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Synthesis and antirhinovirus activity of new 3-benzyl chromene and chroman derivatives

Cinzia Conti [a], Nicoletta Desideri [b,*]

[a] Istituto Pasteur Fondazione Cenci Bolognetti, Dipartimento di Scienze di Sanità Pubblica, Sezione di Microbiologia, Università 'La Sapienza' di Roma, P.le A. Moro, 5, 00185 Roma, Italy
[b] Istituto Pasteur Fondazione Cenci Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, Università 'La Sapienza', P:le A. Moro, 00185 Rome, Italy

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ABSTRACT

A series of 3-benzyl chromenes and chromans were synthesized and tested in vitro against human rhinovirus (HRV) 1B and 14, two representative serotypes for rhinovirus group B and A, respectively. All the new compounds, with the exception of 3-benzyl-2*H*-chromene (**3a**), showed a potent activity against HRV serotype 1B within micro or submicromolar range (IC_{50}s from 0.11 to 6.62 μM). The low cytotoxicity of all the derivatives resulted in compounds with high therapeutic index (TI). On the contrary, HRV 14 infection was only weakly inhibited by the majority of these compounds. The 3-benzylidenechromans **2b** and **2c** showed the highest anti-HRV 1B activity (IC_{50} 0.12 and 0.11 μM, respectively) coupled with remarkable TI (625.00 and 340.91, respectively). Mechanism of action studies on (*Z*)-3-(4-chlorobenzylidene)chroman (**2b**) suggest that the new compounds behave as capsid binders and interfere with very early stages of HRV 1B replication, similarly to related flavanoids.

1. Introduction

The human rhinoviruses (HRV) are important pathogens causing most of the upper respiratory tract infections in humans.[1] Although these infections are often mild and self-limiting, the impact on human productivity and on medical costs is enormous. HRV infections are also associated with several serious medical complications such as otitis media, chronic bronchitis and asthma.[2] Since the more than one hundred serotypes of HRVs make the development of a vaccine impractical, extensive efforts have been focused on the development of effective antiviral agents for the treatment of HRV infections. However, despite the in vitro activity of several compounds, to date only few drugs have shown efficacy in humans and none has been approved for clinical use.[3]

Among the structurally different classes of molecules inhibiting picornavirus replication in vitro, we focused our attention on natural and synthetic flavanoids and flavonoids. Several flavans, isoflavans, 3(2*H*)-isoflavenes and homo-isoflavonoids exhibited a broad spectrum of antipicornavirus activity.[4–9] Studies on the mechanism of action of selected flavanoids on HRV and EV infections suggested that they specifically interfere with some early events of viral replication. In the case of HRV, 4′,6-dicyanoflavan delayed the uncoating kinetic of neutral-red encapsidated virus and prevented mild acid or thermal inactivation of virus infectivity, suggesting an action at the uncoating level due to a stabilizing effect on virion capsid conformation.[10] In the case of poliovirus, 3(2*H*)-isoflavene was found to exert its action during the uncoating step. Analysis of mutations conferring resistance provides strong evidence that 3(2*H*)-isoflavene inserts into a hydrophobic pocket inside capsid protein VP1, increasing the stability of the viral particle and making the virus resistant to uncoating.[11] The capsid protein VP1 is considered a promising therapeutic target since several compounds which have reached clinical evaluation were shown to bind to this viral protein.[12] The presence of so-called capsid-binding molecules appears to stabilize the protein coat, preventing adsorption of the virus to the cell and/or uncoating.[12]

In continuation of the search for more potent and highly selective analogues, we designed (*Z*)-3-benzylidenechromans (**2a–d**), 3-benzyl-2*H*-chromenes (**3a–d**) and 3-benzylchromans (**4a–d**, **6a–d**, **7a**, **7d**) related to the most active synthetic 3(2*H*)-isoflavenes[4–6] and homoisoflavones[7–9] previously studied by us.

2. Results and discussion

2.1. Chemistry

The synthesis of the designed (*Z*)-3-benzylidenechromans **2a–d** was achieved starting from the corresponding (*E*)-3-benzylidench-

Abbreviations: ATCC, American Type Culture Collection; EV, enterovirus; FCS, foetal calf serum; HRV, human rhinovirus; IC_{50}, 50% inhibitory concentration; MEM, minimum essential medium; MNTC, maximum non-cytotoxic concentration; PBS, phosphate buffered saline; PFU, plaque forming unit; TC_{50}, 50% cytotoxic concentration; TI, therapeutic index; VP, viral protein; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt.

* Corresponding author. Tel.: +39 06 49913892; fax: +39 06 491491.
E-mail address: nicoletta.desideri@uniroma1.it (N. Desideri).

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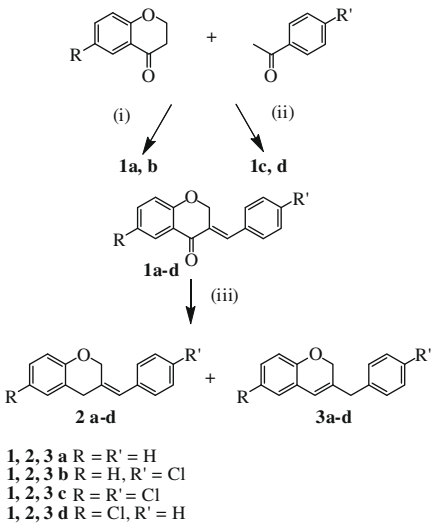
roman-4-ones **1a–d** obtained by condensation of the appropriate chroman-4-one with the suitable benzaldehyde. The reaction was conducted in the presence of 85% phosphoric acid or dry hydrochloric acid in ethyl alcohol following previously described general methods.[7] Subsequent reaction of (*E*)-3-benzylidenchroman-4-ones **1a–d** with lithium aluminium hydride in the presence of aluminium chloride, selectively reduced the carbonyl group to a methylene group with some isomerization of the double bond (Scheme 1). The obtained mixture of (*Z*)-3-benzylidenechromans **2a–d** and 3-benzyl-2*H*-chromenes **3a–d** (about in 3:1 ratio) was separated by column chromatography. The stereochemistry of the double bond of (*Z*)-3-benzylidenechromans **2a–d** was established by 2D NOESY experiments. The strong NOE cross peaks between H4 and vinylic proton, and also between H2 and H2′, H6′ unequivocally indicated the *cis* configuration of this double bond. Therefore, the reduction of (*E*)-3-benzylidenchroman-4-ones **1a–d** to (*Z*)-3-benzylidenechromans **2a–d** occurred with the retention of configuration, but the *E*/*Z* descriptors changed because of the change in priority at C3.

