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A 'new' generation of more potent synthetic antirhinovirus compounds: comparison of their MICs and their synergistic interactions

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Summary

A 'new' generation of synthetic antirhinovirus compounds has recently become available for in vitro evaluation. Thus a new group of compounds from Janssen was found to be 10-fold more active than enviroxime or 57-fold more active than dichloroflavan (DCF), against human rhinovirus 9 (HRV-9). In addition, they were also some 5- and 10-fold more potent than enviroxime and DCF, respectively, against HRV-2. Similarly, a 'new' series of antirhinovirus compounds from Roche, although as active as enviroxime against HRV-9, were found to be 4-fold more potent than DCF against the same virus. Moreover, they were 45- and 90-fold more active than enviroxime and DCF, respectively, when tested against HRV-2. We found that generally HRV-2 was more sensitive to these new compounds than HRV-9.

In this study we also report on the synergistic interaction between these new synthetic substances and also with some of the earlier compounds such as DCF and enviroxime and we discuss the possible implication of this synergistic activity regarding the future prevention and treatment of common colds caused by rhinoviruses.

Antiviral; Rhinovirus; Synergy

Introduction

'Common colds' caused by rhinoviruses are a major cause of morbidity in developed societies (Couch, 1984). Furthermore, rhinoviruses have been implicated

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in the exacerbation of more serious lower respiratory tract symptoms such as bronchitis and asthma (Gregg, 1983). In addition, the virus has been isolated from immunosuppressed infants with lower respiratory tract infections (Krilov et al., 1986).

Most interferons (IFN) have been shown to be effective in preventing experimental rhinovirus infections and more recently IFN-alpha-2 has been shown to prevent naturally occurring rhinovirus infections (Douglas et al., 1986; Hayden et al., 1986; Scott and Tyrrell, 1985). In contrast, although a number of synthetic antirhinovirus compounds have been produced and most were found to be active against rhinoviruses at concentrations of 0.06–0.25 μg/ml, none proved effective in the prevention of experimental or naturally occurring rhinovirus infection (Phillpotts and Tyrrell, 1985).

However, recently a 'new' generation of compounds has become available and we therefore have had the opportunity to study their efficacy in vitro and to compare them with that of some of the earlier substances. In addition we have studied their in vitro interaction to see whether we can demonstrate any synergistic activity. In this paper we report our findings.

Materials and Methods

Compounds

4′,6 Dichloroflavan (BW683C) (DCF) was supplied by the Wellcome Research Laboratories, Beckenham, Kent, U.K. Enviroxime [2-amino-1-(isopropylsulphonyl)-6-benzimidazole-phenyl-ketone-oxime] was prepared and supplied by Eli Lilly Research Laboratories, Indianapolis, U.S.A., while compound R61837 (3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl]-pyridazine) and R60164 (3-Bromo-6-[4-(3-methylphenyl)-1-piperazinyl]-pyridazine) were made available to us by Janssen Pharmaceuticals, Beerse, Belgium. Ro 09-0410 (4′-ethoxy-2′-hydroxy-4,6′-dimethoxy chalcone), Ro 09-0881 (4-ethoxy-2-hydroxy-6-methoxy-N-[4-(methylamino)benzyl]-benzamide), Ro 09-0535 (4-ethoxy-2-hydroxy-6-methoxy-*N*-(*p*-methoxybenzyl)-benzamide) and Ro 09-0696 (2-hydroxy-6-methoxy-*N*-(*p*-methoxybenzyl)-4-[3-methyl-2-butenyl]oxy-benzamide) were supplied by Nippon Roche, Japan, while the compound 89.365 (2-[1-(2,5-dimethylbenzyl)-piperazin-4-yl] = -1,3-thiazol-4-carbonic acid) was submitted to us for evaluation by Sandoz, Austria (Fig. 1). Finally, ribavirin and selenazofurin were a kind gift from Dr. R.K. Robins of the Nucleic Acid Research Institute, U.S.A.

Viruses

Laboratory passaged HRV-9 and 2 were grown in Ohio HeLa cells maintained in Eagle's Basal Medium supplemented with 2% foetal calf serum (FCS). Cultures were harvested when cytopathic effect (CPE) involved most of the cell monolayer. Cells were frozen and thawed 3 times, clarified by centrifugation and the supernatant was titrated to estimate its tissue culture infectivity doses ($TCID_{50}$). Viruses were stored in aliquots at −70°C until used.

