Antiviral Research, 13 (1990) 201–208
Elsevier

AVR 00410

Short Communication

In vitro effect of synthetic flavanoids on astrovirus infection

Fabiana Superti[1], Lucilla Seganti[2], Nicola Orsi[2], Nicoletta Desideri[3], Maria Luisa Stein[3], Antonella Tinari[1], Maria Luisa Marziano[1] and Gianfranco Donelli[1]

[1]*Laboratorio di Ultrastrutture, Istituto Superiore di Sanità,* [2]*Istituto di Microbiologia, Facoltà di Medicina, Università di Roma 'La Sapienza' and* [3] *Dipartimento di Studi Farmaceutici, Facoltà di Farmacia, Università di Roma 'La Sapienza', Rome, Italy*

(Received 16 October 1989; accepted 4 January 1990)

Summary

In this study we investigated the activity of halogeno-, cyano- and amidino-isoflavenes, isoflavans and flavans on the multiplication of human astroviruses. These are naked small round viruses which have been recognized as causative agents of human gastroenteritis, and whose capsid proteins are similar to those of picornaviruses. Although all drugs tested caused a dose-dependent reduction of viral antigen synthesis as monitored by immunofluorescence, the chloro derivatives were the most effective.

Astrovirus; Synthetic flavanoid

Introduction

Human astroviruses are small round viruses (approximately 28 nm diameter) with an ultrastructural star-like morphology (Madeley and Cosgrove, 1975). They are mainly responsible for gastroenteritis in children (Kurtz et al., 1977; Madeley et al., 1977), although infections in adults have also been described (Konno et al., 1982). Among the five serotypes of human astroviruses identified so far, type 1 is the most widely spread (Kurtz and Lee, 1987).

Correspondence to: F. Superti, Istituto Superiore di Sanità, Laboratorio di Ultrastrutture, Via Regina Elena 299, 00161 Rome, Italy

These viruses are not enveloped, have a positive strand RNA genome of approximately 7500 nucleotides and a polypeptide electrophoresis pattern which displays similarities to that of enteroviruses. Because of these features, astroviruses can be considered as members of the Picornaviridae family (Kurtz and Lee, 1987).

Several compounds which inhibit the early phases of picornavirus infection have been identified (Mac Sharry et al., 1979; Ninomiya et al., 1984, 1985; Diana et al., 1985; Fox et al., 1986). Among these, a variety of naturally occurring flavonoids (Beladi et al., 1977; Van Hoof et al., 1984; Kaul et al., 1985; De Meyer et al., 1988) and synthetic flavans, isoflavans and 3(2H)-isoflavenes related to 6,4′-dichloroflavan (Bauer et al., 1981) inhibit disassembly of different enteroviruses and rhinoviruses (Ishitsuka et al., 1982; Tisdale and Selway, 1983; Ninomiya et al., 1984; Burali et al., 1987; Conti et al., 1988; Desideri et al., 1989; Superti et al., 1989).

It has been suggested that compounds which interfere with viral uncoating, without affecting viral attachment and/or penetration, are likely to bind to the same site of picornavirus structural proteins (Eggers and Rosenwirth, 1988; Rossmann, 1989). Taking into account the significant conservation of internal residues of capsid proteins among picornaviruses (Rossmann and Palmenberg, 1988) and the similarity in capsid proteins between astroviruses and picornaviruses (Kurtz and Lee, 1987), we investigated the effects of halo-, cyano-, and amidino-flavanoids (Burali et al., 1987; Desideri et al., 1989) on astrovirus infection in vitro.

Materials and Methods

Test substances

The following substances (shown in Fig. 1) were evaluated: (1a) isoflavene; (1b) 6-chloroisoflavene; (1c) 4′-chloroisoflavene; (1d) 6,4′-dichloroisoflavene; (1e) 6-cyanoisoflavene; (1f) 4′-cyanoisoflavene; (1g) 6,4′-dicyanoisoflavene; (1h) 6-amidinoisoflavene; (1i) 4′-amidinoisoflavene; (1j) 6,4′-diamidinoisoflavene; (1k) 6-bromoisoflavene; (1l) 4′-bromoisoflavene; (2a) isoflavan; (2b) 6-chloroisoflavan; (2c) 4′-chloroisoflavan; (2d) 6,4′-dichloroisoflavan; (2e) 6-cyanoisoflavan; (2f) 4′-cyanoisoflavan; (2g) 6,4′-dicyanoisoflavan; (2h) 6-amidinoisoflavan; (3d) 6,4′-dichloroflavan; (3e) 6-cyanoflavan; (3f) 4′-cyanoflavan; (3g) 6,4′-dicyanoflavan; (3h) 6-amidinoflavan; (3i) 4′-amidinoflavan and (3j) 6,4′-diamidinoflavan. The substances were initially dissolved in absolute ethanol or distilled water at 1 mg/ml and further diluted in cell culture medium before use.