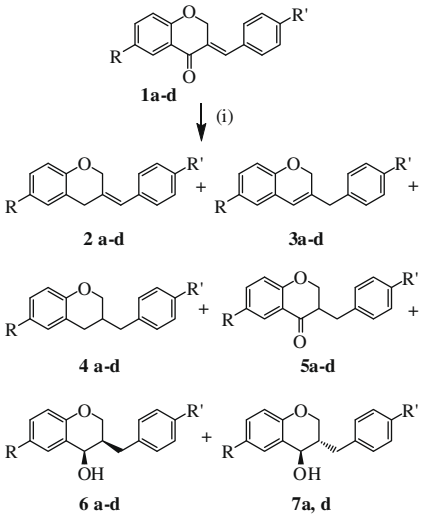
On the contrary, (*E*)-3-benzylidenchroman-4-ones **1a–d** were reduced to a complex mixture of partially and fully reduced products when treated with sodium cyanoborohydride in the presence of zinc iodide (Scheme 2). Complete separation of all the reaction products was achieved by column chromatography. The saturated *cis*-chromanols **6a–d** were the predominant products isolated (about in 40% yield) from this reaction. The stereochemistry of the diastereoisomeric chromanols **6a–d** and **7a**,**d** was assigned on the basis of NMR data which was compared with literature values reported for **6a** and **7a**.[13–15]

Since 3-benzyl-2*H*-chromenes **3a–d** were obtained in poor yield by reduction of (*E*)-3-benzylidenchroman-4-ones **1a–d**, the three-step procedure previously described by us was followed to synthesize these compounds.[16] The synthesis began with the O-alkylation of the appropriate 2-(hydroxymethyl)phenol with 1-bromo-3-phenylpropan-2-one. The intermediate ethers were then converted to the corresponding triphenylphosphonium bromides and finally cyclized by an intramolecular Wittig reaction.[16]

As shown in Scheme 3, 3-benzylchromans **4a–d** were synthesized in good yield by reduction 3-benzylchroman-4-ones **5a–d**[7] with lithium aluminium hydride in the presence of aluminium chloride.

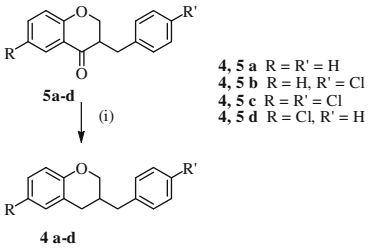


Scheme 1. Reagents and conditions: (i) 85% H_3PO_4, 80 °C, 6 h; (ii) HCl gas, EtOH, rt, 24 h; (iii) $LiAlH_4$, $AlCl_3$, Et_2O, reflux, 30 min.



Compound (Yield %)			
2a (6)	**2b** (7)	**2c** (11)	**2d** (12)
3a (0.5)	**3b** (2)	**3c** (3)	**3d** (0.5)
4a (8)	**4b** (7)	**4c** (4)	**4d** (6)
5a (10)	**5b** (12)	**5c** (11)	**5d** (12)
6a (37)	**6b** (39)	**6c** (39)	**6d** (44)
7a (4)	**7b** (0)	**7c** (0)	**7d** (6)

Scheme 2. Reagents and conditions: (i) $NaCNBH_4$, ZnI_2, $ClCH_2CH_2Cl$, reflux, 20 h.



Scheme 3. Reagents and conditions: (i) $LiAlH_4$, $AlCl_3$, Et_2O, reflux, 30 min.

2.2. Antiviral tests

In preliminary studies, the cytotoxicity of all the new synthesized compounds (**1a–d**, **2a–d**, **3a–d**, **4a–d**, **5a–d**, **6a–d**, **7a**,**d**) was determined by evaluating the effects on morphology, viability and growth of HeLa (Ohio) cells, a human cell line suitable for the replication of HRVs. Morphological alterations were scored microscopically, and the action of the compounds on logarithmic cell growth was determined by the XTT colorimetric method.[17] The inhibitory activity of all the compounds on HRV replication was evaluated in a plaque reduction assay, starting from the maximum non-cytotoxic concentration (MNTC), as already described.[5] A previous systematic evaluation of a panel of capsid-binder compounds against all serotyped HRVs established the existence of two virus groups, called groups A and B, with contrasting susceptibilities for these antivirals. Group B contains twice as many serotypes as group A, and accounts for five times as many colds as group A

serotypes.[18,19] On the basis of these observations, we utilized HRV 1B and 14 as representative serotypes for group B and A, respectively. The results of cytotoxicity and antiviral activity are reported in Table 1. 4′,6-Dichloroflavan (BW683C), an inhibitor of group B serotypes, was included as a control.[20]

With the exception of 3-benzyl-2*H*-chromene **3a**, all chromenes and chromans tested showed high antiviral activity against HRV 1B within micro or submicromolar range (IC_{50}s ranging from 0.11 μM to 6.62 μM). Generally, the potent inhibitory activity on HRV 1B coupled with the low cytotoxicity resulted in compounds with high therapeutic index (TI). In contrast, only a modest inhibition of HRV 14 replication was observed up to MNTCs.

Among the (*Z*)-3-benzylidenechromans **2a–d**, 3-benzyl-2*H*-chromenes **3a–d** and 3-benzylchroman-4-ols **6a–d**, the anti-HRV 1B effect was more pronounced for the 4′-chloro and 4′,6-dichloro analogues (compounds **b** and **c**, respectively) than for the corresponding unsubstituted or 6-chloro substituted derivatives (compounds **a** and **d**, respectively). Therefore, a chlorine at the 4′-position appears a key element for optimum activity. In fact, the (*Z*)-3-benzylidenechromans **2b** and **2c** were the most potent compounds in this novel family of inhibitors, with IC_{50}s of 0.12 μM and 0.11 μM, and TIs of 625.00 and 340.91, respectively.

In contrast, among the 3-benzylchromans **4a–d**, the unsubstituted compound **4a** exhibited the highest activity (IC_{50} = 0.23 μM), the corresponding 4′,6-dichloro derivative **4c** showed comparable potency (IC_{50} = 0.29 μM), while 3-(4-chlorobenzyl)chroman **4b** was threefold less effective (IC_{50} = 0.69 μM) than the parent compound **4a**. In addition, these three 3-benzylchromans combined a very low cytotoxicity with a submicromolar effect on the HRV 1B infection.

Although all the 3-benzylchroman-4-ols (**6a–d**, **7a** and **7d**) exhibited an anti-HRV 1B effect within micromolar range, they showed a marked reduction in potency in comparison with the corresponding chromans unsubstituted at the 4-position (**4a–d**).

