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Short communication

Antiviral activity of substituted homoisoflavonoids on enteroviruses

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Abstract

The antiviral activity of homoisoflavonoids, a class of flavonoids, was determined *in vitro* against a large panel of enteroviruses. The inhibition of viral replication was monitored on BGM (Buffalo Green Monkey) cells, and the concentration required for 50% inhibition (IC_{50}), as well as the selectivity index (SI) were determined. None of the substances were effective against Sabin type 1 poliovirus (PV1), but most of them showed a low cytotoxicity and a marked antiviral activity against Coxsackie virus B1, B3, B4, A9 and echovirus 30.
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Picornaviruses, in particular enteroviruses (EVs) and rhinoviruses (HRVs), are responsible for several human viral diseases, ranging from mild upper respiratory diseases to fatal neurological or cardiac-based illnesses. Enteroviruses cause aseptic meningitis, encephalitis, febrile illness, foot and mouth diseases, myocarditis, and pancreatitis, whereas, rhinoviruses are estimated to cause approximately one-third of all upper respiratory tract viral infections (Pallansch and Roos, 2001).

Due to the widespread nature of the diseases associated with picornaviruses and the difficulty of vaccine development for the majority of these viruses, extensive efforts have been made in the search for effective anti-picornavirus agents. However, despite the in vitro activity of several specific compounds, to date only few drugs have shown efficacy in humans and none have been approved for clinical use (Shih et al., 2004; Pevear et al., 2005; Rawlinson, 2001).

Natural and synthetic flavanoids and flavonoids interfere with picornavirus replication preventing the decapsidation of viral particles and RNA release within cells (Tisdale and Selway, 1984; Castrillo et al., 1986; Conti et al., 1990a; González et al., 1990; Genovese et al., 1995; Salvati et al., 2004) or blocking viral RNA synthesis (Robin et al., 2001).

Several substituted flavanoids (flavans, isoflavans and 3(2H)-isoflavenes) have been reported to have a broad antiviral spectrum of activity, efficiently inhibiting HRV 1B, Sabin type 2 poliovirus, hepatitis A virus, coxsackievirus B4, echovirus 6, and enterovirus 71 infections in vitro (Burali et al., 1987; Conti et al., 1990a,b; Desideri et al., 1990, 1992; Quaglia et al., 1993; Genovese et al., 1995).

Among flavonoids, both natural and synthetic flavanones and flavones presented a large spectrum of activity, although they are less potent than flavanoids. In particular flavones, generally poorly active compounds, achieved good anti-picornavirus potency through the introduction of substituents in position 3 (Van Hoof et al., 1984; De Meyer et al., 1991; Desideri et al., 1995).

Homoisoflavonoids constitute a small class of natural products structurally related to other known anti-picornavirus flavonoids. Synthetic analogues (3-benzylidenechroman-4-ones, 3-benzyl-4-chromones, and 3-benzylchroman-4-ones) (Fig. 1) were prepared and tested for their antiviral activity against picornaviruses. The homoisoflavonoids **1**–**3**(**a**–**g**) were reported to be weakly effective against poliovirus type 2, while they exhibited a variable degree of activity against HRV 1B and 14, selected as representative serotypes for groups B and A of HRVs, respectively (Desideri et al., 1997; Quaglia et al., 1999).

The aim of the present study was to evaluate the antiviral activity of this latter class of flavonoids against a larger panel of pathogenic enteroviruses, including Coxsackie virus B3 (CVB3), one of the major causes of virus-induced acute or chronic heart diseases (Maier et al., 2004), Coxsackie virus B4