Cells and virus

LLC-MK2 cells, a monkey kidney cell line, were grown at 37°C in 1:1 MEM (Minimum Essential Medium) and 199 medium (Flow Laboratories) supplemented with 10% inactivated foetal calf serum, penicillin (100 UI/ml) and streptomycin (100 μg/ml) (growth medium). The virus used was a human astrovirus isolated in Italy (Superti et al., 1988), and identified as serotype 1 by specific antisera kindly provided by Dr Kurtz (Oxford, U.K.).

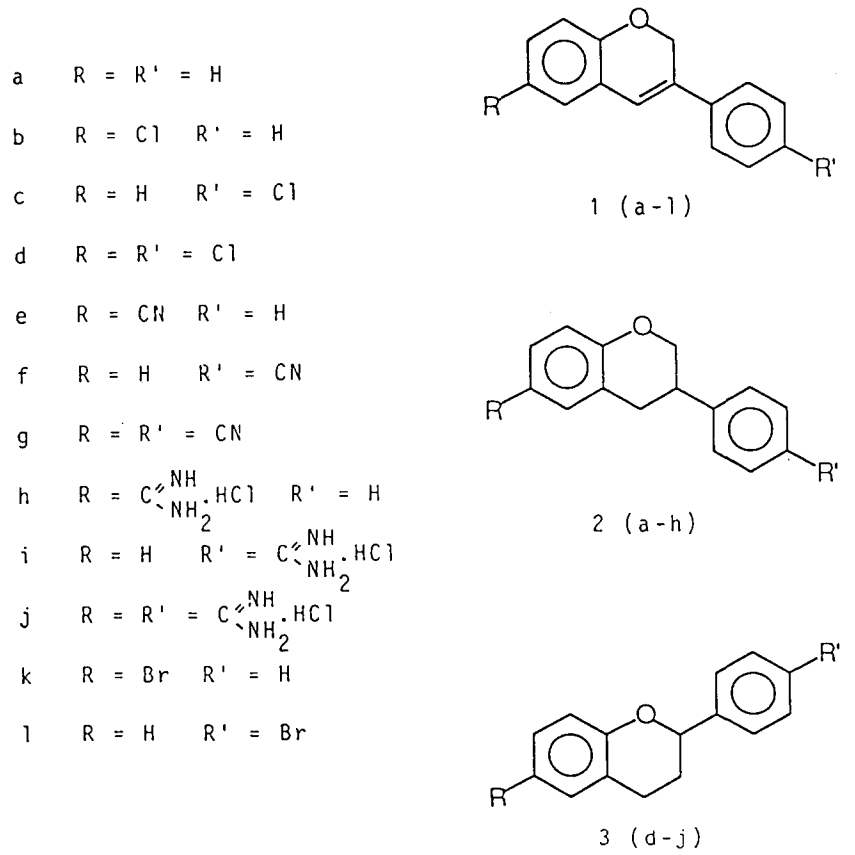

Fig. 1. Structures of isoflavene- (1), isoflavan- (2) and flavan- (3) derivatives.

Virus propagation was carried out in cell monolayers infected with 5 PFU/cell and incubated for 48 h at 37°C in 199 medium containing 20 μg/ml trypsin (Sigma Chemical Co., type III), penicillin (100 UI/ml) and streptomycin (100 μg/ml). Infected cultures were frozen and thawed three times, centrifuged (2000 × *g*/min for 15 min) to remove cellular debris, and the supernatants were stored at −70°C.

Drug cytotoxicity

In order to establish the maximal non-cytotoxic concentrations, two-fold serial dilutions of the drugs in growth medium were incubated with LLC-MK2 cell monolayers grown in 96-well plates (Falcon). The assays were carried out in duplicate.

After an incubation period of 48 h at 37°C in 5% CO_2, cell morphology, cell viability (as determined by neutral red uptake in dispersed cells) and cell number were determined.