2.3. Mechanism of action study

On the basis of both high anti-HRV 1B activity and therapeutic index (IC_{50} = 0.12 μM and TI = 625), (*Z*)-3-(4-chlorobenzylidene)chroman (**2b**) was chosen to clarify the mechanism of antiviral action by evaluating the effects produced either on virus particles or multiplication.

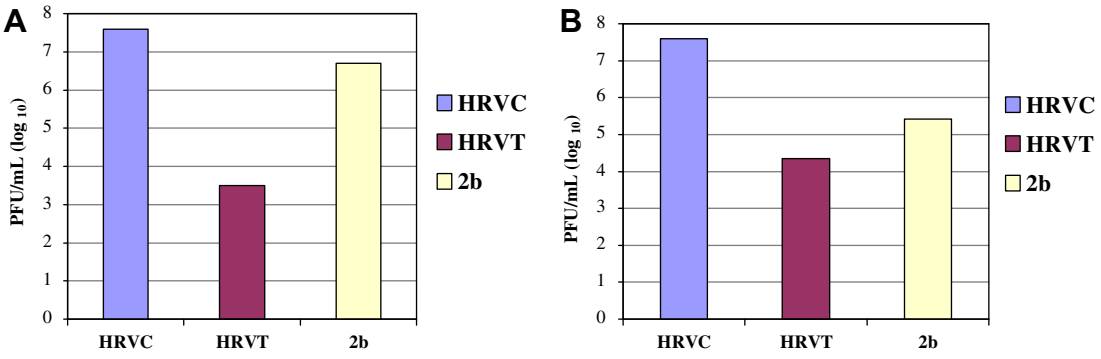
Initially, the direct effect of **2b** on HRV 1B infectivity and stability was investigated. The virus-neutralizing action was studied incubating HRV 1B at high titre with the compound at a concentration of 12 μM, corresponding to 100 times the IC_{50}. After 10-fold dilutions to achieve non-inhibitory concentrations of free compound, the infectivity titres of mock- and **2b**-treated virus suspensions were found to be similar (3.9×10^7 PFU/mL and 4.1×10^7 PFU/mL, respectively). The same results were observed even at a higher concentration (36 μM) (4.0×10^7 PFU/mL). On the contrary, **2b** (12 μM) significantly protected HRV 1B against inactivation by mild acid or heat treatments. Results reported in Figure 1 show that, in the absence of compound, the infectivity of control virus decreased significantly when exposed to either pH 5 (Fig. 1A) or 56 °C (Fig. 1B). In the presence of **2b**, the drop in HRV infectivity was significantly reduced and the protective effect towards low pH and thermal inactivation was 3.2 and 1.4 log, respectively. Taken together, these results indicate that **2b** stabilized the virion capsid conformation without modifying the virus infectivity. Therefore, binding of compound is important to the inhibitory action, although it was reversible by dilution.

The antiviral activity of **2b** (12 μM or 36 μM) towards different stages of HRV 1B multiplication was investigated under one-step growth conditions. Compound was continuously present during the entire time of virus replication, during virus binding to the cell membrane only or added or removed at different time intervals after virus adsorption in the cold. At both concentrations, maximal inhibition (more than 99%) was achieved when compound **2b** was

Table 1
Cytotoxicity and anti-rhinovirus activity of (*Z*)-3-benzylidenechromans (**2a–d**), 3-benzyl-2*H*-chromenes (**3a–d**), 3-benzylchromans (**4a–d**), (*cis*)-3-benzylchroman-4-ols (**6a–d**) and (*trans*)-3-benzylchroman-4-ols (**7a**, **7d**)

Compound	R	R′	MNTC[a] (μM)	TC_{50}[b] (μM)	IC_{50}[c] (μM)		TI[d]
					HRV 1B	HRV 14	
2a	H	H	25.00	150	6.62	21.59	22.66
2b	H	Cl	12.50	75	0.12[e]	12.50 (21.3%)	625.00
2c	Cl	Cl	1.56	37.5	0.11	1.56 (14.2%)	340.91
2d	Cl	H	25.00	150	1.34	25.00 (43.3%)	119.94
3a	H	H	25.00	150	25.00 (36.8%)[e]	25.00 (23.9%)[e]	–
3b	H	Cl	6.25	150	0.34	6.25 (12.8%)	441.18
3c	Cl	Cl	1.56	37.5	0.15	1.56 (11.2%)	250.00
3d	Cl	H	3.12	75	2.40	3.12 (19.3%)	31.25
4a	H	H	25.00	75	0.23[e]	25.00 (41.4%)	326.09
4b	H	Cl	6.25	>200[f]	0.69	6.25 (12.4%)	>289.86
4c	Cl	Cl	12.50	75	0.29[e]	12.50 (33.0%)	258.62
4d	Cl	H	25.00	>200[f]	1.16	25.00 (35.3%)	>172.41
6a	H	H	25.00	150	3.49[e]	25.00 (15.9%)	42.98
6b	H	Cl	12.50	75	1.86[e]	NA[g]	40.32
6c	Cl	Cl	25.00	150	1.48	25.00 (7.0%)	101.35
6d	Cl	H	12.50	75	2.45	12.50 (47.7%)	30.61
7a	H	H	25.00	>200[f]	3.59[e]	NA[g]	>55.71
7d	Cl	H	6.25	150	6.25	6.25 (27.3%)	24.00
BW683C			25.00	>25.00[f]	0.026	NA[g]	>961

[a] The maximum non-cytotoxic concentration (MNTC) was the highest dose tested that did not produce any cytotoxic effect and reduction in viability of HeLa cells, or on cell growth after 3 days of incubation at 37 °C.
[b] The TC_{50} value was the concentration of compound which reduced the cell viability by 50%, as compared with the control.
[c] The IC_{50} value was the dose of compound reducing the plaque number by 50% and was calculated by plotting the log of drug concentration versus the percentage of plaque reduction. When a 50% reduction was not achieved, the percent of inhibition obtained at the MNTC was reported in parentheses.
[d] The therapeutic index (TI) value was equal to TC_{50}/IC_{50} against HRV 1B.
[e] Compound producing a reduction (from 70% to 50%) in the mean viral plaque size, beside an effect on plaque number.
[f] The saturation concentration in cell culture medium was found to be lower than TC_{50}.
[g] Not active up to the highest concentration tested (MNTC).



HRVC: Untreated virus,
HRVT: Virus exposed to pH 5.0,
2b: Virus exposed to pH 5.0 in the presence of **2b**.

HRVC: Untreated virus,
HRVT: Virus heated at 56 ºC,
2b: Virus heated at 56 ºC in the presence of **2b**.

