Antiviral Research, 10 (1988) 117–127
Elsevier

AVR 00313

Effect of isoflavans and isoflavenes on rhinovirus 1B and its replication in HeLa cells

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(Received 3 May 1988; accepted 5 August 1988)

Summary

The effect of newly synthesized halogenated isoflavans and isoflavenes on human rhinovirus 1B (HRV 1B) infection of HeLa cells has been examined. Both series of drugs inhibited virus plaque formation in cell cultures, isoflavans being more effective than isoflavenes. Cells pretreated with compounds before challenge with HRV 1B became resistant to the virus-induced cytopathic effect. The antiviral state induced by the most active compounds persisted for at least 10 h and did not appear to be mediated by interferon production. Experiments whereby the compounds were added at varying times indicated that the isoflavans and isoflavenes interfere with early events of virus replication without affecting virus binding to the cell membrane. In addition to their effects on virus multiplication, the isoflavans were also found to have a direct action on the virus. The inhibitory effect on virus infectivity required extraction with chloroform for reversal. Isoflavans also protected the virions against mild acid or heat inactivation.

Rhinovirus 1B; Isoflavan; Isoflavene

Introduction

Because of the existence of about 100 serotypes of *Rhinovirus* (Melnick, 1980), development of a conventional vaccine is not practical; therefore attention has been

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addressed to chemical agents capable of interfering with viral replication (Korant et al., 1984).

Bauer et al. (1981) described a potent inhibitor of rhinovirus 1B (HRV 1B) infection: 4′,6-dichloroflavan was found to inhibit HRV 1B plaque formation at a 50% inhibitory concentration as low as 0.007 μM. The compound was shown to bind to the viral protein coat of sensitive serotypes and to stabilize the virion against the conformational changes induced by low pH (4.6 to 5.0) or heat (56°C) treatment (Tisdale and Selway, 1984). In infected cells it reduced the formation of subviral particles during virus eclipse and delayed the rate of viral uncoating (Tisdale and Selway, 1984).

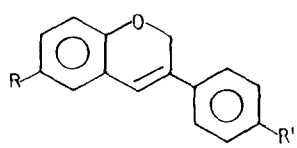
In an earlier study (Burali et al., 1987), we described the synthesis and the in vitro anti-rhinovirus activity of two new series of compounds, halogenated isoflavans and 3(2H)-isoflavenes, related to 4′,6-dichloroflavan. The antiviral concentrations of these drugs were found to be 50-250 times lower than those giving the 50% cytotoxic endpoints on HeLa cells. The inhibitory effect on rhinovirus infection was found to be favored by a non planar molecular structure, as in isoflavans, and enhanced by chlorine substitution at positions 4 and/or 6.

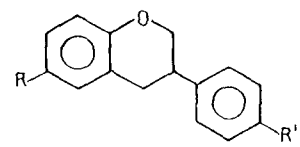
The present study was undertaken to examine further the antiviral efficacy of halogenated isoflavans and isoflavenes towards HRV 1B infection in cell cultures by performing analysis of the effects of the compounds on (a) virus multiplication, (b) cell susceptibility to infection and (c) virus infectivity.

Materials and Methods

Compounds

Compounds were synthesized as described elsewhere (Burali et al., 1987) and their structural formulae are presented in Fig. 1. Stock solutions of 1 mg/ml in ab-





Compound	R	R'
3a	H	H
3b	Cl	H
3c	H	Cl
3d	Cl	Cl
3e	H	Br
3f	Br	Br
3g	Cl	Br

Compound	R	R'
4a	H	H
4b	Cl	H
4c	H	Cl
4d	Cl	Cl

Fig. 1. Structural formulae of 3(2H)-isoflavenes (*3*) and isoflavans (*4*).

solute ethanol were diluted initially in phosphate-buffered saline (PBS), pH 7.4, and subsequently in tissue culture medium.

Cells and virus

HeLa (Mandel) cells were grown in monolayers at 37°C in Eagle's minimum essential medium supplemented with 10% fetal calf serum, antibiotics and glutamine (growth medium). For cell maintenance or for virus growth studies the serum concentration was lowered to 2% (maintenance medium).

