (μg/ml for drugs and IU/ml for IFNs)

Cell system	Drugs or IFNs			
	Ro-0910	Ro-0415	DCF	Enviroxime
Ohio HeLa Cells	0.03	8	0.06	0.06
WISH Cells	0.06	n.t.	0.06	0.125
MRC-5 Cells	0.03	8	0.03	0.06
	HuIFN-α2	HuIFN-β	HuIFN-β × 401	HuIFN-γ
Ohio HeLa Cells	R	R	R	R
WISH Cells	2	2	4	8
MRC-5 Cells	2	2	4	R

R = cell insensitive to IFN.
n.t. = not tested.

Methods we used chequerboard titrations with 2-fold dilutions of drugs starting from 2 MIC (Table 2) and CPE as end-points. Combinations of synthetic compounds showed some evidence of synergy but the synergy between HuIFNs was greater: the greatest synergy was found in combinations of HuIFNs and synthetic compounds and of these the combination of enviroxime and HuIFN-α2 or HuIFN-γ showed the most marked effect. The synergy between synthetic drugs was also shown in plaque reduction assays and Table 3 summarises such results.

TABLE 2

Synergy between antirhinoviral drugs against RV9 (100 $TCID_{50}$/ml) using FIC index[a]

	FIC index of drug combinations					
	HuIFNα-2	HuIFN-β	HuIFN-β × 401	HuIFN-γ	Enviroxime	DCF
HuIFN-β	0.20					
HuIFN-β × 401	0.10	0.13				
HuIFN-γ	0.18	0.12	0.10			
Enviroxime	0.09	0.17	0.18	0.06		
DCF	0.18	0.29	0.18	0.06	0.50	
Ro-09-0410	0.10	0.15	0.13	0.06	0.37	0.50

[a] $\text{FIC index} = \frac{\text{(MIC of drug A in comb.)}}{\text{(MIC of drug A alone)}} + \frac{\text{(MIC of drug B in comb.)}}{\text{(MIC of drug B alone)}}$

The interpretation of the indexes is as follows: FIC index: < 0.5, significant synergism; 0.5–0.9, suggestive of synergism; ≈ 1, effects are additive; 1.1–1.9, indifference or partial antagonism; > 2, antagonism.

TABLE 3

Synergy against RV9 demonstrated by plaque reduction in Ohio HeLa cells

DCF (μg/ml)	Enviroxime (μg/ml)	Chalcone (μg/ml)	No. of plaques	Ratio of plaque formed untreated/treated	Combination index[a]
0	0	0	46	1	N.A.
0.01	0	0	34	1.4	N.A.
0.03	0	0	3	15.3	N.A.
0.06	0	0	0	N.A.	N.A.
0	0	0.01	8	5.8	N.A.
0	0	0.03	0	N.A.	N.A.
0	0	0.06	0	N.A.	N.A.
0.007	0	0.007	0	N.A	N.A.
0.007	0	0.004	7	6.6	1.8
0.004	0	0.007	2	2.3	3.1
0.004	0	0.004	16	2.9	1.0
0	0.06	0	2	2.3	N.A.
0	0.03	0	18	2.6	N.A.
0	0.01	0	36	1.3	N.A.
0.007	0.004	0	0	N.A.	N.A.
0.004	0.004	0	4	11.5	2.4
0.002	0.004	0	12	3.8	1.4
0.007	0.002	0	9	5.1	1.6
0.002	0.002	0	46	1	0
0	0	0.01	8	N.A.	N.A.
0	0	0.03	0	N.A.	N.A.
0	0	0.06	0	N.A.	N.A.
0	0	0.007	44	1.05	N.A.
0	0.015	0.007	0	N.A.	N.A.
0	0.015	0.004	0	N.A.	N.A.
0	0.007	0.007	0	N.A.	N.A.
0	0.007	0.004	6	7.7	2.0

[a] Combination index (C.I.) was calculated as described by Spector et al. (1982), Am. J. Med. 73, suppl. 1A, 36–39, from the equation (Drug 1) (Drug 2)/[(Drug 1 + Drug 2) (VC)]. (Drug 1), (Drug 2), (Drug 1 + Drug 2) and (VC) are yield of treated virus with (Drug 1), (Drug 2) combination of Drug 1 and Drug 2 and untreated virus control respectively. Taking the natural log of the above equation yields the C.I. If C.I. = In 1 = 0 the interaction between the drugs is additive, if C.I. >0 the interaction is synergistic and if C.I. <0 the interaction between drugs is antagonistic.