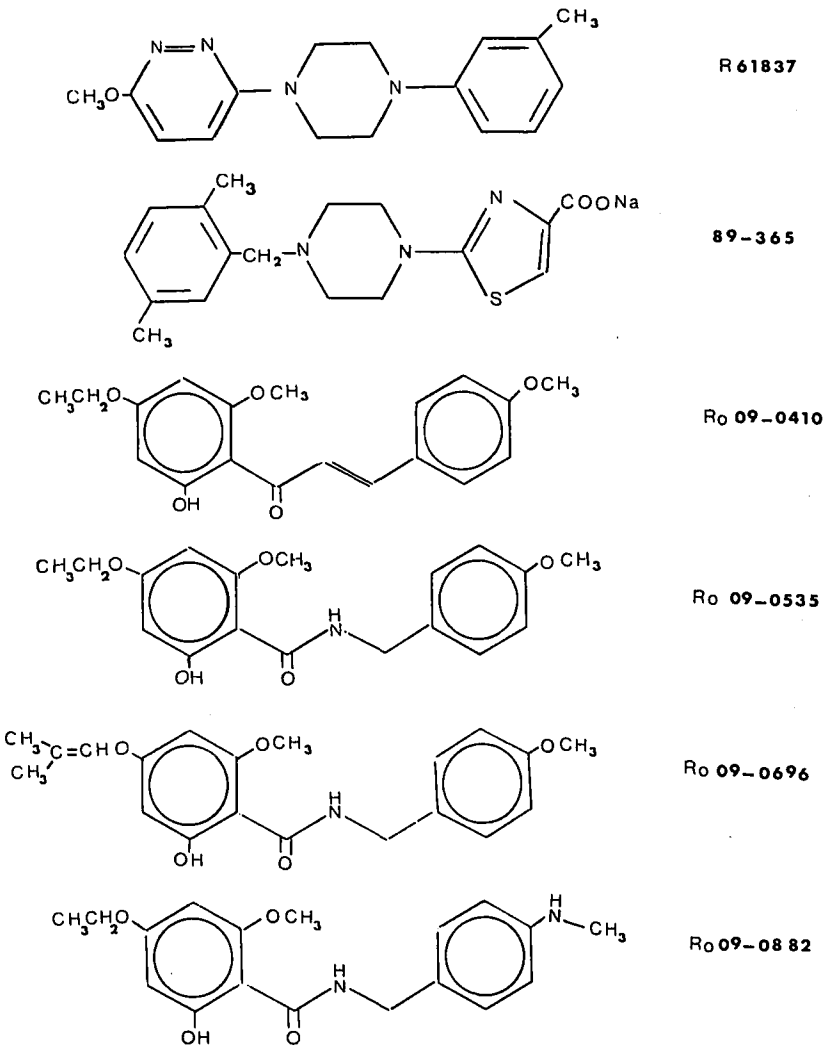

Fig. 1.

Estimation of MIC (variable drug/constant virus)

Fifty microlitres (μl) of Ohio HeLa cell maintenance medium (Eagle's Basal Medium with 2% FCS) were added to all wells of a microtitre or Nunc flat bottomed 96-well tissue culture plate, except cell control wells which received 100 μl. Fifty microlitres of an appropriate starting dilution of each drug (initially dissolved in DMSO, 10 mg/ml) was added to duplicate wells and 2-fold dilutions were made in maintenance medium to cover a wide range of drug concentrations. Fifty microlitres of maintenance medium containing 100 $TCID_{50}$ of virus was added to all wells except cell or drug control wells. Finally, 100 μl of maintenance medium

containing 3×10^4 Ohio HeLa cells was added to all wells. Plates were shaken and incubated in a humidified box at 33°C in the presence of 5% CO_2. Plates were checked daily by light microscopy without staining and read when virus controls (no drug) showed 100% cytopathogenic effect (CPE). The antiviral activity of a compound was defined as the minimal inhibitory concentration (MIC) required to inhibit virus-induced CPE by 50% when 100% of cells were destroyed by the virus in the control virus infected cells monolayer. All the MICs reported in this study were shown to be highly reproducible not just in replicate experiments but also on various occasions during the period of the study.

In a large number of these experiments, the MIC was also determined after cell-fixation in Formal saline and staining with Gentian Violet. However, we consistently found no differences in determining the MIC by either of these 2 methods and therefore we have opted for measurement of MIC by light microscopy reading of CPE without staining.