The 50% cytotoxic concentration (CC_{50}) corresponded to the concentration of test compound at which one of the above mentioned parameters was affected in 50% of cells.

Drug dilutions which did not affect any of these parameters were considered as non-cytotoxic concentrations.

Assay for the effect of synthetic flavanoids on astrovirus infection

Cells were grown in microtissue chamber slides (Lab-tek, Miles Laboratory) for 24 h in 5% CO_2 at 37°C. The monolayers were placed in an ice-water bath, washed with ice-cold medium and infected with astrovirus suspended in precooled medium at an MOI of 1.0 p.f.u./cell. After 1 h at 0°C, the cells were washed with pre-warmed medium and incubated for 24 h in 5% CO_2 at 37°C. The percentage of infected cells was determined by indirect immunofluorescence.

Different types of experiments were carried out: (i) The cells were preincubated with the drugs which were removed just before infection; (ii) The drugs were incubated with the cells during the virus attachment step at 0°C for 1 h, then, they were removed together with the virus inoculum before the temperature was shifted to 37°C; (iii) In other experiments the drugs were incubated with the cells for 24 h after the virus attachment step.

The 50% inhibitory concentrations (IC_{50}) were calculated according to Reed and Münch.

Antisera

Anti-serotype 1 human astrovirus serum was prepared from rabbits immunized with purified virus.

Briefly, viral particles, pelleted by centrifugation at 130000 × *g*, were suspended in PBS (phosphate-buffered saline) (pH 7.4). After treatment with trichloro-trifluoroethane (Carlo Erba), the suspension was layered onto a caesium chloride gradient (0.625 g/ml) and centrifuged at 120000 × *g* (18 h; +4°C). The virus-containing fractions were pooled and dialyzed overnight against PBS.

Rabbits were immunized by three intramuscular inoculations of purified viral particles suspended in complete Freund's adjuvant followed by an intravenous inoculation of the same antigen without adjuvant.

Immunofluorescence

Virus-infected cell monolayers were washed with PBS and fixed with absolute acetone for 10 min at −20°C. After incubation with anti-serotype 1 human astrovirus rabbit serum for 45 min at 37°C, cells were washed with PBS and stained with fluorescein isothiocyanate-conjugated anti-rabbit gammaglobulin antibodies (Sigma Chemical Co.). After 45 min at 37°C, cells were washed with PBS, mounted in buffered glycerol and examined under a Leitz fluorescence microscope. The results are expressed as percentage of infected cells.

Results

Different non-cytotoxic concentrations of flavanoids were added to the LLC-MK2 cell monolayers for 1 h prior to infection and incubated at 37°C. Then, the cell cultures were washed and infected with appropriately diluted stock virus. Pre-exposure of cells to the drugs did not prevent subsequent infection and intracellular replication of human astrovirus serotype 1 as monitored by indirect immunofluorescence.

In other experiments, virus infection was synchronized by a temperature shift as described in Materials and Methods in order to establish whether the putative an-

TABLE 1

Effects of isoflavene, isoflavan and flavan derivatives on astrovirus multiplication in LLC-MK2 cells[a]

Drugs	Concentration (μg/ml)				IC_{50}[b] (μM)	CC_{50}[c] (μM)
	0.001	0.01	0.1	1		
1a	100	100	80	60	9.61	154
1b	100	70	20	0	0.18	65
1c	100	60	50	20	0.41	65
1d	100	100	50	10	0.36	86
1e	100	100	30	0	0.32	68
1f	100	80	50	0	0.43	100
1g	100	80	50	0	0.38	30
1h	100	100	60	40	1.04	7
1i	100	70	60	0	0.87	55
1j	100	70	60	0	0.65	126
1k	100	100	70	60	6.97	111
1l	100	90	60	40	1.92	83
2a	100	100	50	50	1.43	304
2b	100	70	20	0	0.18	131
2c	100	60	40	0	0.28	98
2d	100	80	30	0	0.23	57
2e	100	100	50	0	0.42	102
2f	100	80	40	0	0.32	204
2g	100	70	40	0	0.27	184
2h	100	60	40	0	0.19	55
3d	100	90	30	0	0.25	57
3e	100	70	50	0	0.42	136
3f	100	70	50	0	0.42	136
3g	100	100	50	0	0.38	184
3h	100	100	50	20	0.32	39
3i	100	80	50	20	0.33	107
3j	100	100	60	40	0.76	162

[a]Astrovirus infection was expressed as per cent of fluorescent cells (compared with the untreated cultures) after 24 h incubation at 37°C.

