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Effect of isoflavans and isoflavenes on the infection of Frp/3 cells by hepatitis A virus

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Summary

The effect of 6,4′-dichloroflavan and of isoflavan and isoflavene derivatives on hepatitis A virus (HAV) infection in a monkey cell line (Frp/3 cells) was studied. These compounds were not virucidal and had no measurable effect on the adsorption of virus to the cells at 0°C, whereas they exerted an inhibitory effect on viral antigen synthesis when incubated with the infected cells during HAV multiplication. Among the substances tested, 6,4′-dichloroflavan and 6,4′-dichloroisoflavan showed the highest activity. These compounds are postulated to interact with an early stage (penetration and/or uncoating) of HAV infection.

Hepatitis A virus; Isoflavan; Isoflavene; 6,4′-Dichloroflavan

Introduction

Progress in the knowledge of picornavirus replication made it possible to identify putative chemotherapeutic antiviral agents which specifically interfere with viral functions. Based on this premise several drugs have been selected which exhibit an inhibitory activity on picornavirus uncoating (Diana et al., 1985). Compounds related to arildone (McSharry et al., 1979; Fox et al., 1986), natural and synthetic 3-methoxyflavones (Van Hoof et al., 1984; De Meyer et al., 1988), 4′,6-dichlo-

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roflavan and analogues (Bauer et al., 1981; Ishitsuka et al., 1982; Tisdale and Selway, 1983; Ninomiya et al., 1984) and halogen-substituted isoflavans and isoflavenes (Burali et al., 1987; Conti et al., 1987) were found to be effective against several rhinoviruses and/or enteroviruses. However, no promising candidate inhibitors have so far emerged for the treatment of hepatitis A infection (Widell et al., 1986; Biziagos et al., 1987). Hepatitis A virus (HAV), classified as an enterovirus within the family Picornaviridae (Gust et al., 1983), enters susceptible cells by a mechanism related to receptor-mediated endocytosis followed by endosomal and/or lysosomal uncoating (Superti et al., 1987). In previous reports the effect of different lipophilic amines (Superti et al., 1987) and of an ionophore (Superti et al., 1989) on the early stages of HAV infection has been demonstrated; these compounds probably exert their effect by impeding viral RNA delivery in the cytosol. The aim of the present study was to investigate the effect of different isoflavan and isoflavene derivatives related to 6,4′-dichloroflavan (Bauer et al., 1981) on HAV multiplication in a monkey cell line (Frp/3) infected by a fast growing strain of HAV (Venuti et al., 1985).

Materials and Methods

Test substances

The activity of 6,4′-dichloroflavan (1) and of different isoflavans and isoflavenes, shown in Fig. 1, has been tested: isoflavene (2a), isoflavan (3a), 6-chloroisoflavene (2b), 6-chloroisoflavan (3b), 4′-chloroisoflavene (2c), 4′-chloroisoflavan (3c), 6,4′-dichloroisoflavene (2d), 6,4′-dichloroisoflavan (3d), 4-bromoisoflavene (2e), 6,4′-dibromoisoflavene (2f), 6-chloro,4′-bromoisoflavene (2g). The substances were initially dissolved in ethanol at 1 mg/ml and further diluted with cell culture medium before use.

Virus

Human hepatitis A virus (HAV) was first isolated from stools of a young patient with acute hepatitis A and propagated on Frp/3 cells. Confluent monolayers grown in Nunc flasks (175 cm^2) were infected with 5 ml HAV at high multiplicity of infection (2×10^5 $TCID_{50}$/ml) in Stoker-McPherson medium at 34°C. The medium was changed every 3 days until the cytopathic effect appeared. The flasks were frozen and thawed three times; then supernatants were collected, centrifuged at 4000 rpm for 15 min to remove cellular debris and stored at −70°C.