Rhinovirus 1B was propagated in HeLa cells at 33°C at an input multiplicity of 1 PFU/cell. When an extensive cytopathic effect was recorded, infected cells were freeze-thawed three times and cellular debris was removed by low speed centrifugation (700 × *g*, 30 min). HRV 1B and HeLa cells were originally supplied by Dr. J.W.T. Selway, Wellcome Research Laboratories, U.K.

Plaque assay

Plaque assay was performed on monolayers of HeLa cells as previously described (Burali et al., 1987).

Determination of cytotoxic activity in uninfected cells

Cytotoxicity was monitored by determining the effect of different concentrations of each compound on cellular RNA and protein synthesis. HeLa cells (3×10^5 cells/well) in 24-well plates were incubated with compounds for 24 h at 37°C, washed and then labeled (2 h, 37°C) with 5 μCi/ml [5-^{3}H]uridine (Amersham International, 27 Ci/mmol) or 7 μCi/ml [^{35}S]methionine (Amersham International, 800 Ci/mmol), respectively. For the [^{35}S]methionine incorporation study, cells were incubated for 2 h in methionine-free medium before the labeling period. The medium was then discarded, the cells were washed three times with PBS and successively lysed with 1% SDS in 10 mM Tris-HCl, pH 7.4. After precipitation with 10% trichloroacetic acid in the cold, samples were filtered and the filters were added to 5 ml of Filtercount (United Technologies Packard) for scintillation counting. In parallel cultures, cytotoxicity was determined after 3 days of incubation at 33°C in the presence of compounds by (i) microscopic examination of cell morphology and (ii) counting the cell number in treated and untreated wells.

Determination of the antiviral effect by plaque reduction

To evaluate the effect of compounds on virus plaque formation, two-fold dilutions of drugs were incorporated into the agar overlay medium of infected cells and maintained until plaques developed (72 h, 33°C).

Determination of the antiviral effect after preincubation of cells with compounds

To evaluate the protective effect of drugs on cell cultures against virus-induced cytopathic effect (CPE), confluent monolayers were preincubated for 2 or 4 h at 37°C with different concentrations of the compounds in growth medium. After washing three times with PBS, the cells were infected with HRV 1B in order to obtain in the control cultures a complete (100%) CPE after two days of incubation at 33°C.

To investigate the reversibility of the protective effect, HeLa cells were incubated with the test compounds (10 μM) for 24 h at 37°C. After washing three times with PBS, the cells were incubated at 37°C in growth medium (free of compounds) for 2, 4, 6, 8, and 10 h. After a second set of washings, monolayers were infected with virus as described above. Tissue culture media of drug-treated cells were assayed for interferon-beta (IFN-β) production in WISH cells, as described previously (Antonelli et al., 1985). In some experiments, HeLa cells were cultured for 24 h at 37°C in the presence of compounds (10 μM) and 50 neutralizing units/ml (U/ml) of sheep anti-human IFN-β antibodies. The cells were then infected with HRV 1B (1 h, 33°C) and successively incubated in the presence of 50 U/ml of anti-human IFN-β antisera. Forty-eight hours later, the cell cultures were examined for the appearance of virus-induced CPE.

Effects of different times of addition of compounds on virus yield following a single growth cycle

HeLa cell monolayers in 24-well plates were infected with HRV 1B at a multiplicity of infection of 10. Virus adsorption was carried out at 4°C for 1 h to synchronize the infection process. After washing out the viral inoculum, 1 ml of maintenance medium was added and the temperature shifted to 33°C. Drugs (final concentration: 0.5 μM) were added to the culture medium immediately or at different times after the viral binding step and the cells were further incubated for 8 h. Cells were then freeze-thawed three times, extracted with chloroform to remove the antiviral agents and titrated for total virus yield by plaque assay.

Direct inactivation of virus

A suspension of HRV 1B (6×10^6 PFU/ml) virions was exposed to compounds (final concentration 0.5 μM) for 60 min at 37°C (pH 7.2). Ten-fold dilution series of the mixtures were prepared and titrated by plaque assay. As the compounds were diluted out, they did not directly interfere with virus titration. Statistical analysis was carried out using the Student's *t*-test.