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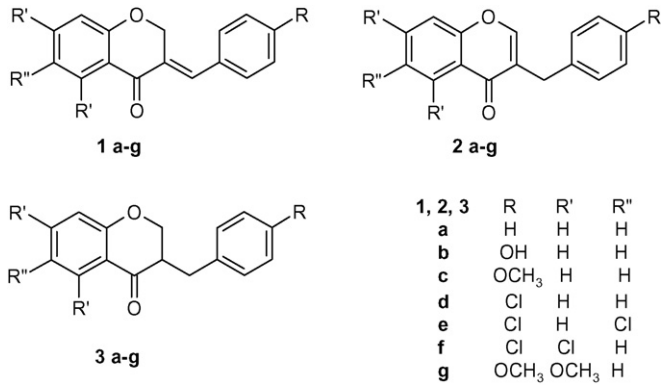

Fig. 1. Chemical structures of homoisoflavonoids: (E)-3-benzylidenechroman-4-ones (**1a**–**g**), 3-benzyl-4-chromones (**2a**–**g**) and 3-benzylchroman-4-ones (**3a**–**g**).

(CVB4) and A9 (CAV9), correlated with pancreatitis (Roivainen et al., 2000; Huber and Ramsingh, 2004) and echovirus 30 (Echo30) associated with meningitis (Savolainen et al., 2001). Among the three series of homoisoflavonoids described above, 3-benzyl-4-chromones (**2a**–**g**) (Fig. 1) were selected since they proved less cytotoxic in HeLa cell cultures (Desideri et al., 1997).

Initially, we confirmed the low cytotoxicity of these compounds on BGM (Buffalo Green Monkey) cells, widely used in enterovirus isolation because of their susceptibility to most of picornaviruses. The 50% cytotoxic concentration (CC_{50}) of the compounds, defined as the concentration reducing the viability of untreated cell cultures by 50%, was determined (Table 1). Confluent cell monolayers grown in 96-well plates were incubated with 10-fold serial dilutions (from 10 to 100 μM) of compounds for 3 days (37 °C, 5% CO_2) in D-MEM supplemented with 2% FCS. Cells were then fixed and stained with a methanol solution of crystal violet, as previously described (Sugarman et al., 1987; Horn et al., 1990). After dye extraction, the optical density of individual wells was quantified spectrophotometrically at 590 nm with a microplate reader. Cell viability in individual compound-treated wells was determined as the percentage of the mean value of optical density resulting from the average of three replicates with respect to the mock-treated cell control set as 100%.

The CC_{50} values of compounds **2a**–**d**, **2f** and **2g** ranged from 39 to 89 μM, while derivative **2e** was not toxic up to the highest concentration tested (100 μM). These data indicated that all the substances had a low cytotoxicity for BGM cells, comparable or lower than WIN 51711, a broad-spectrum anti-picornavirus compound used as reference compound (Otto et al., 1985). Cytotoxicity was dependent on the nature of substituents, the chloro-substituted derivatives **2d**–**f** being the least toxic among the 3-benzyl-4-chromones tested (Table 1).

The inhibitory activity of the homoisoflavonoids **2a**–**g** was evaluated against Sabin type 1 poliovirus (PV1), Coxsackie virus B1 (CVB1), B3 (CVB3), B4 (CVB4), A9 (CAV9) and echovirus 30 (Echo30) by focus reduction neutralization assay, as previously described (Di Lonardo et al., 2002). Briefly, BGM cell monolayers were grown in 96-well microtiter plates. After

Table 1
Cytotoxicity and antiviral activity of 3-benzyl-4-chromones (**2a**–**g**), 3-(4′-hydroxybenzyliden)chroman-4-one (**1b**) and 3-(4′-hydroxybenzyl) chroman-4-one (**3b**) against Sabin type 1 poliovirus (PV1), coxsackievirus B1 (CVB1), coxsackievirus B3 (CVB3), coxsackievirus B4 (CVB4), coxsackievirus A9 (CAV9) and echovirus 30 (Echo30)