Figure 1. Protective effect of **2b** on acid (A) and thermal inactivation (B) of HRV 1B infectivity.

added to cells together with the virus and maintained until the end of HRV multiplication. When the compound was added to infected HeLa cells immediately after virus binding, the reduction in virus yield was still 94%. Instead, when **2b** was added 30 min after virus binding, inhibition dropped to 44% at 36 μM and 6% at 12 μM (Fig. 2). At the same time, removal of **2b** from infected cells after only 30 min of contact still produced more than 93% suppression in virus production. Similar levels of inhibition were achieved when the compound was removed after longer incubation times (Fig. 3).

Remarkably, maximal reduction of virus yield was also observed when **2b** was present during the time of virus adsorption only (1 h, 4 °C) (Fig. 4). Instead, treatment of HeLa cells (1 h, 4 °C) with **2b** before HRV infection, produced only a moderate level of virus yield reduction (Fig. 4). Together, these results suggest that the compound interferes with virus attachment to the cell surface and that the inhibitory effect on HRV 1B binding is not mediated by an interaction of compound with cellular receptors.

The overall analysis of data from inactivation and stabilization studies of HRV infectivity are consistent with **2b** acting as a capsid-binder, although binding was reversible by dilution. This mechanism of action is shared with several structurally related antipicornavirus agents such as 3(2*H*)-isoflavene.[11] Previous research on capsid-binding compounds demonstrate that optimum activity is associated with a high degree of occupancy of the pocket. Therefore, the difference in activity by **2b** observed against HRV 1B and

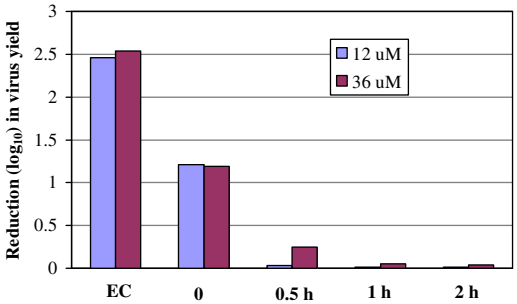


Figure 2. Effect of varying the time of addition of **2b** (12 μM or 36 μM) on the inhibition of HRV 1B replication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was 3.8×10^5 PFU/mL. **EC**: **2b** was present during the entire infection cycle (1 h at 4 °C and 10 h at 33 °C). 0, 0.5 h, 1 h, 2 h: compound was added at different times (0, 0.5, 1, 2 h) after the virus adsorption period (1 h at 4 °C, time 0) and maintained until the end of virus multiplication (up to 10 h).

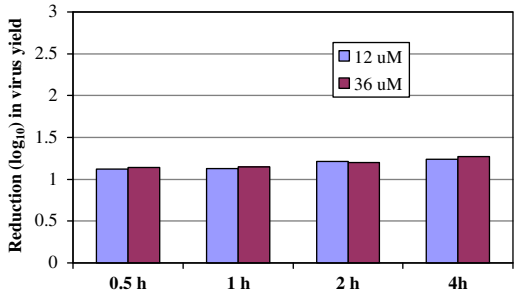


Figure 3. Effect of varying the time of removal of **2b** (12 μM or 36 μM) on the inhibition of HRV 1B replication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was 3.8×10^5 PFU/mL. Compound was added after virus adsorption period (1 h at 4 °C, time 0) and removed at different lengths of incubation (0.5, 1, 2 h).

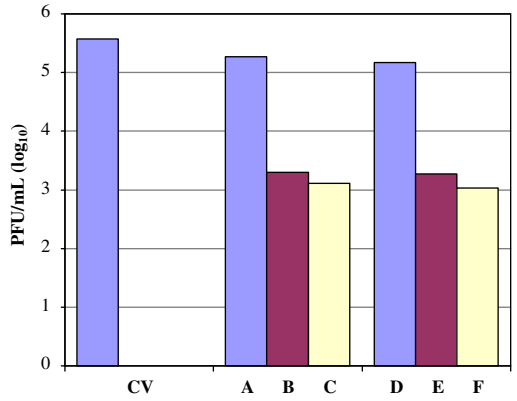


Figure 4. Effect of **2b** on HRV 1B attachment to the cell surface and on membrane receptors for virus. Virus yield was determined by plaque assay after one cycle of virus growth (1 h at 4 °C and 10 h at 33 °C). **CV**: Virus control. **A** (12 μM) and **D** (36 μM): Cells exposed to **2b** for 1 h at 4 °C before virus attachment (1 h at 4 °C). **B** (12 μM) and **E** (36 μM): Cells exposed to **2b** during the time of virus attachment (1 h at 4 °C). **C** (12 μM) and **F** (36 μM): Cells exposed to **2b** during virus attachment (1 h at 4 °C) and replication (10 h at 33 °C).

14 could be attributed to the relative size of the binding site within the capsid protein VP1. In fact, the viral group B binding site accommodates shorter molecules, while longer compounds were routinely more active against serotype 14.[21] In agreement with the capsid-binder hypothesis, data from time of addition/removal

studies indicate that **2b** interferes with very early event(s) of virus infection process, as already reported for several related compounds.[6,10,11,22] Moreover, the finding that an equal efficacy is achieved when **2b** is present during the entire replication time or during virus binding only, strongly suggests that this compound exerts its antiviral effect by a direct interaction with virus particles during HRV attachment to the cell surface. This observation is further supported by the absence of a direct action on cell membrane receptors for virus in pre-treatment studies.

Mechanism of action studies of previously synthesized compounds, such as 4′,6-dicyanoflavan and isoflavans, suggested an interference with the uncoating of viral genome without an action on HRV adsorption.[10,22] Remarkably, (*Z*)-3-(4-chlorobenzylidene)chroman (**2b**), besides an effect at the uncoating level, as suggested by protection of virus infectivity against low pH and thermal inactivation, demonstrated a significant activity also during the HRV attachment step to the host cell membrane.

3. Conclusion

A series of 3-benzyl chromene and chroman derivatives was synthesized and tested for its anti HRV activity and cytotoxicity in vitro. Several members of this new family of inhibitors showed submicromolar potency against HRV 1B coupled with a high therapeutic index.

Similarly to related flavanoids, the new compounds behaved as capsid-binders and interfered with very early events of virus multiplication.

4. Experimental

4.1. Chemistry

Chemicals were purchased from Sigma–Aldrich or Alfa Aesar and used without further purification. Melting points were determined on a Stenford Research Systems OptiMelt (MPA-100) apparatus and are uncorrected. ^{1}H NMR spectra were detected with a Bruker AM-400 spectrometer, using TMS as internal standard. IR spectra were recorded on a FT-IR PerkinElmer Spectrum 1000. All compounds were routinely checked by thin-layer chromatography (TLC) and ^{1}H NMR. TLC was performed on silica gel or aluminium oxide fluorescent coated plates (Merck, Kieselgel or Aluminium oxide 60 F254). Components were visualized by UV light. Elemental analyses (C, H, Cl) of all new compounds were within ±0.4% of theoretical values. (*E*)-3-Benzylidenechroman-4-ones (**1a**–**d**) were synthesized following the procedure previously described.[7] 3-Benzyl-2*H*-chromenes (**3a**–**d**)[16] and 3-benzylchroman-4-ones (**5a**–**d**)[7] were prepared with high yields according to the methods previously reported.