Reversal of the inhibitory effect on virus

Extraction of the antiviral agents bound to HRV was carried out at room temperature by adding an equal volume of chloroform and shaking for 1 min. After centrifugation at $700 \times g$ for 5 min, the aqueous supernatants were collected and titrated by plaque assay.

Effect of compounds on mild acid inactivation of virus

HRV 1B (6×10^6 PFU/ml) was incubated with or without compounds (final concentration 2 μM) for 1 h at 37°C (pH 7.2). The pH of the mixtures was then adjusted to 5.0 by adding 0.3 vol of 0.2 M acetate buffer (pH 5.0). After incubation at 37°C for 30 min the pH was brought back to neutrality by addition of 0.3 vol of 1 M Hepes buffer. After extraction of compounds with chloroform, samples were diluted and titrated for infectivity by plaque assay.

Effect of compounds on heat inactivation of virus

HRV 1B (6×10^6 PFU/ml) with or without compounds (final concentration 2 μM) was incubated for 20 min at 56°C (pH 7.2). After cooling on ice to halt the reaction, samples were extracted with chloroform, diluted and titrated in HeLa cells by plaque assay.

Results

Effect of isoflavans and isoflavenes on virus plaque formation

The activity of each compound on HRV 1B replication in HeLa cell cultures was initially studied by examining their ability to interfere with virus plaque formation. Fig. 2 shows that all compounds exhibited a dose-dependent inhibitory effect on virus multiplication when added to the medium after virus adsorption. Isoflavans were found to be more active, by weight, than the corresponding isoflavenes. At the concentrations tested, the compounds did not cause any apparent alteration in cell viability, morphology and growth during a 72 h exposure period (data not shown).

Effect of isoflavans and isoflavenes on cell susceptibility to infection

In order to verify whether isoflavans and isoflavenes could protect cell cultures from the viral infection, HeLa cells were exposed to various concentrations of each compound for 2 or 4 h at 37°C before challenge with HRV 1B. Pretreatment of

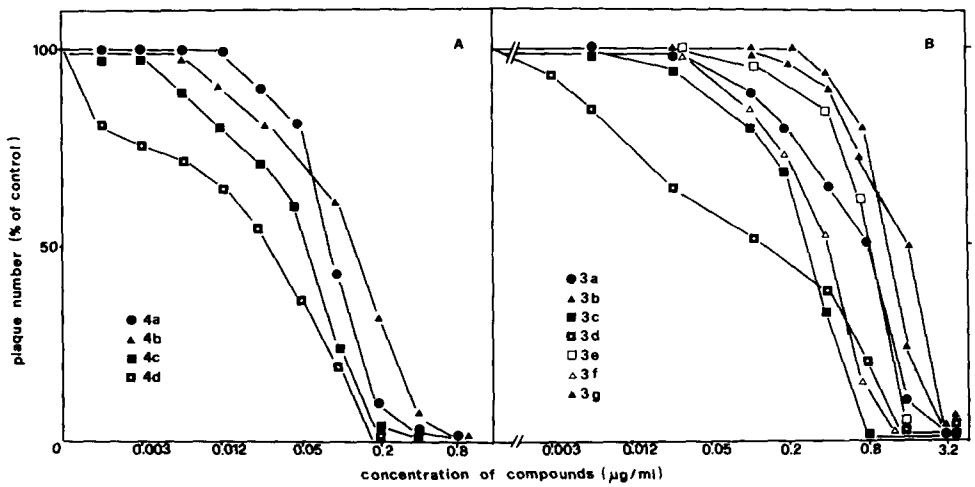


Fig. 2. Effect of increasing concentrations of isoflavans (A) and isoflavenes (B) on plaque formation by HRV 1B in HeLa cell cultures. Two-fold dilutions of each compound were incorporated into the agar overlay medium of infected cells. Plaques were counted 72 h after infection. The values represent the mean of triplicate vials for each point. The number of plaques counted in untreated infected control cultures was about 80.

cells with compounds at 0.5 μM did not modify their susceptibility to infection. At 5 μM, cells treated with isoflavans and some isoflavenes became partially resistant (approximately 50% reduction in virus CPE) to subsequent infection by the virus. A complete protection was observed with the same compounds at 10 μM (Table 1), a concentration which had no effect on either RNA or protein synthesis or vesicular stomatitis virus infection in HeLa cell cultures (data not shown).

