Bioorganic & Medicinal Chemistry 18 (2010) 6480–6488

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

New 4*H*-chromen-4-one and 2*H*-chromene derivatives as anti-picornavirus capsid-binders

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ARTICLE INFO

Article history:
Received 19 April 2010
Revised 24 June 2010
Accepted 29 June 2010
Available online 30 July 2010

Keywords:
2*H*-Chromenes
4*H*-Chromen-4-ones
Antivirals
Human rhinoviruses
Enterovirus 71
Capsid binder

ABSTRACT

Substituted (*E*)-3-styryl-4*H*-chromen-4-ones **1a–d**, 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-ones **2a–d**, (*E*)-3-styryl-2*H*-chromenes **3a–d** and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes **4a–d** were designed and synthesized to improve the anti-picornavirus activity of previously tested analogues. The new compounds were evaluated in vitro against human rhinovirus (HRV) serotypes 1B and 14 and enterovirus (EV) 71. All the compounds interfered with the replication of picornaviruses, although considerable differences were observed in the sensitivity of viruses to each compound. Generally, both HRVs were more susceptible than EV71 and their sensitivity was dependent upon the linker chain length as well as upon the oxidation state of the heterocyclic ring. (*E*)-3-Styryl-2*H*-chromene (**3a**) emerged as the most effective inhibitor of both HRVs showing IC_{50} values of 0.20 μM and 1.38 μM towards serotype 1B and 14, respectively. The potent activity was also coupled with low cytotoxicity resulting in high therapeutic indexes (250 and 36, respectively). Mechanism of action studies indicated that **3a**, like structurally related compounds, behaves as a capsid binder interfering with the early stages of rhinovirus infection, probably at the adsorption and/or uncoating level.

1. Introduction

Picornaviruses are a group of small, non-enveloped, positive stranded RNA viruses that constitute one of the largest virus families and comprise several pathogens implicated in an extensive range of clinical manifestations which affect humans as well as animals. Human rhinoviruses (HRVs) and enteroviruses (EVs) are two related genera within this virus family.

HRVs represent the most important aetiological agents of common cold.[1] The over 100 viral serotypes can be divided into two distinct groups, A and B, on the basis of their susceptibility to capsid-binding compounds.[2,3] Although HRV infections are often mild and self-limiting in healthy adults, the socioeconomic impact caused by missed school or work days is enormous. Moreover, increasing evidences describe the link between HRV infections and several serious upper and lower tract complications such as asthma, chronic bronchitis and otitis media.[4]

EVs comprise poliovirus, echoviruses, coxsackieviruses, and numbered enteroviruses. The clinical manifestations associated with EVs range from mild illnesses such as fever and rash to potentially severe and life-threatening infections, such as meningitis, encephalitis, myocarditis, poliomyelitis, and neonatal sepsis.[5] After the eradication of poliovirus from developed countries, EV71 has emerged as major cause of neurologic threat in the world. Although EV71 usually causes hand, foot and mouth disease, a mild exanthematous infection occurring mainly in young children, it is also associated with acute neurological diseases including aseptic meningitis, acute flaccid paralysis and even fatal neurogenic pulmonary edema.[6] In the last decades a significant increase in epidemic activity of EV71 has been observed and severe outbreaks have been reported in various parts of the world, particularly in the Asia-Pacific region.[7–10]

The difficulty of vaccine development for the majority of picornaviruses makes the search of efficacious drugs a necessity. Although extensive efforts have been devoted to the search for effective agents, to date no drug has been approved by the FDA for clinical use, and the patient care remains symptomatic. Each step during the life cycle of picornaviruses has been considered a potential target for antiviral compounds, however only inhibitors of capsid functions and of 3C protease demonstrated in vivo activity. Since it is well recognized that the structures of viral capsid and 3C protease are relatively conserved among different serotypes, molecules acting on each of these targets could be promising anti-picornavirus agents with a broad-spectrum of activity.[11]

Recently, we prepared and tested against HRV serotype 1B and 14 a series of (*Z*)-3-benzylidenechromans, 3-benzyl-2*H*-chromenes and 3-benzylchromans[12] related to the most active synthetic

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doi:10.1016/j.bmc.2010.06.103