4.1.1. General procedure for the reduction of 1a–d with lithium aluminium hydride and aluminium chloride

A solution of the appropriate (*E*)-3-benzylidenechroman-4-one (**1a**–**d**) (10 mmol) in dry ethyl ether (120 mL) was added dropwise to a suspension of lithium aluminium hydride (17.5 mmol) and aluminium chloride (35.0 mmol) in dry ethyl ether (20 mL). After complete addition, the mixture was refluxed for 30 min. Excess of reducing reagent was destroyed by adding ethyl acetate at 0 °C, and the mixture was poured into 2 N hydrochloric acid. The ether layer was washed with brine, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The obtained mixture of the corresponding (*Z*)-3-benzylidenechromans (**2a**–**d**) and 3-benzyl-2*H*-chromenes (**3a**–**d**) (about 3:1 ratio) were separated by column chromatography on silica gel eluting with light petroleum.

4.1.1.1. (*Z*)-3-Benzylidenechroman (2a). Yield: 37%, colourless oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.37–7.18 (m, 5H, H2′–H6′), 7.13–7.08 (m, 2H, H5, H7), 6.90 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.4 Hz, J_{6-8} = 1.1 Hz), 6.84 (dd, 1H, H8, J_{7-8} = 8.0 Hz, J_{6-8} = 1.1 Hz), 6.69 (s, 1H, H9), 4,83 (s, 2H, H2), 3.66 (s, 2H, H4). Anal. Calcd for $C_{16}H_{14}O$: C, 86.45; H, 6.35. Found: C, 86.73; H, 6.30.

4.1.1.2. (*Z*)-3-(4-Chlorobenzylidene)chroman (2b). Yield: 42%, mp = 77–79 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.32 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.6 Hz), 7.13–7.08 (m, 4H, H2′, H6′, H5, H7), 6.91 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.4 Hz, J_{6-8} = 1.1 Hz), 6.84 (dd, 1H, H8, J_{7-8} = 8.0 Hz, J_{6-8} = 1.1 Hz), 6.63 (s, 1H, H9), 4,79 (s, 2H, H2), 3.65 (s, 2H, H4). Anal. Calcd for $C_{16}H_{13}ClO$: C, 74.85; H, 5.10; Cl, 13.81. Found: C, 74.98; H, 5.25; Cl, 14.00.

4.1.1.3. (*Z*)-6-Chloro-3-(4-chlorobenzylidene)chroman (2c). Yield: 28%, mp = 71–72 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.33 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.3 Hz), 7.11 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.4 Hz), 7.07–7.05 (m, 2H, H5, H7), 6.76 (d, 1H, H8, J_{7-8} = 8.6 Hz), 6.64 (s, 1H, H9), 4,77 (s, 2H, H2), 3.61 (s, 2H, H4). Anal. Calcd for $C_{16}H_{12}Cl_2O$: C, 66.00; H, 4.15; Cl, 24.35. Found: C, 66.12; H, 4.22; Cl, 24.15.

4.1.1.4. (*Z*)-3-Benzylidene-6-chlorochroman (2d). Yield: 32%, mp = 68–70 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.40–7.25 (m, 3H, H3′–H5′), 7.18 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.2 Hz), 7.07–7.04 (m, 2H, H5, H7), 6.76 (d, 1H, H8, J_{7-8} = 8.6 Hz), 6.70 (s, 1H, H9), 4,81 (s, 2H, H2), 3.61 (s, 2H, H4). Anal. Calcd for $C_{16}H_{13}ClO$: C, 74.85; H, 5.10; Cl, 13.81. Found: C, 74.99; H, 5.08; Cl, 13.96.

4.1.1.5. 3-Benzyl-2*H*-chromene (3a). Yield: 12%, colourless oil (lit.[23] 31 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.29–7.16 (m, 5H, H2′–H6′), 7.02 (ddd, 1H, H7, J_{6-7} = 7.4 Hz, J_{7-8} = 8.0 Hz, J_{5-7} = 1.7 Hz), 6.88 (dd, 1H, H5, J_{5-6} = 7.4 Hz, J_{5-7} = 1.7 Hz), 6.80 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.4 Hz, J_{6-8} = 1.2 Hz), 6.74 (dd, 1H, H8, J_{7-8} = 8.0 Hz, J_{6-8} = 1.2 Hz), 6.11 (s, 1H, H4), 4,59 (s, 2H, H2), 3.34 (s, 2H, H9). Anal. Calcd for $C_{16}H_{14}O$: C, 86.45; H, 6.35. Found: C, 86.67; H, 6.23.

4.1.1.6. 3-(4-Chlorobenzyl)-2*H*-chromene (3b). Yield: 17%, mp = 74–75 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.29 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.6 Hz), 7.16 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.6 Hz), 7.07 (ddd, 1H, H7, J_{6-7} = 7.4 Hz, J_{7-8} = 8.0 Hz, J_{5-7} = 1.6 Hz), 6.92 (dd, 1H, H5, J_{5-6} = 7.4 Hz, J_{5-7} = 1.6 Hz), 6.84 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.4 Hz, J_{6-8} = 1.1 Hz), 6.76 (dd, 1H, H8, J_{7-8} = 8.0 Hz, J_{6-8} = 1.1 Hz), 6.14 (s, 1H, H4), 4,62 (s, 2H, H2), 3.38 (s, 2H, H9). Anal. Calcd for $C_{16}H_{13}ClO$: C, 74.85; H, 5.10; Cl, 13.81. Found: C, 74.65; H, 5.24; Cl, 13.99.

4.1.1.7. 6-Chloro-3-(4-chlorobenzyl)-2*H*-chromene (3c). Yield: 8%, mp = 105–106 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.30 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.6 Hz), 7.15 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.6 Hz), 7.00 (dd, 1H, H7, J_{7-8} = 8.6 Hz, J_{5-7} = 2.5 Hz), 6.89 (d, 1H, H5, J_{5-7} = 2.5 Hz), 6.68 (d, 1H, H8, J_{7-8} = 8.6 Hz), 6.06 (s, 1H, H4), 4,62 (s, 2H, H2), 3.38 (s, 2H, H9). Anal. Calcd for $C_{16}H_{12}Cl_2O$: C, 66.00; H, 4.15; Cl, 24.35. Found: C, 66.25; H, 4.13; Cl, 24.46.