Cell monolayers

Frp/3 cells is a subline derived from the Virology Laboratory of the Department of Public Health of II University of Rome from FrhK/4 (Foetal rhesus Kidney) cells (Venuti et al., 1985). The cells were grown at 37°C in Stoker-McPherson me-

dium supplemented with 1% non-essential aminoacids, 100 IU/ml penicillin, 100 μg/ml streptomycin and either 10% calf serum plus 0.11% bicarbonate (growth medium) or 2% serum and twice the normal amount of bicarbonate (maintenance medium).

Inhibitor assays on HAV infection

To determine the effect of the isoflavans and isoflavenes on HAV infection, Frp/3 cells were grown in microtissue chamber/slide (80 000 cells/well) during 24 h in 5% CO_2 at 37°C. The monolayers were placed in an ice-water bath and infected with 100 μl of HAV at 3.5×10^6 $TCID_{50}$/ml suspended in precooled medium in order to infect approximately 100% of cells. After 1 h incubation at 0°C, the cells were washed with prewarmed medium and incubated for 72 h in 5% CO_2 at 34°C. The percentage of infected cells was determined by indirect immunofluorescence staining.

Different types of experiments were carried out: (a) the cells were preincubated with the compounds which were then removed before infection; (b) the cells were incubated with the inhibitors during the attachment step at 0°C for 1 h and the drugs were removed together with the virus inoculum before the temperature was shifted to 34°C; (c) in other experiments the drugs were added for various periods of time after the virus attachment step.

Immunofluorescence

HAV infection was monitored by immunofluorescence as previously described (Seganti et al., 1986). The results were expressed as the percentage of infected cells for both treated and untreated cell cultures.

Results

Drug cytotoxicity

The substances were studied in parallel for their influence on Frp/3 cell morphology, viability (as determined by neutral red uptake in dispersed cells) and yield. Drug cytotoxicity evaluation was performed in uninfected cells treated with different doses of the substances for 72 h, as reported in Table 1. For none of the drugs tested up to the concentrations of 1.56 μg/ml, cytotoxicity was observed in Frp/3 cells.

Effect of different concentrations of 6,4'-dichloroflavan, isoflavans and isoflavenes on HAV infection in Frp/3 cells

To determine the dose-response effect of the compounds on HAV replication in Frp/3 cells, experiments were carried out to establish the ability of the virus to

TABLE 1

Toxicity of different isoflavans and isoflavenes for Frp/3 cells[a]

Compounds	Concentration (μg/ml)						
	1.56	3.12	6.25	12.5	25	50	100
1	−	−	−	+	+	++	++
2a	−	−	−	−	+	+	++
3a	−	−	+	+	++	++	++
2b	−	−	−	+	+	++	++
3b	−	−	−	−	+	++	++
2c	−	−	−	−	+	++	++
3c	−	+	+	+	++	++	++
2d	−	−	−	+	+	++	++
3d	−	+	+	+	+	++	++
2e	−	−	+	+	+	++	++
2f	−	−	+	+	+	++	++
2g	−	−	−	+	+	++	++

[a]To determine the cytotoxic effect, cells were incubated with the drugs at different concentrations. After an incubation period of 72 h, cell morphology, viability and yield were examined. Results are expressed as complete cytotoxicity (++) when at least one of the parameters was affected in 100% of cells, or partial cytotoxicity (+) when one parameter was affected in 50% of cells, or absence of cytotoxicity (−), when none of the parameters was affected.

multiply in the presence of various non-cytotoxic concentrations of the drugs. In these experiments the infection was synchronized by a temperature shift: virus was first allowed to bind to the cell membrane at 0°C during 1 h and then to enter the cell by raising the temperature to 34°C. The drugs were added to the infected cul-

TABLE 2

Effect of different isoflavans and isoflavenes on HAV multiplication in Frp/3 cells[a]