3(2*H*)-isoflavenes[13–15] and homoisoflavones[16–18] previously studied by us. Several members within these families of inhibitors showed submicromolar potency against HRV 1B coupled with a high therapeutic index. On the contrary, HRV 14, a representative serotype A, was only weakly inhibited.[12] Mechanism of action studies indicated that, similarly to related flavanoids,[19,20] the new compounds behave as capsid-binders and interfere with very early events of virus multiplication.[12] The different activity against HRV 1B and 14 has been attributed to the size of the compound-binding site into the capsid protein VP1 varying with the specific serotype. Previous research on capsid-binding compounds indicated that the viral group B binding site accommodates molecules with shorter chains, while long-chained compounds are routinely more active against viral group A rhinoviruses.[21] Subtle differences in the size and shape of the hydrophobic pocket have been described also among EVs. In the case EV71, the binding pocket was shown to accommodate long molecules such as pyridyl imidazolidinones.[22,23] Therefore, we planned to increase the length of the linker chain between the heterocycle and the phenyl moiety in order to occupy more efficiently the capsid pocket of picornaviruses.

In this paper, we describe the synthesis of (*E*)-3-styryl-4*H*-chromen-4-ones **1a–d**, 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-ones **2a–d**, (*E*)-3-styryl-2*H*-chromenes **3a–d** and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes **4a–d** designed to improve the anti-picornavirus spectrum of activity of the previously tested analogues. The antiviral potency of the new derivatives was evaluated against HRV 1B and 14, representative serotypes for group B and A of HRVs, and against EV-71 selected among EVs for its clinical significance.

2. Results and discussion

2.1. Chemistry

The method described by Silva et al.[24] was used for the synthesis of (*E*)-3-styryl-4*H*-chromen-4-ones **1a–d**. This procedure involves the condensation of 4-oxo-4*H*-chromene-3-carbaldehydes with 5 M equiv of phenylacetic acids in the presence of potassium *tert*-butoxide and refluxing dry pyridine. Under these conditions, a Knoevenagel-type reaction, followed by decarboxylation, took place and the (*E*)-3-styryl-4*H*-chromen-4-ones **1a–d** were diastereoselectively obtained (Scheme 1). The coupling constant values of the vinylic protons ($J_{\alpha-\beta}$ ~16 Hz) indicate the *trans* configuration of this double bond.

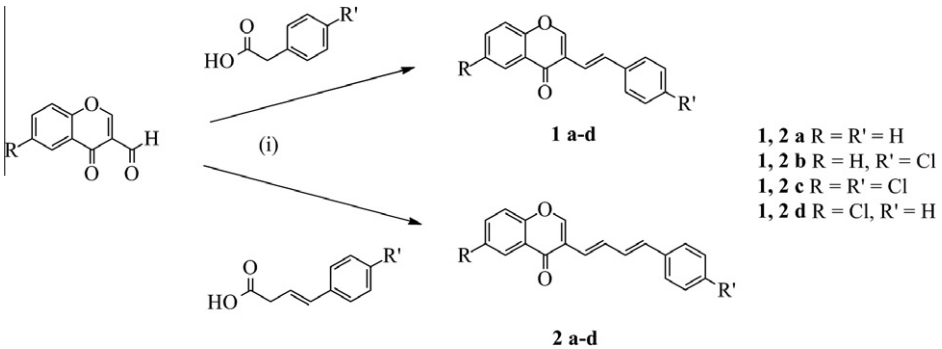
The same experimental conditions were also successfully applied to the condensation of 4-oxo-4*H*-chromene-3-carbaldehydes with (*E*)-4-phenylbut-3-enoic acids in order to prepare 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-ones **2a–d** (Scheme 1). The stereoselectivity of the methods was confirmed by the coupling constant values of the vinylic protons ($J_{\alpha-\beta}$ and $J_{\gamma-\delta}$ ~16 Hz) that prove the *trans* configuration for both double bonds of the dienic system.