4.1.1.8. 3-Benzyl-6-chloro-2*H*-chromene (3d). Yield: 10%, mp = 99–100 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34–7.19 (m, 5H, H2′–H6′), 6.99 (dd, 1H, H7, J_{7-8} = 8.5 Hz, J_{5-7} = 2.5 Hz), 6.88 (d, 1H, H5, J_{5-7} = 2.5 Hz), 6.66 (d, 1H, H8, J_{7-8} = 8.5 Hz), 6.07 (s, 1H, H4), 4,63 (s, 2H, H2), 3.40 (s, 2H, H9). Anal. Calcd for $C_{16}H_{13}ClO$: C, 74.85; H, 5.10; Cl, 13.81. Found: C, 74.57; H, 5.06; Cl, 14.08.

4.1.2. General procedure for the synthesis of the 3-benzylchromans (4a–d)

A solution of the appropriate 3-benzylchroman-4-one (**5a–d**) (10 mmol) in dry ethyl ether (120 mL) was added dropwise to a suspension of lithium aluminium hydride (17.5 mmol) and aluminium chloride (35.0 mmol) in dry ethyl ether (20 mL). After complete addition, the mixture was refluxed for 30 min. Excess of reducing reagent was destroyed by adding ethyl acetate at 0 °C, and the mixture was poured into 2 N hydrochloric acid. The ether layer was washed with brine, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with light petroleum, to give the corresponding 3-benzylchromans (**4a–d**).

4.1.2.1. 3-Benzylchroman (4a). Yield: 64%, mp = 46–48 °C from *n*-hexane (lit.[24] 52 °C from ethyl alcohol). ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.23–7.08 (m, 5H, H2′–H6′), 6.98 (ddd, 1H, H7, J_{6-7} = 7.4 Hz, J_{7-8} = 8.0 Hz, J_{5-7} = 1.6 Hz), 6.89 (dd, 1H, H5, J_{5-6} = 7.4 Hz, J_{5-7} = 1.6 Hz), 6.75–6.70 (m, 2H, H6, H8), 4.08 (dd, 1H, H2, J_{gem} = 10.6 Hz, J_{2-3} = 3.1 Hz), 3.71 (dd, 1H, H2, J_{gem} = 10.6 Hz, J_{2-3} = 8.4 Hz), 2.69 (dd, 1H, H4, J_{gem} = 16.2 Hz, J_{3-4} = 4.9 Hz), 2.60 (dd, 1H, H9, J_{gem} = 13.6 Hz, J_{3-9} = 7.4 Hz), 2.51 (dd, 1H, H9, J_{gem} = 13.6 Hz, J_{3-9} = 7.6 Hz), 2.42 (dd, 1H, H4, J_{gem} = 16.2 Hz, J_{3-4} = 8.6 Hz), 2.22 (m, 1H, H3). Anal. Calcd for $C_{16}H_{16}O$: C, 85.68; H, 7.19. Found: C, 85.79; H, 7.33.

4.1.2.2. 3-(4-Chlorobenzyl)chroman (4b). Yield: 72%, mp = 71–72 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.3 Hz), 7.13 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.3 Hz), 7.09 (ddd, 1H, H7, J_{6-7} = 7.4 Hz, J_{7-8} = 8.0 Hz, J_{5-7} = 1.6 Hz), 6.99 (dd, 1H, H5, J_{5-6} = 7.4 Hz, J_{5-7} = 1.6 Hz), 6.83 (dt, 1H, H6, J_{5-6} =J_{6-7} = 7.4 Hz, J_{6-8} = 1.1 Hz), 6.80 (dd, 1H, H8, J_{7-8} = 8.0 Hz, J_{6-8} = 1.1 Hz), 4.16 (dd, 1H, H2, J_{gem} = 10.7 Hz, J_{2-3} = 3.0 Hz), 3.82 (dd, 1H, H2, J_{gem} = 10.7 Hz, J_{2-3} = 8.0 Hz), 2.80 (dd, 1H, H4, J_{gem} = 16.2 Hz, J_{3-4} = 5.3 Hz), 2.67 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.5 Hz), 2.59 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.7 Hz), 2.50 (dd, 1H, H4, J_{gem} = 16.2 Hz, J_{3-4} = 8.3 Hz), 2.27 (m, 1H, H3). Anal. Calcd for $C_{16}H_{15}ClO$: C, 74.27; H, 5.84; Cl, 13.70. Found: C, 74.31; H, 5.76; Cl, 13.88.

4.1.2.3. 6-Chloro-3-(4-chlorobenzyl)chroman (4c). Yield: 20%, mp = 75–78 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.26 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.08 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.5 Hz), 7.01 (dd, 1H, H7, J_{7-8} = 8.7 Hz, J_{5-7} = 2.5 Hz), 6.95 (d, 1H, H5, J_{5-7} = 2.5 Hz), 6.71 (d, 1H, H8, J_{7-8} = 8.7 Hz), 4.11 (dd, 1H, H2, J_{gem} = 10.6 Hz, J_{2-3} = 3.0 Hz), 3.77 (dd, 1H, H2, J_{gem} = 10.6 Hz, J_{2-3} = 8.1 Hz), 2.73 (dd, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 5.2 Hz), 2.64 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.6 Hz), 2.54 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.6 Hz), 2.43 (dd, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 8.2 Hz), 2.23 (m, 1H, H3). Anal. Calcd for $C_{16}H_{14}Cl_2O$: C, 65.55; H, 4.81; Cl, 24.18. Found: C, 66.80; H, 4.97; Cl, 24.03.

4.1.2.4. 3-Benzyl-6-chlorochroman (4d). Yield: 56%, mp = 54–55 °C from *n*-hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.38–7.17 (m, 5H, H2′–H6′), 7.03 (dd, 1H, H7, J_{7-8} = 8.6 Hz, J_{5-7} = 2.4 Hz), 7.00 (d, 1H, H5, J_{5-7} = 2.4 Hz), 6.73 (d, 1H, H8, J_{7-8} = 8.6 Hz), 4.17 (dd, 1H, H2, J_{gem} = 10.7 Hz, J_{2-3} = 3.1 Hz), 3.81 (dd, 1H, H2, J_{gem} = 10.7 Hz, J_{2-3} = 8.4 Hz), 2.79 (dd, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 5.1 Hz), 2.70 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.5 Hz), 2.61 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.7 Hz), 2.49 (dd, 1H, H4, J_{gem} = 16.4 Hz, J_{3-4} = 8.6 Hz), 2.30 (m, 1H, H3). Anal. Calcd for $C_{16}H_{15}ClO$: C, 74.27; H, 5.84; Cl, 13.70. Found: C, 74.03; H, 5.98; Cl, 13.63.