To evaluate the reversibility of the antiviral state, cells were exposed to the test compounds (10 μM) for 24 h, washed and successively incubated in growth medium (free from drugs) for different lengths of time (2, 4, 6, 8 and 10 h) before challenge with virus. Data reported in Table 2 show that only the cells treated with 4′-chloroisoflavan (*4c*) and 4′,6-dichloroisoflavan (*4d*) retained a distinct resistance to the viral infection for up to 10 h after their removal.

Attempts were made to clarify the mechanism(s) responsible for the establishment of the antiviral state. To verify the possibility that the antiviral process triggered within the cells was mediated by IFN, we looked for the presence of IFN in culture media of cells incubated (a) 24 h with compounds and (b) 24 h with compounds, followed by drug-free medium for 2–10 h. In none of the samples was any amount of IFN (< 5 IU/ml) detected.

To eliminate the possibility that undetectable levels of IFN (less than 5 IU/ml) could be responsible for the resistance state to HRV 1B infection, a second set of experiments was carried out in which HeLa cells were incubated (24 h, 37°C) in the presence of active compounds (10 μM) and antiserum against human IFN-β (50 U/ml) before challenge with HRV 1B. After virus infection, cells were incubated in medium containing 50 U/ml of anti-human IFN-β antibodies until the appearance of viral CPE in control infected cultures (48 h, 33°C). Even under these conditions the protection conferred on the cell cultures by the compounds was not

TABLE 1

Resistance to HRV 1B infection induced in HeLa cell cultures by pretreatment with isoflavenes (*3*) and isoflavans (*4*)

Compound (10 μM)	Hours of treatment	
	2	4
None	–	–
3a	++	++
3b	+	++
3c	++	++
3d	–	+
3e	–	–
3f	+	++
3g	–	–
4a	++	++
4b	+	++
4c	++	++
4d	++	++

The virus-induced cytopathic effect was 100% in control cultures 48 h after infection. Results are expressed as: ++, complete protection; +, 50% protection; –, absence of protection.

TABLE 2

Persistence of the antiviral state towards HRV 1B infection induced by isoflavenes (*3*) and isoflavans (*4*)

Compound (10 μM)	Recovery time (h)				
	2	4	6	8	10
None	−	−	−	−	−
3a	−	−	−	−	−
3b	−	−	−	−	−
3c	+	+	−	−	−
3d	−	−	−	−	−
3e	−	−	−	−	−
3f	+	+	−	−	−
3g	−	−	−	−	−
4a	−	−	−	−	−
4b	+	+	−	−	−
4c	++	++	++	++	++
4d	++	++	++	++	++

HeLa cells were pretreated with compounds for 24 h at 37°C. After washing off the drugs, cells were incubated in growth medium for different times before challenge with HRV 1B. For explanation of symbols see footnote to Table 1.

overcome by the presence of anti-IFN-β antisera (data not shown). Moreover, HeLa cells incubated for 24 h at 37°C with 3–30 U/ml of IFN-β before addition of virus were as susceptible to infection as untreated cells (data not shown).

Effect of isoflavans on the different stages of HRV 1B growth cycle

On the basis of the results reported above, isoflavans, i.e. the most active compounds, were utilized in one-step multiplication cycle experiments in order to determine which stage of virus replication was affected by the compounds.

To study the effect of the compounds on the virus binding step, HeLa cells were exposed simultaneously to virus and drugs (final concentrations 0.5 μM) in the cold, in order to prevent virus internalization. After washing out the inoculum, the cells were overlaid with medium and incubated at 33°C until plaques developed. Under these conditions, the presence of compounds did not modify the virus plaque number (data not shown). In control experiments we ascertained that HeLa cells incubated for 1 h at 4°C with compounds before virus infection (1 h, 4°C) showed the same susceptibility to infection as untreated cells (data not shown).