(*E*)-3-Styryl-2*H*-chromenes **3a–d** and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes **4a** and **4d** were synthesized according to the two step procedure shown in Scheme 2. For this purpose, 2*H*-chromene-3-carbaldehydes **5a** and **5d** were prepared with some modifications of the literature procedures,[25,26] by refluxing the appropriate 2-hydroxybenzaldehyde and acrolein in the presence of potassium carbonate. The subsequent Horner–Wadsworth–Emmons reaction of 2*H*-chromene-3-carbaldehydes **5a** and **5d** with commercially available diethyl benzylphosphonates or diethyl *trans*-cinnamylphosphonate, carried out using sodium hydride as base, provided only the (*E*) isomers **3a–d**, **4a** and **4d**. The stereochemistry of compounds was established on the basis of the coupling constant values of the protons in the chain ($J_{\alpha-\beta}$ = 16.5 Hz and ~15.6 Hz for **3a–d** and **4a,d**, respectively).

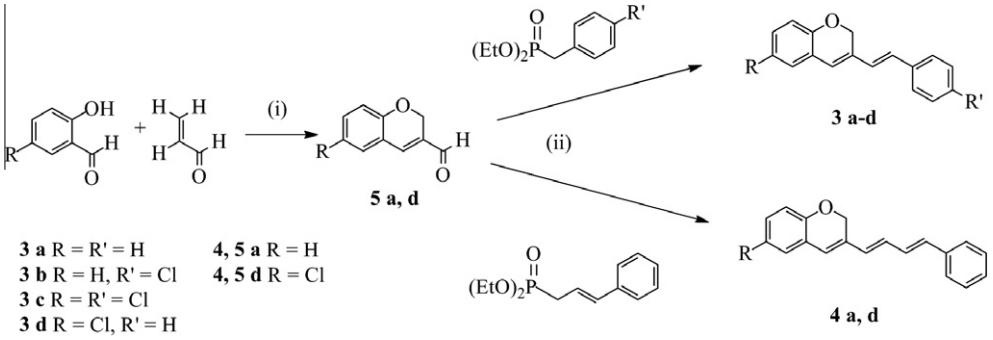
As diethyl *trans*-4-chlorocinnamylphosphonate is not commercially available, an alternative method was adopted to prepare 3-[(1*E*,3*E*)-4-(4-chlorophenyl)buta-1,3-dienyl]-2*H*-chromenes **4b** and **4c** (Scheme 3). The target compounds (**4b** and **4c**) were obtained by the Horner-Wadsworth-Emmons reaction of diethyl 4-chlorobenzylphosphonate with the appropriate (*E*)-3-(2*H*-chromen-3-yl)acrylaldehyde (**6a** and **6d**), obtained by the Wittig reaction of the corresponding 2*H*-chromene-3-carbaldehyde (**5a** and **5d**) with [(1,3-dioxolan-2-yl)methyl]triphenylphosphonium bromide, followed by removal of the protecting group accomplished with oxalic acid. Also this method provided only the isomers with *trans* configuration of both double bonds ($J_{\alpha-\beta}$ and $J_{\gamma-\delta}$ ~15.6 Hz).

2.2. Antiviral tests

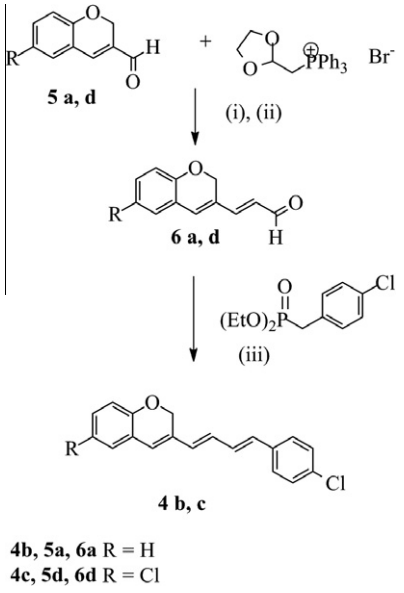
Initially, all the synthesized compounds (**1a–d**, **2a–d**, **3a–d** and **4a–d**) were evaluated for effects on morphology, viability and growth of HeLa and Hep-2 cells, human cell lines suitable for the replication of HRVs and EVs, respectively. Morphological alterations were scored microscopically, and the action of compounds on logarithmic cell growth was determined by the XTT colorimetric method.[27] The maximum non-cytotoxic concentration (MNTC) and the 50% cytotoxic concentration (TC_{50}) of compounds are reported in Table 1. The MNTC was expressed as the highest dose tested that did not produce any toxic effect or reduction of cell growth after 3 days incubation at 37 °C. The TC_{50} was indicated as the concentration of compound reducing the cell viability by 50% as compared with the control. Generally, compounds presented low cytotoxicity against HeLa cells, with the exception of 6-chloro-3-[(1*E*,3*E*)-4-(4-chlorophenyl)buta-1,3-dienyl]-4*H*-chromen-4-one **2c** and 3-[(1*E*,3*E*)-4-(4-chlorophenyl)buta-1,3-dienyl]-2*H*-chromene **4b**. The majority of the compounds exhibited a similar toxicity against Hep-2 cells, while **1a**, **3a** and **3c** were more toxic against this cell line.