[b]Concentration of compounds required to reduce infection by 50%.

[c]Concentration of compounds able to affect morphology or reduce viability or cell count by 50% after 48 h incubation.

tiviral effect took place during the attachment step or the subsequent phases of virus multiplication. Non-cytotoxic ten-fold dilutions of the drugs were added to the cells together with the virus during the incubation at 0°C and then removed after 1 h. Alternatively, they were added after the removal of virus inoculum and were left on the cells for 24 h.

In the first set of experiments it was demonstrated that the compounds did not prevent virus adsorption. Results obtained in the second set of experiments provided evidence that the synthetic flavanoids tested inhibited astrovirus multiplication in a dose-dependent fashion (Table 1). As it could be deduced from their IC_{50}, isoflavene (1a), isoflavan (2a) and bromoisoflavenes (1k) (1l) were the least active. The chloro derivatives caused the highest inhibition of astrovirus antigen synthesis with an IC_{50} ranging from 0.18 to 0.41 μM.

In all cases, with the exception of 6-amidino-isoflavene (1h), the CC_{50} was markedly higher than the IC_{50}.

Discussion

The present study was aimed at evaluating the ability of different flavanoids, i.e. isoflavene and chloro-, cyano-, amidino- and bromo-isoflavenes; isoflavan and chloro-, cyano- and amidino-isoflavans and chloro-, cyano- and amidino-flavans to interfere with astrovirus infection.

The drugs impaired viral antigen synthesis in a dose-dependent fashion. The chloro derivatives were more active than the other compounds. In the isoflavene series, the presence of bromine in the 6- or 4′-position did not significantly enhance the activity, while the presence of a cyano- or amidino- group made the compounds more active.

Flavanoids have been found active against several viruses of the Picornaviridae family (Burali et al., 1987; Conti et al., 1988; Desideri et al., 1989; Superti et al., 1989). The present data add indirect proof to a structural similarity among astroviruses and picornaviruses.

Although the mechanism of action of the flavanoids has not yet been elucidated, some derivatives bind reversibly to virions (Tisdale and Selway, 1983). Their activity seems to take place shortly after virion entry into the cells (Superti et al., 1989). These observations suggest that the flavanoids, like other small lipophilic molecules, bind to proteins with remarkable similarity among a wide range of virus families (Rossmann, 1989).

Experiments performed by preincubating cell monolayers with the drugs or leaving the drugs with the virus inoculum only during the attachment step indicated that the anti-astrovirus activity of flavanoids takes place after virus adsorption. It may be hypothesized that for astroviruses, as for polioviruses (Longberg-Holm et al., 1975), viral capsids undergo conformational alterations after virus internalization. This could generate a new conformation of the surface proteins, thus allowing interaction with the antiviral compounds.

Acknowledgements

This study was supported by grants of Progetto Finalizzato C.N.R. Controllo Malattie da Infezione (87.00656.52), Ministero Pubblica Istruzione and Istituto Pasteur-Fondazione Cenci Bolognetti.

References

Bauer, D.J., Selway, J.W.T., Batchelor, J.F., Tisdale, M., Caldwell, I.C. and Young, D.A.B. (1981) 4',6-dichloroflavan (BW 683C), a new anti-rhinovirus compound. Nature (London) 292, 369–370.

Beladi, I., Pusztai, R., Mucsi, I., Bakay, M. and Gabor, M. (1977) Activity of some flavonoids against viruses. Ann. N.Y. Acad. Sci. 284, 358–364.

Burali, C., Desideri, N., Stein, M.L., Conti, C. and Orsi, N. (1987) Synthesis and antirhinovirus activity of halogen substituted isoflavenes and isoflavans. Eur. J. Med. Chem. 22, 119–123.

Conti, C., Orsi, N. and Stein, M.L. (1988) Effect of isoflavans and isoflavenes on rhinovirus 1B and its replication in HeLa cells. Antiviral Res. 10, 117–127.

De Meyer, N., Van Hoof, L., Pandey, H.K., Mishra, L., Vanden Berghe, D., Vlietinck, A.J. and Haemers, A. (1988) Antiviral activity of synthetic 3-methoxyflavones. In: Abstracts International Symposium of Chemotherapy, Catania, p. 213.