Compound	CC_{50} (μM)	IC_{50} (μM)						SI[a]				
		PV1 (Sab)	CVB3	CVB4	CAV9	CVB1	Echo30	PV1 (Sab)	CVB3	CVB4	CAV9	CVB1	Echo30
2a	53.6	Not active	20.0 ± 1.8	12.0 ± 1.1	23.3 ± 1.5	4.0 ± 0.2	8.0 ± 0.5	–	2.7	4.5	2.3	13.4	6.7
2b	46.8	30.0 ± 1.6	10.0 ± 0.8	14.0 ± 1.3	38.0 ± 1.8	4.0 ± 0.3	13.0 ± 1.1	1.6	4.7	3.3	1.2	11.7	3.6
2c	55.5	>55.5[b]	4.0 ± 0.2	8.0 ± 0.5	20.0 ± 1.3	2.5 ± 0.1	13.0 ± 1.0	–	13.9	6.9	2.8	22.2	4.3
2d	85.0	50.0 ± 2.1	20.0 ± 1.6	15.0 ± 1.2	2.5 ± 0.1	3.0 ± 0.1	2.5 ± 0.1	1.7	4.3	5.7	34.0	28.3	34.0
2e	>100.0	43.0 ± 1.8	30.0 ± 2.1	30.0 ± 1.8	39.3 ± 1.4	7.5 ± 0.4	25.0 ± 1.6	>2.3	>3.3	>3.3	>2.5	>13.3	>4.0
2f	88.6	Not active	20.0 ± 1.6	10.0 ± 0.6	7.5 ± 0.5	20.0 ± 1.4	5.0 ± 0.3	–	4.4	8.9	11.8	4.4	17.7
2g	39.1	>39.0[b]	25.0 ± 1.5	20.0 ± 1.5	36.5 ± 1.7	17.5 ± 1.3	15.0 ± 1.3	–	1.6	2.0	1.1	2.2	2.6
WIN51711	53.2	2.0 ± 0.1	5.0 ± 0.3	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	26.6	10.6	26.6	26.6	26.6	26.6
1b	20.0	15.0 ± 1.3	15.0 ± 1.4	13.0 ± 1.2	8.5 ± 0.4	5.0 ± 0.3	3.5 ± 0.2	1.3	1.3	1.5	2.4	4.0	5.7
3b	91.4	30.0 ± 1.6	26.0 ± 1.7	20.0 ± 1.6	20.0 ± 1.3	14.0 ± 1.3	7.5 ± 0.5	3.0	3.5	4.6	4.6	6.5	12.2

All experiments were performed in triplicate on BGM cells. WIN 51711 was included as control.
[a] SI: ratio CC_{50}/IC_{50}.
[b] $>CC_{50}$: when IC_{50} value was higher than the corresponding CC_{50}.

removal of culture medium, 100 μl of drug solution and a constant amount of virus (150–200 focus forming units (FFU) for each virus), in a volume of 100 μl, were added to the cells. Non-infected and infected cells in the absence of compounds served as cell and virus control, respectively. WIN 51711 was used as positive control (Otto et al., 1985).

The 50% inhibitory concentration (IC_{50}) of antiviral compounds **2a**–**g**, defined as the drug concentration required for 50% virus inhibition, was determined from the mean dose–response curves of three replicates (Table 1). To better evaluate the toxicological profile of the molecules, the selectivity index (SI), expressed as CC_{50} versus IC_{50} ratio, was considered.

None of the substances showed significant inhibition towards Sabin PV1, but all of them were effective against the other EVs tested (Table 1). In contrast with the results previously reported on HRVs, where chloro-substituted compounds (**2d**–**f**) generally showed the highest potency (Desideri et al., 1997), in this study the antiviral effect against CVB3 and CVB4 was more pronounced in the presence of homoisoflavones (**2b** and **2c**) with electron-donating substituents at the 4′-position. 3-(4′-Methoxybenzyl)-4-chromone (**2c**) was the most effective inhibitor of both these CVs with an IC_{50} of 4 and 8 μM, respectively. Moreover, compound **2c** combined a high antiviral activity with a rather low cytotoxicity resulting in a SI against CVB3 higher than that of the reference compound (WIN 51711). The introduction of two additional methoxy groups in positions 5 and 7 gave compound **2g**, with a lower activity against all the EVs tested and a greater cytotoxicity with respect to the 4′-methoxy analogue (**2c**). Although the chloro-substituted homoisoflavones (**2d**–**f**) were the least toxic compounds of this series, they generally exhibited a moderate selectivity against CVB3 and CVB4 due to lower potency.