Scheme 1. Reagents: (i) *tert*-BuOK, dry Py, reflux.



Scheme 2. Reagents and conditions: (i) K_2CO_3, dry dioxane, reflux, 4 h; (ii) NaH, dry THF, rt 20 h.



Scheme 3. Reagents and conditions: (i) *tert*-BuOK, dry THF, rt 24 h and reflux 6 h; (ii) $(COOH)_2$, H_2O, rt, overnight; (iii) NaH, dry THF, rt 22 h.

The inhibitory activity of compounds on the replication of HRV 1B, HRV 14 and EV71 was evaluated by a plaque reduction assay, starting from the MNTC. HRV 1B and 14 were utilized as representative serotypes for group B and A of HRVs, while EV71 was chosen as a clinically relevant member within EVs. The compound concentration required to produce a 50% reduction of plaque number with respect to mock-treated virus-infected cultures (IC_{50}) is reported in Table 1. When the IC_{50} value was higher than the MNTC, the percentage inhibition at this dose is reported in parentheses. The therapeutic index (TI), expressed as TC_{50} versus IC_{50} ratio, was calculated (Table 1). 4′,6-Dichloroflavan (**BW683C**), an inhibitor of group B serotypes, was included as a control.[28]

The new compounds interfered with the replication of both HRV serotypes and EV71 dose-dependently, although considerable differences were observed in sensitivity of each virus. EV71 was generally less susceptible than both HRVs and the percent reduction of plaque number was found always lower than 50% up to the MNTC. Despite the low potency of all compounds against EV71, derivatives **2a–d** and **4a–d**, with a longer linker chain between the heterocycle and the phenyl moiety, produced a higher percent of inhibition with respect to the corresponding shorter analogues **1a–d** and **3a–d**. Unfortunately, the most active

Table 1
Cytotoxicity and anti-picornavirus activity of (*E*)-3-styryl-4*H*-chromen-4-ones (**1a–d**) and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-ones (**2a–d**), (*E*)-3-styryl-2*H*-chromenes (**3a–d**) and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes (**4a–d**)

Compd	R	R′	MNTC[a] (μM)	TC_{50}[b] (μM)	IC_{50}[c] (μM) HRV 1B	TI[d]	IC_{50}[c] (μM) HRV 14	TI[d]	MNTC[e] (μM)	IC_{50}[c] (μM)E71
1a	H	H	>200.00[f]	>200.00[f]	3.12	>64.16	4.36	>45.87	50.00	50.00 (34.6%)
1b	H	Cl	25.00	50.00	4.54	11.01	5.52	9.06	25.00	25.00 (21.6%)
1c	Cl	Cl	25.00	50.00	2.19	22.19	25 (24.1%)	—	25.00	25.00 (35.7%)
1d	Cl	H	25.00	50.00	3.22	15.53	25 (33.1%)	—	25.00	25.00 (37.0%)
2a	H	H	25.00	50.00	5.27	9.49	15.00	3.33	25.00	25.00 (39.8%)
2b	H	Cl	25.00	50.00	4.09	12.22	4.07	12.28	25.00	25.00 (49.1%)
2c	Cl	Cl	3.12	6.25	0.71	8.80	3.12 (36.2%)	—	3.12	3.12 (46.6%)
2d	Cl	H	50.00	100.00	1.6	62.5	50.00 (48.3%)	—	50.00	50.00 (46.7%)
3a	H	H	25.00	50.00	0.20	250.00	1.38	36.23	12.50	12.50 (36.1%)
3b	H	Cl	50.00	100.00	0.40	250.00	18.39	5.44	50.00	50.00 (49.7%)
3c	Cl	Cl	25.00	50.00	7.13	7.01	8.28	6.04	12.50	12.50 (46.6%)
3d	Cl	H	50.00	100.00	11.20	8.93	11.17	8.95	50.00	50.00 (30.8%)
4a	H	H	50.00	100.00	30.83	3.24	50.00	2.00	50.00	50.00 (29.5%)
4b	H	Cl	3.12	6.25	3.10	2.00	3.12 (30.4%)	—	3.12	3.12 (32.9%)
4c	Cl	Cl	6.25	12.50	0.54	23.15	6.25 (12.8%)	—	6.25	6.25 (39.1%)
4d	Cl	H	12.50	25.00	6.27	3.99	12.50 (37.2%)	—	12.50	12.50 (25.4%)
BW683C			25.00	>25.00	0.026		NA[g]	—	10.00	10.00 (14%)