N.A., not applicable.

The results of the experiments shown in Table 2 are shown in more detail by isobolograms. Fig. 1 is an example of these. The complete results are plotted and synergy is shown by the marked displacement of the curve which reflects the low values of the FIC index. The size of the synergistic effect varied from combination to combination but was usually observed over a wide range of both absolute and relative concentrations of both substances.

We wished to show that this effect was due to a reduction in virus yield rather than to

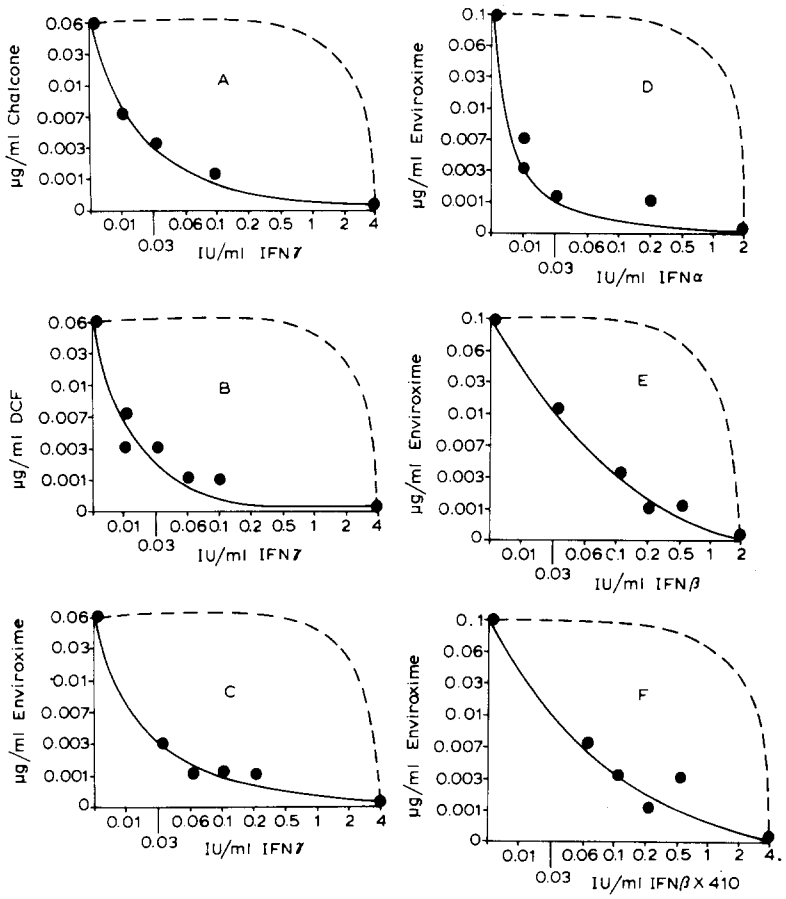

Fig. 1. Synergistic activity between HuIFNs and synthetic antirhinoviral compounds, HuIFN concentrations IU/ml and drug concentrations µg/ml, that inhibit 100 $TCID_{50}$ of RV9 in WISH cells. Broken line shows expected results if drug effects are merely additive. In the usual isobologram employing an arithmetic scale this would be a straight line. The effect however is so large that it is impossible to plot the results clearly without using a logarithmic scale. (A) HuIFN-γ versus chalcone Ro-09-0410. (B) HuIFN-γ versus DCF. (C) HuIFN-γ versus enviroxime. (D) HuIFN-α versus enviroxime. (E) HuIFN-β versus enviroxime. (F) HuIFN-β × 410 versus enviroxime.

an effect on cell survival. We therefore studied the yield of virus in the presence of selected concentrations of HuIFN-γ and enviroxime in WISH cells. This was greatly reduced by both drugs separately as shown in Table 4. However, the amount of antiviral required to produce a given reduction was reduced more than 100-fold when they were combined than when they were used alone.