Cytotoxicity tests

The method was basically the same as that used for the antiviral assay to measure the MIC except that virus was not added. Plates were read 4–6 days after the addition of cells. Cytotoxicity was examined microscopically and defined as the minimal concentration of a drug that altered normal cell morphology in about 50% of the cells in the monolayer, e.g. rounding up, shrinking and detachment of cells when compared with cells from the same batch grown at the same time but not exposed to drug. As for the antiviral assay, in a large number of experiments, plates were also read after fixation and staining with Gentian Violet but again we consistently found no advantage in cell staining and therefore abandoned this method.

Measurement of synergy

The procedure was a modification of the method used for measuring the MIC of single drugs except that Ohio HeLa cells were first grown to confluence in microtitre plates. Medium was removed from the plates and 50 μl of each drug, diluted to the appropriate concentration with Ohio HeLa maintenance media, was added to 1 row of wells on a microtitre plate. Therefore, each dilution of each drug was tested in combination with different concentrations of the other drugs. One hundred microlitres of virus (HRV-9 or 2 at 100 $TCID_{50}$) was then added to the appropriate test wells. Cell control (no drug or virus), drug control (no virus) and virus control (no drug) were included in each plate. Furthermore, using the outer wells of each plate, the MIC of each drug being tested on that plate was established by conducting a 2-fold dilution of the drug and then challenging the cells with 100 $TCID_{50}$ of either HRV-9 or 2. This enabled us to monitor the MIC of the various drugs very closely and exclude results where we considered the variation unsatisfactory, e.g. >4-fold from that normally found for that drug. Plates were read when 100% CPE was seen in the virus control wells (no drug). Synergy was estimated from the Fractional Inhibitory Concentration (FIC) index (Allen et al., 1982) calculated from the following formula.

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

An FIC index of <0.5 was taken to indicate significant synergism; 0.5–0.9 suggestive of synergism; 1.0 effects are additive; 1.1–1.9 indifference or partial antagonism and ≥2.0 indicating antagonism.

All FIC indices reported in this study were shown to be reproducible on at least 2 different occasions. The cytotoxicity of the various drug combinations was also investigated by studying the effect of drug combinations on healthy Ohio HeLa cells in the absence of virus challenge. Cytotoxicity was scored as described earlier for the single drug assay.

Results

MIC and cytotoxicity of antirhinovirus compounds

Table 1 shows the MIC for HRV-9 and 2 and cytotoxicity of the various antirhinovirus compounds when tested in Ohio HeLa cells. This shows that the new series of compounds from Janssen, R61837 and R60164 and the new generation of chalcones from Roche, Ro 09-0881, Ro 09-0535 and Ro 09-0696 were extremely active against both HRV-9 and 2. They were active against HRV-9 and 2 at an MIC in the range of 0.001–0.063 μg/ml. In addition, their cytotoxic concentration for Ohio HeLa cells was usually in excess of 25 μg/ml. Thus they have a high in vitro therapeutic index. They were clearly more active than the earlier compounds such as DCF, enviroxime or the chalcone Ro 09-0410. Thus, the new Janssen com-

TABLE 1

MICs and cytotoxicity of various antirhinovirus compounds in Ohio HeLa cells.

Compound	MIC (μg/ml) against		Cytotoxicity (μg/ml)
	HRV-9	HRV-2	
1) BW683C (DCF)	0.25	0.12 – 0.25	12.5
2) Enviroxime	0.03 – 0.06	0.06 – 0.12	6.25 – 12.5
3) 89.365	> 1.0	0.5	≥ 25
4) R61837	0.005	0.032	> 50
5) R60164	0.0037	0.006	12.5 – 25
6) Ro 09-0881	0.063	0.002	≥ 25
7) Ro 09-0535	0.063	0.002	≥ 25
8) Ro 09-0696	0.063	0.001 – 0.002	≥ 25
9) Ro 09-0410	0.13	0.0078	≥ 6.25
10) Ribavirin	> 8.0	N.T.	12.5 – 25
11) Selenazofurin	> 4.0	N.T.	3.125 – 6.25

Cytotoxicity was scored as morphological alteration, e.g. rounding up, shrinking, detachment, in about 50% of the cells.

Potency was scored as 50% cell protection when challenged with 100 $TCID_{50}$ of HRV-9 or 2.

TABLE 2

Synergy between antirhinovirus compounds against HRV-9 (100 $TCID_{50}$/ml) expressed as FIC* index.