[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 HeLa 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}.
[e] The maximum non-cytotoxic concentration (MNTC) was the highest dose tested that did not produce any cytotoxic effect and reduction in viability of Hep cells, or on cell growth after 3 days of incubation at 37 °C.
[f] The saturation concentration in cell culture medium was found to be lower than MNTC and TC_{50}.
[g] Not active up to the highest concentration tested (MNTC).

compounds, **2c** and **4b**, were also the most toxic for Hep-2 cells (MNTC = 3.12 μM). Therefore, it was impossible to test both analogues at concentrations higher than 3.12 μM.

On the contrary, several compounds of these series showed significant antiviral activity against both HRV serotypes, although they generally exhibited a higher potency against serotype 1B.

Within the chromen-4-one series (**1a–d** and **2a–d**), 6-chloro-3-[(1*E*,3*E*)-4-(4-chlorophenyl)buta-1,3-dienyl]-4*H*-chromen-4-one **2c** was the most potent compound against HRV 1B, with an IC_{50} of 0.71 μM. Potency was reduced when one (**2b** and **2d**) or both the chlorine atoms (**2a**) were removed or when the linker chain was shortened (**1c**). Despite the high in vitro anti-HRV 1B potency, **2c** showed a modest therapeutic index (TI = 8.80) coupled with a weak activity against HRV 14. On the contrary, although both unsubstituted chromones (**1a** and **2a**) and 4′-chloro substituted chromones (**1b** and **2b**) exhibited a lower activity than **2c** against serotype 1B, they showed comparable potency against both HRV serotypes. In addition to the broader spectrum of anti-HRV activity, (*E*)-3-styryl-4*H*-chromen-4-one **1a** exhibited low cytotoxicity resulting in high therapeutic indexes (TI >64.1 and >45.87 against HRV 1B and 14, respectively).

Compared to compound **2c**, the replacement of the chromen-4-one moiety with the chromene ring (**4c**) produced a slight improvement in anti-HRV 1B potency (IC_{50} = 0.54 μM) and an higher selectivity (TI = 23.15), while the anti-HRV 14 inhibitory activity became even lower.

Remarkably, all the 3- styryl-2*H*-chromenes tested (**3a–d**) displayed a broad-spectrum of anti-HRV activity. 3-Benzyl-2*H*-chromene **3a** showed the highest level of activity against both serotypes (IC_{50} of 0.20 μM and 1.38 μM towards HRV 1B and 14, respectively) and significant selectivity (TI = 250 and 36.23, respectively). All modifications in this series resulted in diminished activity compared with **3a**. In particular, the introduction of a chlorine atom at the 4′ position (**3b**) caused a slight decrease in the inhibitory effect against HRV 1B and a dramatic loss of activity against HRV 14 (2- and 13-fold, respectively). When a chlorine atom was introduced at the 6 position (**3d**) or at both 4′ and 6 positions (**3c**), the reduction in potency and selectivity was substantial for both serotypes.