Compounds	Concentration (μg/ml)						MIC[b]	
	0.00048	0.0024	0.012	0.06	0.3	1.5	(μg/ml $\times 10^{-3}$)	(μM $\times 10^{-1}$)
1	100	30	20	10	10	0	1.7	0.06
2a	100	100	70	20	20	0	35.9	1.73
3a	100	100	70	10	5	5	28.1	1.34
2b	100	50	60	25	5	0	24.3	1.00
3b	100	100	60	5	5	0	22.0	0.90
2c	100	100	60	15	10	5	23.0	0.95
3c	100	100	40	30	10	5	11.0	0.45
2d	100	100	60	10	10	5	22.7	0.82
3d	100	30	25	5	5	0	1.7	0.06
2e	100	100	50	10	5	5	14.5	0.45
2f	100	100	50	15	10	10	10.0	0.35
2g	100	100	25	20	10	5	3.3	0.09

[a]HAV infection was expressed as per cent of fluorescent cells as compared to untreated cultures following an incubation period of 72 h at 34°C.

[b]Minimum inhibitory concentrations of compound required to inhibit infection by 50%.

tures after the attachment step and left on the cells for 72 h. Table 2 presents the comparative effect of five-fold serial dilutions of drugs on HAV infection and the minimal concentrations required to inhibit viral antigen synthesis by 50% as compared with control infected cultures. Results show that all the drugs tested exerted an inhibitory effect on HAV multiplication, although to a different extent; 6,4′-dichloroflavan (1) and 6,4′-dichloroisoflavan (3d) were the most active: at 0.006 μM, 50% inhibition of viral antigen synthesis was obtained (Table 2). Drug concentrations ranging between 0.06 μg/ml and 1.50 μg/ml almost completely prevented viral antigen synthesis. Binding of HAV to Frp/3 cells was not affected by these drugs because no inhibition was observed when these compounds were preincubated with the cells and removed before viral infection, or when they were added only during the virus attachment step.

Lack of direct virucidal effect of the drugs on the infectivity of HAV

The putative virucidal effect of 6,4′-dichloroflavan, isoflavans and isoflavenes on HAV was tested by incubating the virus (3.5×10^6 $TCID_{50}$/ml) in culture medium containing the drugs at 0.06 μg/ml during 2 h at 37°C. Then Frp/3 monolayers were infected with virus-inhibitor mixtures for 1 h at 0°C. After this time the cells were washed and incubated for 72 h at 34°C. The residual infectivity of HAV was measured by immunofluorescence. Results obtained (data not shown) indicated that, under these experimental conditions, none of the compounds affected virus infectivity.

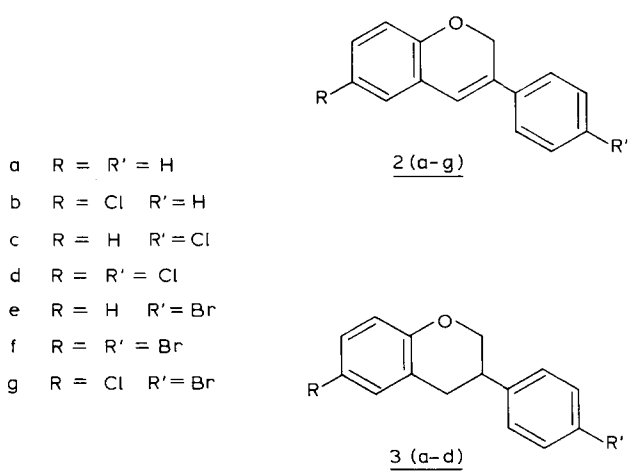

Fig. 1. Structure of isoflavenes (2a-g) and isoflavans (3a-d).