4.1.3. General procedure for the reduction of 1a–d with sodium cyanoborohydride in the presence of zinc iodide

Solid zinc iodide (15 mmol) and sodium cyanoborohydride (75 mmol) were added at room temperature to a solution of (*E*)-3-benzylidenechroman-4-one (**1a–d**) (10 mmol) in 1,2-dichloroethane (50 mL). The reaction mixture was refluxed for 20 h. After cooling to room temperature, the mixture was poured into an ice-cold mixture of saturated ammonium chloride solution containing 10% of 6 N hydrochloric acid (200 mL). After stirring until the gas evolution ceased, the mixture was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The residue was chromatographed on silica gel eluting with light petroleum to separate (*Z*)-3-benzylidenechroman (**2a–d**), 3-benzyl-2*H*-chromene (**3a–d**) and 3-benzylchroman (**4a–d**). Further elution with ethyl acetate and light petroleum (1:10) allowed the isolation of 3-benzylchroman-4-one (**5a–d**), (*cis*)-3-benzylchroman-4-ol (**6a–d**) and, eventually, (*trans*)-3-benzylchroman-4-ol (**7a**, **7d**). The yields of all the compounds are reported in Scheme 2.

4.1.3.1. (*cis*)-3-Benzylchroman-4-ol (6a). Yield: 37%, mp = 113–114 °C from *n*-hexane (lit.[13] 114 °C from benzene–hexane). IR (KBr): 3440 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.37–7.17 (m, 7H, H2′–H5′, H5, H7), 6.89 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.4 Hz, J_{6-8} = 1.0 Hz), 6.84 (dd, 1H, H8, J_{7-8} = 8.2 Hz, J_{6-8} = 1.0 Hz), 4.52 (t, 1H, H4, J_{3-4} = J_{4-OH} = 4.6 Hz), 4.14–4.07 (m, 2H, H2), 2.89 (dd, 1H, H9, J_{gem} = 13.6 Hz, J_{3-9} = 8.4 Hz), 2.68 (dd, 1H, H9, J_{gem} = 13.6 Hz, J_{3-9} = 7.3 Hz), 2.33 (m, 1H, H3), 1.65 (d, 1H, OH, J_{4-OH} = 4.6 Hz). Anal. Calcd for $C_{16}H_{16}O_2$: C, 79.97; H, 6.71. Found: C, 80.07; H, 6.83.

4.1.3.2. (*cis*)-3-(4-Chlorobenzyl)chroman-4-ol (6b). Yield: 39%, mp = 130–132 °C from *n*-hexane. IR (KBr): 3528 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.15 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.06–7.03 (m, 4H, H2′, H6′, H5, H7), 6.74 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.5 Hz, J_{6-8} = 1.2 Hz), 6.70 (dd, 1H, H8, J_{7-8} = 8.1 Hz, J_{6-8} = 1.2 Hz), 4.31 (d, 1H, H4, J_{3-4} = 2.9 Hz), 3.93–3.88 (m, 2H, H2), 2.68 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 8.6 Hz), 2.45 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.2 Hz), 2.19 (m, 1H, H3), 1.90 (br s, 1H, OH). Anal. Calcd for $C_{16}H_{15}ClO_2$: C, 69.95; H, 5.50; Cl, 12.90. Found: C, 70.03; H, 5.63; Cl, 12.98.

4.1.3.3. (*cis*)-6-Chloro-3-(4-chlorobenzyl)chroman-4-ol (6c). Yield: 39%, mp = 149–150 °C from *n*-hexane. IR (KBr): 3530 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.30 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.3 Hz), 7.21–7.19 (m, 3H, H2′, H6′, H5), 7.15 (dd, 1H, H7, J_{7-8} = 8.7 Hz, J_{5-7} = 2.5 Hz), 6.78 (d, 1H, H8, J_{7-8} = 8.7 Hz), 4.46 (m, 1H, H4), 4.09–4.05 (m, 2H, H2), 2.85 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 8.5 Hz), 2.63 (dd, 1H, H9, J_{gem} = 13.7 Hz, J_{3-9} = 7.4 Hz), 2.25 (m, 1H, H3), 1.70 (d, 1H, OH, J_{4-OH} = 4.5 Hz). Anal. Calcd for $C_{16}H_{14}Cl_2O_2$: C, 62.15; H, 4.56; Cl, 22.93. Found: C, 62.03; H, 4.77; Cl, 23.02.

4.1.3.4. (*cis*)-6-Chloro-3-benzylchroman-4-ol (6d). Yield: 44%, mp = 113–115 °C from *n*-hexane. IR (KBr): 3380 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.38–7.24 (m, 5H, H2′–H6′), 7.19 (d, 1H, H5, J_{5-7} = 2.6 Hz), 7.14 (dd, 1H, H7, J_{7-8} = 8.7 Hz, J_{5-7} = 2.6 Hz), 6.78 (d, 1H, H8, J_{7-8} = 8.7 Hz), 4.48 (d, 1H, H4, J_{3-4} = 3.2 Hz), 4.12–4.04 (m, 2H, 2H2), 2.86 (dd, 1H, H9, J_{gem} = 13.6 Hz, J_{3-9} = 8.4 Hz), 2.68 (dd, 1H, H9, J_{gem} = 13.6 Hz, J_{3-9} = 7.3 Hz), 2.30 (m, 1H, H3), 1.66 (br s, 1H, OH). Anal. Calcd for $C_{16}H_{15}ClO_2$: C, 69.95; H, 5.50; Cl, 12.90. Found: C, 70.11; H, 5.68; Cl, 12.73.

4.1.3.5. (*trans*)-3-Benzylchroman-4-ol (7a). Yield: 5%, mp = 143–144 °C from *n*-hexane (lit.[13] 144 °C from benzene–hexane). IR (KBr): 3380 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34–7.17 (m, 7H, H2′–H5′, H5, H7), 6.96 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.4 Hz, J_{6-8} = 1.0 Hz), 6.88 (dd, 1H, H8, J_{7-8} = 8.2 Hz, J_{6-8} = 1.0 Hz), 4.55 (t, 1H, H4, J_{3-4} = J_{4-OH} = 4.6 Hz), 4.23 (dd, 1H, H2, J_{gem} = 11.1 Hz, J_{2-3} = 2.6 Hz), 3.98 (dd, 1H, H2, J_{gem} = 11.1 Hz, J_{2-3} = 4.5 Hz), 2.71 (dd, 1H, H9, J_{gem} = 13.8 Hz, J_{3-9} = 6.5 Hz), 2.54 (dd, 1H, H9, J_{gem} = 13.8 Hz,

J_{3-9} = 9.2 Hz), 2.23 (m, 1H, H3), 1.82 (d, 1H, OH, $J_{4\text{-OH}}$ = 4.6 Hz). Anal. Calcd for $C_{16}H_{16}O_2$: C, 79.97; H, 6.71. Found: C, 79.75; H, 6.84.