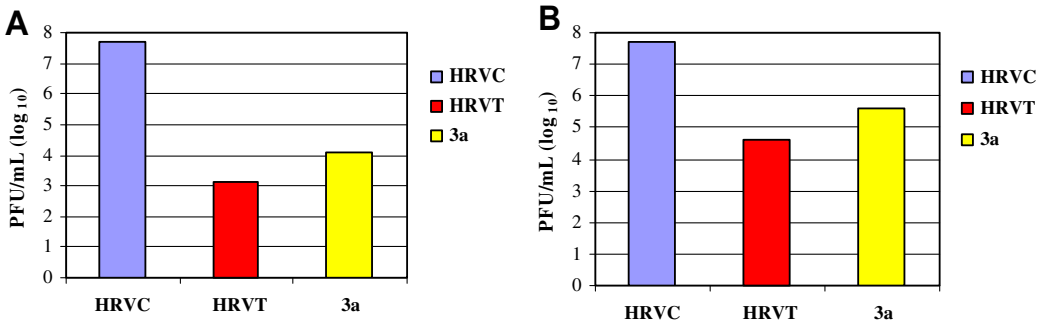
Surprisingly, despite the larger size of the HRV 14 capsid binding site, increasing the linker chain length from two (**3a–d**) to four (**4a–d**) carbon atoms resulted in a loss of activity against this serotype. Moreover, the chromen-4-one derivatives **4a–d** displayed a variable degree of potency against HRV 1B (IC_{50} ranging from 30.83 μM to 0.54 μM) and only the 4′,6-dichloro analogue **4c** was active within a submicromolar range.

2.3. Mechanism of action study

Due to its potent in vitro activity against both HRV serotypes (IC_{50} = 0.20 μM and 1.38 μM, respectively) and high therapeutic indexes (TI = 250.00 and 36.23, respectively), (*E*)-3-styryl-2*H*-chromene **3a** was chosen to investigate the mechanism of the antiviral action. The effects produced either on HRV 1B virus particles or HRV 1B multiplication have been evaluated.

Initially, the action of **3a** on virus infectivity and stability was investigated. The virus-neutralizing potential was studied by incubating HRV 1B at high titre with the compound at the concentration of 20 μM, corresponding to 100 times the IC_{50}. After serial 10-fold dilutions to achieve non-inhibitory concentrations of free compound, the infectivity titres of mock- and **3a**-treated virus suspensions were found to be similar (2.86×10^7 PFU/mL and 2.66×10^7 PFU/mL, respectively), indicating that **3a** does not damage virus particles. In stabilization studies, **3a** (20 μM) was found to protect HRV 1B against inactivation by mild acid or heat treatment. As shown in Figure 1, 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) (4.5 and 3.1 log PFU/mL, respectively). In the presence of **3a**, the drop in virus infectivity was reduced (3.5 and 2.1 log PFU/mL, respectively) and the protective effect towards inactivation was 1.0 log for both treatments. It is known that exposure to mild acid or heat would cause conformational changes of the virion capsid structure similar to those produced during the uncoating of the viral genome.[29,30] Binding of so-called capsid-binders within the hydrophobic pocket of VP1 protein results in resistance to acid and thermal inactivation due to a reduction of capsid flexibility.[31,32] Taken together, our results on stabilization of HRV infectivity suggest that **3a** could act as a capsid-binder. However, binding of **3a** was reversible by dilution as indicated by results on virus infectivity. A similar behaviour has also been observed for 4′,6-dicyanoflavan and (*Z*)-3-(4-chlorobenzylidene)chroman, related compounds previously studies by us.[10,12]

The antiviral activity of **3a** (20 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.



HRVC: untreated virus,
HRVT: virus exposed to pH 5.0,
3a: virus exposed to pH 5.0 in the presence of **3a**.

HRVC: untreated virus,
HRVT: virus heated at 56 °C,
3a: virus heated at 56 °C in the presence of **3a**.

Figure 1. Protective effect of **3a** on acid (**A**) and thermal inactivation (**B**) of HRV 1B infectivity.