Compound	Sandoz 89.365	BW 683C	Ro 09-0410	Ro 09-0535	Ro 09-0696	Ro 09-0881	Enviroxime
Sandoz 89.365	–	–	–	–	–	–	–
BW683C (DCF)	0.25	–	–	–	–	–	–
Ro 09-0410	0.38	0.28	–	–	–	–	–
Ro 09-0535	0.50	0.26	0.30	–	–	–	–
Ro 09-0696	0.50	0.50	0.30	0.25	–	–	–
Ro 09-0881	0.26	0.27	0.28	0.37	0.37	–	–
Enviroxime	1.0	0.10	0.18	0.37	0.75	0.28	–
Ribavirin	1.25	1.0	0.60	0.53	0.88	0.75	1.25
Selenazofurin	>2.0	>2.0	0.79	1.0	0.28	0.53	1.0

*FIC index: <0.5 Significant synergism; 0.5–0.9 Suggestive of synergism; ~1.0 Effects are additive; 1.1–1.9 Indifference or partial antagonism; >2.0 Antagonism.

pounds were approximately 10- and 57-fold more active against HRV-9 than enviroxime and DCF, respectively. In addition, they were 5- and 10-fold more potent than enviroxime or DCF, respectively, against HRV-2. Moreover, the new series of chalcones, although as active as enviroxime against HRV-9 were 4-fold more active than DCF when tested against HRV-9. Furthermore, they were some 45- and 90-fold more potent than enviroxime and DCF when tested against HRV-2. Generally, HRV-2 appeared to be more sensitive to these new antivirals than HRV-9. Both ribavirin and selenazofurin had no effect on these rhinoviruses while the Sandoz compound 89.365 was only moderately active.

Synergy between various antirhinovirus compounds

Tables 2 and 3 show the FIC index when these synthetic antirhinovirus compounds (with the exception of the Janssen compounds) were tested in combination. The tables show that synergistic effects occurred with many of these combi-

TABLE 3

Synergy between antirhinovirus compounds against HRV-2 (100 $TCID_{50}$/ml) expressed as FIC* index.

Compound	Sandoz 89.365	BW 683C	Ro 09-0410	Ro 09-0535	Ro 09-0696	Ro 09-0881	Enviroxime
Sandoz 89.365	–	–	–	–	–	–	–
BW683C (DCF)	0.38	–	–	–	–	–	–
Ro 09-0410	0.31	0.19	–	–	–	–	–
Ro 09-0535	0.75	0.55	0.50	–	–	–	–
Ro 09-0696	0.50	0.75	0.50	0.15	–	–	–
Ro 09-0881	0.38	0.28	0.29	0.20	0.35	–	–
Enviroxime	0.36	0.38	0.25	1.0	1.0	0.57	–
Ribavirin	>2.0	>2.0	>2.0	0.75	>2.0	>2.0	>2.0
Selenazofurin	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0

*FIC index: <0.5 Significant synergism; 0.5–0.9 Suggestive of synergism; ~1.0 Effects are additive; 1.1–1.9 Indifference or partial antagonism; >2.0 Antagonism.

nations. The strongest and most consistent synergistic effects were seen with the chalcones Ro 09-0410, Ro 09-0535, Ro 09-0696 and Ro 09-0881. They not only demonstrated significant or suggestive synergism with compounds of other series such as 89.365 and BW683C (DCF) but also significant synergism with each other. This phenomenon was generally seen for both HRV-9 and HRV-2. The MICs of the compounds in combination were some 4- to 64-fold lower than those of the compounds alone. Both 89.365 and BW683C (DCF) also produced consistent synergistic effects with other antirhinovirus compounds against both HRV-9 and 2. The 89.365 did not interact synergistically with enviroxime against HRV-9 although it did so when tested against HRV-2. Interestingly, enviroxime was not consistent in producing synergistic effects with other antivirals and this was observed using both HRV-9 and 2.

Ribavirin and selenazofurin generally produced indifferent or antagonistic effects when combined with the majority of the antirhinovirus agents, although some synergistic effect was seen between ribavirin and Ro 09-0535 and between selenazofurin and Ro 09-0696 or Ro 09-0881 against HRV-9.