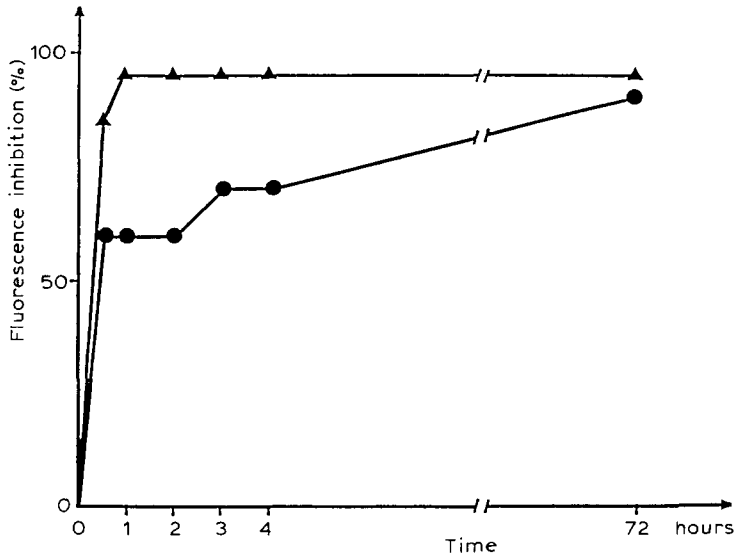

Fig. 2. Effect of 6,4'-dichloroflavan (●) and 6,4'-dichloroisoflavan (▲) on HAV infection in Frp/3 cells. The compounds were added to the cell culture medium immediately after the virus attachment step. The concentration of the compounds was 0.06 μg/ml. The cells were incubated for varying times as indicated on the x-axis.

Effect of 1 and 3d on HAV multiplication in Frp/3 cells

We then tried to establish which phase of HAV infection was affected by 1 and 3d. The latter compound was chosen as representative for the active isoflavan derivatives.

In these experiments, the drugs were used at a concentration of 0.06 μg/ml, added to the medium immediately after the viral attachment step and incubated for different periods of time (30 min, 1,2,3 or 4 h). After these incubation intervals, the drugs were removed and fresh medium was added to the cell monolayers. Seventy-two hours after the virus attachment step, the cultures were examined for the percentage of infected cells by immunofluorescence staining.

Fig. 2 shows that 1 and 3d effected a strong inhibition of HAV infection and that this action occurred soon after the attachment step. When the substances were present for 1 h after the temperature shift to 34°C, an inhibition of 60 and 90% of viral antigen synthesis was observed with 1 and 3d, respectively. Although an incubation of 30 min with 3d was sufficient to reduce the number of infected cells to 15%, 3 h of incubation in the presence of 6,4'-dichloroflavan were necessary to obtain a 70% inhibition of viral antigen synthesis.

Discussion

The results described here demonstrate that 6,4′-dichloroflavan (Bauer et al., 1981) and different isoflavan and isoflavene derivatives (Burali et al., 1987) protect Frp/3 cells against HAV infection. This inhibitory effect was shown by the different compounds, varied from one compound to another, and was not pronounced for compounds 1 and 3d. The minimum concentration required to reduce HAV infection by 50% was 0.006 μM for both substances. None of the drugs tested showed a virucidal effect or significantly altered virus binding to Frp/3 cells when added to the cells before or during the viral attachment step at 0°C.

The experiments in which inhibitory concentrations of 1 and 3d were present for different times after the attachment step suggest that the inhibition took place at an early time (penetration and/or uncoating) of HAV infection. It is known that HAV uncoating is a slow and gradual process and up to 9 h after infection non-eclipsed virus is still present (Anderson et al., 1987). As to the delay in the inhibitory activity of compound 1 as compared to 3d (Fig. 2), this might be due to a weaker interaction of compound 1 with the virus or a slower penetration of the compound across the cell membrane.

According to the mechanism of action of these drugs against human rhinoviruses (Tisdale and Selway, 1983; Ninomiya et al., 1985) and polioviruses (Fiore et al., 1987) and based on the present results, it is inferred that the drugs affect HAV infection at the uncoating stage. However, an inhibition of the penetration step cannot be ruled out. Further research will be required to verify whether these lipophilic substances bind to the virion capsid and inhibit conformational changes needed for viral uncoating.

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