The effect of the addition of compounds to HeLa cells at various times after infection is shown in Fig. 3. When compounds were added immediately or within 30 min post infection (p.i.), virus growth was inhibited by 40 to 65%. Addition of 3(2H)-isoflavan (*4a*) and 4′-chloroisoflavan (*4c*) at 1 h p.i. was still effective, whereas an apparent loss of activity was observed if the compounds were added at about 2 h p.i. These results indicated that isoflavans interfere with an early stage of the virus replicative cycle.

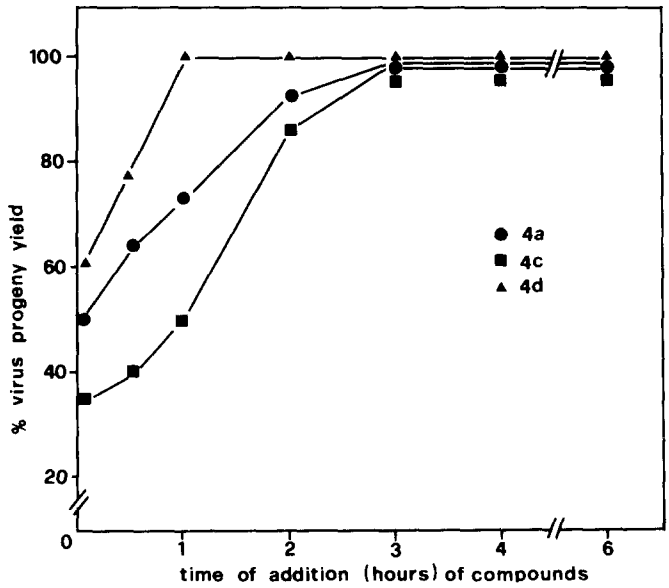


Fig. 3. Effect on virus yield of time of addition of isoflavans to HRV 1B-infected cells. After virus attachment for 1 h at 4°C (0 time), cells were shifted to 33°C and drugs (final concentration 0.5 μM) were added at different times. Cell cultures were incubated for 8 h. After three cycles of freezing and thawing, samples were centrifuged to remove cell debris, extracted with chloroform and titrated by plaque assay. Virus yield from untreated infected cells was 2×10^4 PFU/ml.

Effect of isoflavans on virus infectivity

To investigate a possible direct virucidal effect of compounds, HRV 1B was exposed to drugs for 1 h at 37°C (pH 7.2), diluted ten-fold serially and titrated by plaque assay on HeLa cell monolayers. All isoflavans effected a slight reduction (about 0.4 $\log_{10}$) of viral infectivity ($P < 0.05$). The reduction of the virus infectious titer was found to be dependent on the incubation temperature since no effect could be observed when the mixtures of virus and drugs were incubated at 4°C before inoculation on the cell cultures.

The infectivity of HRV 1B, reduced by exposure to isoflavans, was completely restored to the original value after extraction of drugs with chloroform at room temperature. The reversal of the inhibition could not be observed if the chloroform treatment was carried out at low temperature (data not shown).

Effect of compounds on mild acid or heat inactivation of HRV 1B

Since the inactivation of virus infectivity by the test compounds seemed to be related to binding of the compounds to the virions, it was of interest to study the behavior of compound-treated HRV 1B virions with respect to mild acid or heat inactivation. For this purpose, HRV 1B virus suspensions treated with or without isoflavans were incubated at pH 5 (30 min, 37°C) or at 56°C (20 min, pH 7.2). The virus titer was assayed after extraction of the drugs with chloroform. The results

obtained showed that the infectivity of HRV 1B decreased significantly ($P < 0.05$) after both treatments (1.9 $\log_{10}$ for pH 5 treatment and 4.7 $\log_{10}$ for heat treatment). However, in the presence of isoflavan (*4a*), 6-chloroisoflavan (*4b*), 4′-chloroisoflavan (*4c*) and 4′,6-dichloroisoflavan (*4d*) the drop in infectivity was only 0.1, 0.3, 0.1 and 0 $\log_{10}$ for pH 5 treatment ($P < 0.05$) and 2.1, 3.8, 2.0 and 2.7 $\log_{10}$ for heat treatment ($P < 0.05$), suggested that isoflavans (with the exception of *4b* for thermal inactivation) exerted a stabilizing effect on HRV 1B infectivity.