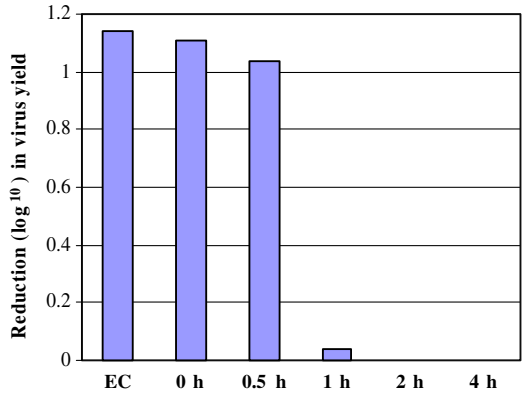


Figure 2. Effect of varying the time of addition of **3a** (20 μM) on the inhibition of HRV 1B replication under one-step growth conditions. Virus yield was determined by plaque assay. Virus control titre was 3.82×10^6 PFU/mL. **EC**: **3a** was present during the entire infection cycle (1 h at 4 °C and 10 h at 33 °C). 0, 0.5, 1, 2 and 4 h: compound was added at different times (0, 0.5, 1, 2 and 4 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 at 33 °C).

Maximal inhibition (92%) was achieved when **3a** was added to cells together with the virus and maintained until the end of HRV multiplication. Also when the compound was added to infected HeLa cells immediately after virus binding or 30 min after virus binding, a strong reduction in virus yield (91.4% and 90.0%, respectively) was still observed. However, when **3a** was added 1 h after virus binding, inhibition dropped to 7.7%. Virus yield was unaffected when the compound was added 2 or 4 h post infection (Fig. 2). These data indicate that **3a** interferes with early event(s) of virus replication.

Remarkably, when **3a** was present during the time of virus adsorption only (1 h, 4 °C) a 91.7% reduction of virus yield was still observed (Fig. 3). At the same time, the incubation (1 h, 4 °C) of HeLa cells with **3a** before virus infection did not modify the virus yield (data not shown), thus ruling out an effect at the cellular receptor level. Therefore, interaction of **3a** with HRV during the adsorption step probably hinders capsid structure(s) responsible for successful virus binding to the host cell membrane.

When **3a** was added immediately after virus adsorption and removed after only 30 min of contact at 33 °C, it still produced a suppression in virus yield of 91.8%, confirming an early activity of this compound. Similar levels of inhibition were achieved when **3a** was removed after 1 h or longer incubation times (Fig. 3).

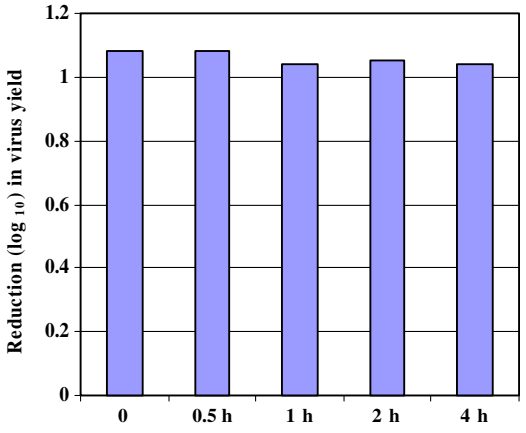


Figure 3. Effect of varying the time of removal of **3a** (20 μM) on the inhibition of HRV 1B replication under one-step growth conditions. Virus yield was determined by plaque assay after 10 h of incubation at 33 °C. Virus control titre was 3.82×10^6 PFU/mL. 0: **3a** was present during the time of virus adsorption only (1 h, 4 °C) and removed at the time indicated as 0. 0.5, 1, 2 and 4 h: compound was added after virus adsorption period (1 h at 4 °C, time 0) and removed after different lengths of incubation (0.5, 1, 2 and 4 h) at 33 °C.

The analysis of data from time of addition/removal studies are in agreement with the capsid-binder hypothesis, as suggested by the interfering activity of **3a** during virus binding and the very early events of virus life cycle. In fact, the finding that a comparable efficacy is achieved when **3a** is present during the entire replication time or during virus binding only, strongly suggests a direct interaction with virus particle. A similar behaviour has already been described for the previously studied (*Z*)-3-(4-chlorobenzylidene)chroman.[12] However, differently from (*Z*)-3-(4-chlorobenzylidene)chroman, the activity of **3a** is significantly exerted also within 30 min following virus attachment (91.8% inhibition). At this incubation time, (*Z*)-3-(4-chlorobenzylidene)chroman produced only a 44% reduction of virus yield even at a higher concentration (36 μM vs 20 μM). This finding could reflect a better ability of **3a** to enter the cells and reach the intracellular virus target.