Desideri, N., Conti, C., Orsi, N. and Stein, M.L. (1989) Synthesis and antiviral activity of substituted flavanoids. In: Abstracts 16th International Congress of Chemotherapy, Jerusalem, June 11–16, p. 120.

Diana, G.D., Otto, M.J. and McKinlay, M.A. (1985) Inhibitors of picornavirus uncoating as antiviral agents. Pharmacol. Ther. 29, 287–297.

Eggers, H.J. and Rosenwirth, B. (1988) Isolation and characterization of an arildone-resistant poliovirus 2 mutant with an altered capsid protein VP1. Antiviral Res. 9, 23–36.

Fox, M.P., Otto, M.J. and McKinlay, M.A. (1986) Prevention of rhinovirus and poliovirus uncoating by WIN 51711, a new antiviral drug. Antimicrob. Agents Chemother. 30, 110–116.

Ishitsuka, H., Ohsawa, C., Ohiwa, T., Umeda, I. and Suhara, Y. (1982) Antipicornavirus flavone Ro 09-0179. Antimicrob. Agents Chemother. 22, 611–616.

Kaul, T.N., Middleton, E. and Ogra, P.L. (1985) Antiviral effect of flavonoids on human viruses. J. Med. Virol. 15, 71–79.

Konno, T., Suzuki, H., Ishida, N., Ohiba, R., Moshisuki, K. and Tsunoda, A. (1982) Astrovirus-associated epidemic gastroenteritis in Japan. J. Med. Virol. 9, 11–17.

Kurtz, J.B., Lee, T.W. and Pickering, D. (1977) Astrovirus associated gastroenteritis in a children ward. J. Clin. Pathol. 30, 948–952.

Kurtz, J.B. and Lee, T.W. (1987) Astroviruses: human and animal. Ciba Foundation Symp. 128, 92–187.

Lonberg-Holm, K., Gosser, L.B. and Kauer, J.C. (1975) Early alteration of poliovirus in infected cells and its specific inhibition. J. Gen. Virol. 27, 329–342.

Madeley, C.R. and Cosgrove, B.P. (1975) 28 nm particles in faeces in infantile gastroenteritis. Lancet 2, 451–452.

Madeley, C.R., Cosgrove, B.P., Bell, E.J. and Fallon, R.J. (1977) Stool viruses in babies in Glasgow. J. Hyg. (London) 78, 261–273.

McSharry, J.J., Caliguiri, L.A. and Eggers, H.J. (1979) Inhibition of uncoating of poliovirus by arildone, a new antiviral drug. Virology 97, 307–315.

Ninomiya, Y., Ohsawa, C., Aoyama, M., Umeda, I., Suhara, Y. and Ishitsuka, H. (1984) Antivirus agent, Ro 09-0410, binds to rhinovirus specifically and stabilizes the virus conformation. Virology 134, 269–276.

Ninomiya, Y., Aoyama, M., Umeda, I., Suhara, Y. and Ishitsuka, H. (1985) Comparative studies on the modes of action of the antirhinovirus agents Ro 09-0410, Ro 09-0179, RMI-15'731, 4',6-dichloroflavan, and enviroxime. Antimicrob. Agents Chemother. 27, 595–599.

Rossmann, M.G. and Palmenberg, A.C. (1988) Conservation of the putative receptor attachment site in Picornaviruses. Virology 164, 373–382.

Rossmann, M.G. (1989) The structure of antiviral agents that inhibit uncoating when complexed with viral capsids. Antiviral Res. 11, 3–14.

Superti, F., Tinari, A. and Donelli, G. (1988) A new approach for laboratory diagnosis of astrovirus infections. In: Abstracts XIII International Symposium on Intestinal Microecology. Porto Conte (Sassari), September 11–14, p. 102.

Superti, F., Seganti, L., Orsi, N., Divizia, M., Gabrieli, R., Panà, A. and Stein, M.L. (1989) Effect of isoflavans and isoflavenes on the infection of Frp/3 cells by hepatitis A virus. Antiviral Res. 11, 247–254.

Tisdale, M. and Selway, J.W.T. (1983) Inhibition of an early stage of rhinovirus replication by dichloroflavan (BW 683C). J. Gen. Virol. 64, 795–803.

Van Hoof, L., Vanden Berghe, D.A., Hatfield, G.M. and Vlietinck, A.J. (1984) Plant antiviral agents V. 3-methoxyflavones as potent inhibitors of viral induced block of cell synthesis. Planta Med. 50, 513–517.