Since many substances that inhibit influenza viruses in tissue culture failed to inhibit them in organ cultures of ferret trachea [5] it was very important to see if the synergy detected in tissue culture also occurred in organ culture of human respiratory epithelium. Synergy between HuIFN-γ and enviroxime was therefore studied in HENE and HETC. Table 5 summarises the results, which showed that they act synergistically and that again there is a large effect.

TABLE 4

Synergy between HuIFN-γ and enviroxime against RV2 yield from WISH cells

HuIFN-γ (unit/ml)	Enviroxime (μg/ml)	Virus yield (log 10)	Ratio of virus yield untreated/treated	Combination index[a]
0	0	6.5	1	N.A.
8	0	1.5	100 000	N.A.
4	0	4.0	320	N.A.
2	0	4.5	100	N.A.
1	0	5.0	32	N.A.
0.5	0	6.0	3	N.A.
0.25	0	6.5	1	N.A.
0	0.125	1.5	100 000	N.A.
0	0.06	2.0	32 000	N.A.
0	0.03	4.0	320	N.A.
0	0.015	5.0	32	N.A.
0	0.0075	6.5	1	N.A.
0.2	0.0015	1.5	100 000	11.5
0.1	0.0008	1.5	100 000	11.5
0.06	0.004	1.5	100 000	11.5
0.06	0.002	3	3200	8.0
0.06	0.001	5	32	3.4
0.03	0.0004	3.5	1000	6.9
0.03	0.0002	5.5	10	2.3
0.01	0.0001	6	3	1.1
0.007	0.0001	6.5	1	0
0.01	0.00008	6.5	1	0

[a] Combination index was calculated as described in Table 3, except that HuIFN-γ was used instead of drug 1.

N.A., not applicable.

In summary, the MIC of HuIFN-γ is reduced by at least 100-fold in the presence of enviroxime, and the MIC of enviroxime is reduced to a similar extent in the presence of HuIFN-γ, whether they are measured by inhibition of CPE in monolayers, by virus yield in WISH cells or by virus yield in human respiratory epithelium. A similar reduction was seen using HuIFN-α2 and enviroxime.

Discussion

A simple theory of the effect of drug combinations on drug synergy suggests that those drugs with identical modes of action will have an effect which at the most will be additive when used in combination, whereas those with different modes of action could exhibit synergism. It appears that DCF and Ro-09-0410 are similar chemically and act on viral peptide synthesis [4,12] but, in spite of this, some synergy can be detected; possibly this is because they act differently in detail, i.e. their precise mode or

TABLE 5

Synergistic effect of HuIFN-γ and enviroxime on yield of RV2 in human embryo nasal (HEN) or tracheal epithelium (HET) in organ culture

Conc. of drug in medium		$\log_{10}$ virus yield from indicated culture on day									
HuIFN-γ (U/ml)	Enviroxime (μg/ml)	Nasal					Tracheal				
		1	2	3	4	5	1	2	3	4	5
0	0	4.8	6.5	5.1	5.25	5	0	4.5	5.5	4.7	4.6
0.3	0	4.0	6.0	6.1	6.5	–	0	4.5	4.5	5.0	–
2.5	0	3.5	3.75	3.2	4.0	4	0	3	3.2	4.2	4.5
10	0	0	2.5	1.0	3.5	4	0	0.5	1.8	4.2	4.8
40	0	0	0.5	0.5	1.0	1.5	0	0	0.6	2.5	3
160	0	0	0	0	0	0	0	0	0	0	0
0	0.004						0	5.5	4.5	5.7	–
0	0.125						0	0	0.5	1.0	–
0.3	0.004	0	0	0	0	–	0	0	0	0	–
0.15	0.002	0	0	0	0	–	0	0.25	0	0.3	–
0.03	0.002	2	3.7	4.5	5.0	–	0	3.4	4.5	5.0	–