Discussion

The results presented here provide evidence that the newly synthesized halogenated isoflavans and isoflavenes inhibited HRV 1B infection in HeLa cells.

The data of experiments in which drugs were added to cells at different times of the viral growth cycle suggest that compounds interfered with early stages of viral infection without affecting the viral binding step to the cell membrane. In this respect isoflavans appeared to have a behavior similar to that of 4′,6-dichloroflavan which was also shown to inhibit rhinovirus infection at an early stage (Tisdale and Selway, 1983).

Although our compounds were found to possess only a modest activity (40–65% reduction in virus yield) against HRV 1B infection, as compared to 4′,6-dichloroflavan at the same concentration (Tisdale and Selway, 1983), we considered them worthy of further investigation because they were about ten times less inhibitory to HeLa cell growth (data not shown) and, moreover, they were also found to be active against poliovirus infection (Conti et al., 1987).

An interesting property of some of the compounds was their ability to protect pretreated cells from the subsequent challenge by HRV 1B. In the case of isoflavans the resistance towards the virus infection was not reversed after the drugs were removed, the cells shifted to reagent-free medium and further incubated for up to 10 h before inoculation with HRV 1B. The induction of the antiviral state did not appear to be the consequence of IFN production. In fact, IFN was not detected in cell culture media of drug-treated cells, and IFN-β treatment of cells was not able to protect them against virus infection. Moreover, the presence of anti-IFN-β antibodies during the period that the cells were treated with compounds and during the post-infection period failed to prevent the establishment of the antiviral state. Therefore, this protection appears more likely to be due to the ability of residual compound, not removed by washing, to insert into the cell membrane because of their lipophilic nature. This has also been suggested by Tisdale and Selway (1983) for 4′,6-dichloroflavan. An alternative explanation could be the intracellular accumulation of drugs.

In addition to their effect on virus multiplication in cell cultures, the compounds were also able to directly inactivate HRV 1B. The infectious titer of virus was restored to the original value after extraction of the agents with chloroform. The latter finding indicates that no irreversible damage of virus particle took place and that inactivation may be due to the binding of compounds to some specific site(s)

of the rhinovirus capsid. Moreover, these data seem also to suggest that the inhibitory effect of compounds on virus replication is most probably secondary to their direct interaction with the virus capsid proteins.

Instability of infectivity at mildly acidic pH (below 6) or at 56°C is a common characteristic of rhinoviruses (Stott and Killington, 1972; Newman et al., 1973). Both treatments are known to modify the virion size, conformation and native antigenicity (Korant et al., 1972, 1975; Lonberg-Holm and Yin, 1973), similarly to changes occurring during the viral uncoating (Lonberg-Holm and Korant, 1972). The presence of isoflavans decreased the susceptibility of HRV 1B to inactivation by heat or low pH, indicating a stabilizing action on virion structure. An analogous effect on picornaviruses has been suggested for various other compounds such as SDS (Lonberg-Holm and Nobel-Harvey, 1973), arildone (McSharry et al., 1979; Caliguiri et al., 1980), Ro 09-0410 (Ishitsuka et al., 1982) and 4′,6-dichloroflavan (Tisdale and Selway, 1984).

In conclusion, the stabilizing effect on virion capsid conformation shown by our compounds and the finding that they act at early stages of infection point towards the involvement of viral uncoating as the most likely target for HRV inhibition, but this contention needs to be supported by further research.

Acknowledgements

We wish to thank Professor F. Dianzani for helpful suggestions towards improvement of the manuscript and for supplying human IFN-β and sheep antihuman IFN-β antibodies and Dr. G. Antonelli for interferon assay. The skilful technical assistance of Mr. R. Sampalmieri is also acknowledged. This research was supported by a grant from Istituto Pasteur-Fondazione Cenci Bolognetti.

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