Discussion

In this paper we report that a number of new synthetic antirhinovirus compounds have been produced and that they have MICs in the range of 0.001–0.063 μg/ml. They are about 1- to 10- and 4- to 68-fold more active against HRV-9 than enviroxime and DCF, respectively. Furthermore, they are 3- to 45- and 36- to 90-fold more active against HRV-2 than enviroxime and DCF, respectively. In addition, the newer Roche compounds Ro 09-0881, Ro 09-0535 and Ro 09-0696 were found to be about 4-fold more active against HRV-2 than the earlier chalcone Ro 09-0410. However, it must be noted that our study was limited to only HRV-9 and 2 since these are the 2 most commonly used serotypes in human volunteer experiments at the Common Cold Unit. Hence, a compound such as 89.365, although not found to be active against HRV-9 and 2, is in fact very active against many other rhinovirus serotypes (Dr. B. Rosenwirth, personal communication). Therefore, clearly further development had led to the synthesis and identification of compounds with much increased antirhinovirus activity. However, it remains to be seen whether any of these new compounds will prove more active in vivo in the prevention of rhinovirus infection and/or illness in man than the earlier compounds, enviroxime, DCF or Ro 09-0415 which were not found to be effective (Phillpotts and Tyrrell, 1985). Therefore, provided they can be suitably formulated they probably merit trial as intranasal spray against experimental rhinovirus infections in volunteers. Our results suggest that additional potency could be achieved by administering them as synergistic mixtures, as has already been suggested for combination of interferons and synthetic antivirals (Ahmad and Tyrrell, 1986). However, it is our experience that greater potency alone may not mean that compounds will prove effective in vivo. In our opinion we also need to develop more effective methods to deliver these drugs to the target tissues, the nasal epi-

thelium at effective antiviral concentrations and to ensure that such concentrations are maintained despite continuous clearance by the mucociliary mechanism. This might be achieved if these compounds or some modification can be given safely by mouth and are both absorbed and then reach the nasal mucosa at effective inhibitory concentrations. Indeed, recent studies with rimantadine and a new anti-influenza compound, ICI 130,685 suggest that it is possible to produce molecules that although administered orally produce drug concentrations in nasal secretions that are 2- and 4- to 7-fold higher than that in the blood, respectively (Al-Nakib et al., 1986; Hayden et al., 1985; Hayden and Monto, 1986). Indeed, recent clinical evaluation showed that both compounds were very effective in preventing and treating an influenza A virus infection in man (Al-Nakib et al., 1986; Hayden and Monto, 1986).

The mechanism of action of dichloroflavan, 89.365, the Janssen and Roche compounds is thought to be similar to that recently elucidated for the Sterling-Winthrop compounds WIN51711 and WIN52084 (Smith et al., 1986). Using detailed X-ray crystallography studies, Smith et al. (1986) showed that these Winthrop compounds interact directly with the viral capsid protein VP1 in the 'Canyon' and inhibit viral uncoating by preventing the collapse of VP1 hydrophobic pocket or by blocking the flow of ions in the virus interior (Smith et al., 1986).

In contrast, enviroxime is thought to interfere with rhinovirus replication by affecting the synthesis or action of viral RNA (Swallow and Kampfner, 1985). However, in the absence of detailed studies on the exact mechanisms of action of any of these new synthetic antivirals, it would be difficult to speculate as to how each of these antivirals interacts with rhinoviruses or how any combination of these compounds may interact synergistically. Therefore, it would be interesting and perhaps important to conduct further studies, this time on the mechanism by which these drugs interact synergistically when in combination.

Nevertheless, the data of the present study confirm our earlier observations on the synergy between interferon and synthetic antivirals (Ahmad and Tyrrell, 1986) that synergy against rhinoviruses often occurs between antivirals, although the phenomenon is by no means universal and varies a great deal quantitatively.

We see some possible further implications of this phenomenon of synergy between antirhinovirus agents as applied to the prevention and/or treatment of the common cold. For example, apart from enviroxime the compounds examined were only active against certain serotypes of rhinoviruses. It is possible that by combining drugs it will be possible to achieve a clinical effect against even the relatively resistant serotypes. On the other hand, it may be possible to obtain clinical effects with lower concentrations and smaller total doses of individual drugs than would be required if they were used alone – this would reduce the cost. In vitro tests indicated that cytotoxicity is not increased in mixtures that show antirhinovirus synergy (data not shown). If reflected in vivo this suggests that such mixtures would show increased safety and an enhanced therapeutic ratio. Finally rhinoviruses may become resistant to, or even dependent upon some of these compounds (e.g. chalcone) and it is likely that such resistance would be less likely if synergistic mixtures were used, but this has yet to be shown. If it is, it could be another benefit of using mixed drugs.

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