4.1.3.6. (*trans*)-3-Benzyl-6-chlorochroman-4-ol (7d). Yield: 6%, mp = 137–139 °C from *n*-hexane. IR (KBr): 3380 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.33–7.15 (m, 7H, H2′–H6′, H5, H7), 6.81 (d, 1H, H8, J_{7-8} = 8.7 Hz), 4.46 (d, 1H, H4, J_{3-4} = 4.2 Hz), 4.21 (dd, 1H, H2, J_{gem} = 11.2 Hz, J_{2-3} = 2.9 Hz), 3.97 (dd, 1H, H2, J_{gem} = 11.2 Hz, J_{2-3} = 4.5 Hz), 2.72 (dd, 1H, H9, J_{gem} = 13.8 Hz, J_{3-9} = 6.4 Hz), 2.52 (dd, 1H, H9, J_{gem} = 13.8 Hz, J_{3-9} = 9.0 Hz), 2.22 (m, 1H, H3), 1.68 (sa, 1H, OH). Anal. Calcd for $C_{16}H_{15}ClO_2$: C, 69.95; H, 5.50; Cl, 12.90. Found: C, 70.21; H, 5.43; Cl, 12.98.

4.2. Virology

4.2.1. Cells

HeLa (Ohio) cells were routinely grown at 37 °C using Eagle's Minimum Essential Medium (MEM) supplemented with 100 μg mL^{-1} of streptomycin and 100 U/mL of penicillin G and 8% heat-inactivated foetal calf serum (FCS) (growth medium). The concentration was reduced to 2% for cell maintenance (maintenance medium).

4.2.2. Compounds

Stock solutions were prepared in ethanol (1, 0.5 or 0.1 mg mL^{-1}) and further diluted in tissue culture medium shortly before use.

4.2.3. Virus

Reference strains of HRV type 1B and 14 were purchased from American Type Culture Collection (ATCC). Virus stocks were prepared by inoculating HeLa (Ohio) cell monolayers at a low multiplicity of infection (0.1 PFU/cell). Infected cells were incubated at 33 °C. When the viral-induced cytopathic effect involved most of the cells, the cultures were freeze-thawed three times and the clarified supernatants titrated by plaque assay, essentially as described by Fiala and Kenny.[25] The virus was stored in aliquots at −80 °C until used.

4.2.4. XTT assay for cellular cytotoxicity

A tetrazolium-based (XTT) colorimetric assay was used to measure the cytotoxicity of compounds. This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by viable, active cells. The formazan dye formed is soluble in aqueous solution and is directly quantified using a scanning multiwell spectrophotometer.[17] HeLa cells were seeded in 96 well tissue culture plates at 2×10^3 cells for well in 100 μL of growth medium with or without compounds in twofold dilutions, starting from the maximum soluble concentration in cell culture medium. Quadruplicate wells were used for each drug concentration to be tested. The plates were incubated at 37 °C in 5% CO_2–air until the untreated monolayers were confluent (3 days). Then, 50 μL of XTT labelling mixture was added to each well (final XTT concentration 0.15 mg mL^{-1}) and the cells incubated for 4 h at 37 °C. The spectrophotometric absorbance of the samples was measured using an ELISA reader at 492 nm with a reference wavelength at 690 nm. Cytotoxicity was also scored microscopically as morphological alterations on the third day of incubation in the presence of compounds. The highest concentration of compound that did not produce any modification of morphology and viability on 100% of cells was the maximum non-cytotoxic concentration. The 50% cytotoxic concentration (TC_{50}) was indicated as the concentration of compound reducing the cell viability by 50%, as compared with mock-treated cells.

4.2.5. Determination of the 50% inhibitory concentration (IC_{50})

The IC_{50} values were determined as described previously.[5] Briefly, confluent monolayers of HeLa cells in 6-well plates were infected with a virus suspension producing approximately 100 plaques per well. After 1 h of incubation at 33 °C, the virus inoculum was removed and the cells were overlaid with medium for plaques, in the presence or absence of fourfold dilutions of drugs. After three days of incubation at 33 °C, the cells were stained with a neutral red solution at 0.2 mg mL^{-1} in pH 7.4 phosphate buffered saline (PBS) and the plaques were counted. The IC_{50} was expressed as the concentration of drug reducing the plaque number by 50% as compared with mock-treated control. It was calculated from a dose/response curve obtained by plotting the percentage of plaque reduction, with respect to the control plaque count, versus the logarithm of compound dose. Triplicate wells were utilized for each drug concentration.

4.2.6. Virus inactivation and stabilization

For virus inactivation studies, HRV 1B suspensions with or without **2b** at different concentrations (12 μM and 36 μM) were incubated at 33 °C for 1 h. After serial 10-fold dilutions, virus titres were measured by plaque assay on HeLa cell monolayers.

For virus stabilization studies, the virus was incubated with or without **2b** at different concentrations (12 μM and 36 μM) for 1 h at 33 °C before mild acid or thermal treatment. For mild acid treatment, the pH of the mixtures was adjusted to 5 by adding 0.2 M acetate buffer (pH 5). After incubation at 33 °C for 30 min, the mixtures were neutralized with 0.85 M Tris base. For thermal treatment, the mixtures were incubated for 20 min at 56 °C (pH 7.2) and then refrigerated on ice. All samples were diluted 10-fold serially and titrated by plaque assay on HeLa cell monolayers.

4.2.7. Virus yield reduction assays

Confluent monolayers of HeLa cells in 24-well plates were infected at a multiplicity of 5 in the presence or absence of **2b** (12 μM or 36 μM). The infection was synchronized by allowing HRV 1B to attach in the cold (4 °C). After 1 h, the inoculum was removed by washing thrice with PBS. The end of virus binding is indicated as 0 time. Then, MEM with or without the compound (12 μM or 36 μM) was added and the temperature raised to 33 °C to permit internalization. Single-cycle conditions were achieved by incubating the cells at 33 °C for 10 h post infection (p.i.). The cultures were freeze-thawed three times, cell debris removed by low-speed centrifugation in the cold and the supernatants titrated by plaque assay on HeLa cell monolayers. To determine which stage of virus replication was affected by **2b**, the drug was added or removed from HRV-infected cells at various times p.i. (0.5, 1, 2, 4 h) and the cultures incubated at 33 °C up to 10 h p.i. The virus yield was determined as above.

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