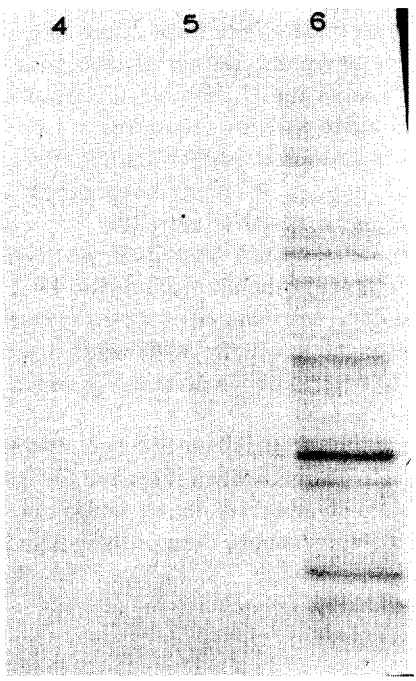

Fig. 2. Autoradiogram of electrophoretically separated ^{35}S-methionine-labelled proteins from WISH cells infected with RV9. Cells were infected at 10 PFU per cell in absence or presence of drugs and analysed by SDS-PAGE. (4) 0.2 μg/ml chalcone Ro-09-0410; (5) 10 IU/ml HuIFN-γ treated cells 18 h before virus inoculation; (6) untreated. Similar suppression of specific protein synthesis was seen in cultures treated with 0.2 μg/ml enviroxime and 0.2 μg/ml of DCF.

site of action is different although the end result is the same; perhaps they act on different parts of the peptide molecules or at different stages of replication. Enviroxime, on the other hand, probably inhibits in a different manner by acting on the viral polymerase and this compound, as might be expected, shows some synergy with the other two synthetic drugs [10,24].

Interferons have complex effects but probably the main antiviral action is to reduce the translation of viral mRNA. It is surprising in some ways that different IFNs are synergistic, but this may be partly because IFN-γ uses different receptors from IFN-α and -β [1,19,22] so that synergy occurs at the receptor rather than the effector level. However we have no ready explanation for synergy between IFN-α and IFN-β.

Whatever the explanation the most marked synergism has been demonstrated with HuIFN both with other IFNs and the synthetic compounds studied. The best synergism was seen with HuIFN-γ and enviroxime; which, incidentally, also has the advantage that enviroxime acts effectively against all serotypes of RV, whereas the other two drugs inhibit only about two thirds of them. It is obviously of great interest to discover how this synergy is mediated, but we think much more work is needed to solve this problem.

We have done some experiments in order to decide whether this synergy might be clinically useful, since synergy between HuIFN and antiherpes drugs may be valuable in the treatment of virus infections of the eye [23]. In the first place, the synergy was seen over a wide range of absolute and relative concentrations of both drugs so it is plausible that such synergy might occur in a patient where the concentrations cannot be controlled strictly. Secondly, in the case of enviroxime and HuIFN we have shown that synergism occurs in human respiratory epithelium in vitro and, therefore, is likely to occur in such cells in vivo as well. Thirdly, there is no evidence that the toxic effects of the compounds on cells are altered by combining them, so, in effect, the therapeutic ratio of each drug is enhanced to the same degree as its antiviral activity.

Although IFN resistant rhinoviruses have not so far been described we have produced and selected many chalcone resistant variants in the laboratory and DCF resistant viruses have also been reported [14]. In theory, combined drug therapy with drugs with different modes of action should reduce the frequency with which such variants develop but further work will be needed to determine whether this is true in practice.

Experiment shows that the intranasal administration of HuIFN-α prevents rhinovirus infections of volunteers and, since enviroxime has already been shown to have a weak effect when given prophylactically or therapeutically, we think that it would be of great interest to look for clinical synergy by giving volunteers both these antivirals and then challenging with a rhinovirus.

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