Interestingly, as observed in previous studies with HRVs (Desideri et al., 1997), 3-(4′-chlorobenzyl)-4-chromone (**2d**) was found to be the most active homoisoflavone against CAV9 and Echo30, with an IC_{50} of 2.5 μM. In addition, compound **2d** showed a better selectivity index against these two viruses than that found for WIN 51711 with a same potency.

The introduction of a second chlorine atom in position 6 resulted in the 4′,6-dichloro derivative **2e**, which was not cytotoxic, but showed a moderate activity against CAV9 and Echo30. The substitution of compound **2d** with two additional chlorine atoms in positions 5 and 7 led to compound **2f** with a cytotoxicity comparable to that of compound **2d** and a slightly lower activity against these two viruses (IC_{50} = 7.5 and 5.0 μM for CAV9 and Echo30, respectively).

The unsubstituted 3-benzyl-4-chromone (**2a**) and all the 4′-substituted analogues (**2b**–**d**) were potent and selective inhibitors of CVB1, 3-(4′-methoxybenzyl)-4-chromone (**2c**) being the most active compound (IC_{50} = 2.5 μM) and 3-(4′-chlorobenzyl)-4-chromone (**2d**) the most selective (SI = 28.3).

To verify whether the chemical modifications of the 3-benzyl-4-chromone skeleton could influence the activity observed against EVs, we extended our study to the **2b** analogues: 3-benzylidenechroman-4-one (**1b**), characterized by the presence of an exocyclic instead of an endocyclic double bond, and to the more flexible 3-benzylchroman-4-one (**3b**). The compound **2b** was selected for its broad spectrum of anti-EV activity, including PV1. The cytotoxicity and the antiviral activity of these compounds against the EVs selected was evaluated, and the results are reported in Table 1. Both analogues (**1b** and **3b**), as well as the parent compound (**2b**), interfered with the replication of all the EVs tested, including PV1. The isomerization of an endo to an exo double bond led to compound **1b**, which showed a higher potency against the viruses tested but also an increased cytotoxicity with respect to the compound **2b**. In contrast, the reduction of the double bond resulted in the less toxic compound **3b**, with a moderate activity against all EVs tested. Concerning selectivity, both analogues (**1b** and **3b**) exhibited a selectivity index equal or lower than **2b** against PV1, CVB1, CVB3 and CVB4, while they showed a higher SI against CAV9 and Echo30 (Table 1).

In summary, we have identified a series of homoisoflavonoids with low cytotoxicity and a good antiviral activity against all the Coxsackie viruses studied, and Echo30, which might prove useful as additional antiviral drugs against these virus infections. Furthermore, this family of new flavonoids shows a broad spectrum of activity against both genera of HRVs and EVs. On the basis of these results, further structure–activity relationship studies are currently under investigation to identify more potent and selective analogues.

Like previously studied flavanoids and WIN compounds, these new analogues are likely to inhibit an early stage of the enterovirus replication cycle. Elucidation of the antiviral mechanism of these compounds will be the subject of further research and should facilitate understanding of the complex interactions between viruses, compounds and cells and the different susceptibility of HRVs and EVs to these substituted homoisoflavonoids.

Novel molecules and different approaches for the treatment of picornavirus diseases such as the common cold, aseptic meningitis, febrile illness, and, ultimately, the persistent infections of polio in immunodeficient subjects (MacLennan et al., 2004) that could in the future compromise polio eradication efforts, are urgently needed.

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