It would be interesting to evaluate if **3a** can produce similar effects against HRV 14 infection. The results could shed more lights to the capsid-binding hypothesis.

3. Conclusion

New series of (*E*)-3-styryl-4*H*-chromen-4-ones **1a–d**, 3-[(1*E*, 3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-ones **2a–d**, (*E*)-3-styryl-2*H*-chromenes **3a–d** and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes **4a–d** have been synthesized and tested for their cytotoxicity and anti-HRV or EV71 activity in vitro. Some derivatives displayed a broad-spectrum of anti-HRV activity whereas only a modest efficacy was observed towards EV71. (*E*)-3-Styryl-2*H*-chromene **3a** was the most active anti-HRV agent.

Mechanism of action studies suggest that the new compounds affect functions associated with the viral capsid, including virus attachment to cell membrane and probably the uncoating of viral genome.

4. Experimental

4.1. Chemistry

Chemicals were purchased from Sigma–Aldrich and used without further purification. Melting points were determined on a Stanford 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 Perkin–Elmer 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 visualised by UV light. Elemental analyses (C, H, Cl) of all new compounds were within ±0.4% of theoretical values. (*E*)-4-(4-Chlorophenyl)but-3-enoic acid was prepared as previously described.[33]

4.1.1. General procedure for the synthesis of the (*E*)-3-styryl-4*H*-chromen-4-ones (1a–d) and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-ones (2a–d)

To a solution of the appropriate 4-oxo-4*H*-chromene-3-carbaldehyde (10 mmol) and phenylacetic acid or (*E*)-4-phenylbut-3-enoic acid (50 mmol) in dry pyridine (110 mL), *tert*-BuOK (15 mmol) was added. The mixture was refluxed until complete disappearance of 4-oxo-4*H*-chromene-3-carbaldehyde. After cooling the mixture was diluted with ice and water, and acidified to pH 2 with 2 N HCl. The precipitate was filtered, washed with water

and purified by column chromatography on silica gel eluting with a 1:2 mixture of ethyl acetate and light petroleum.

4.1.1.1. (*E*)-3-Styryl-4*H*-chromen-4-one (1a). Yield: 57%, mp = 167–168 °C (lit. 168–169 °C)[34] from ethyl alcohol. The spectroscopic data of the compound thus obtained were identical to those previously reported.[34]

4.1.1.2. (*E*)-3-(4-Chlorostyryl)-4*H*-chromen-4-one (1b). Yield: 72%, mp = 154–156 °C (lit. 159–160 °C)[34] from ethyl alcohol. The compound exhibited spectroscopic data identical to those previously reported.[34]

4.1.1.3. (*E*)-6-Chloro-3-(4-chlorostyryl)-4*H*-chromen-4-one (1c). Yield: 47%, mp = 204–206 °C from ethyl acetate. IR (KBr): 1642 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.25 (d, 1H, H5, J_{5-7} = 2.5 Hz), 8.09 (s, 1H, H2), 7.63–7.58 (m, 2H, H7, Hβ), 7.46–7.44 (m, 3H, H2′, H6′, H8), 7.32 (d, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 8.4 Hz), 6.90 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.3 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 175.3, 154.1, 153.2, 135.7, 133.8, 133.7, 131.4, 131.0, 128.9, 127.8, 125.6, 125.0, 121.6, 119.8, 119.2. Anal. Calcd for $C_{17}H_{10}Cl_2O_2$: C, 64.38; H, 3.18; Cl, 22.36. Found: C, 64.50; H, 3.15; Cl, 22.60.

4.1.1.4. (*E*)-6-Chloro-3-styryl-4*H*-chromen-4-one (1d). Yield: 52%, mp = 198–202 °C from ethyl alcohol. IR (KBr): 1640 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.26 (d, 1H, H5, J_{5-7} = 2.5 Hz), 8.11 (s, 1H, H2), 7.63–7.58 (m, 2H, H7, Hβ), 7.52 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.9 Hz), 7.44 (d, 1H, H8, J_{7-8} = 8.9 Hz), 7.36 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.9 Hz), 7.27 (t, 1H, H4′, $J_{3'-4'}$ = 7.9 Hz), 6.96 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.4 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 175.4, 154.2, 153.0, 137.2, 133.7, 132.2, 131.3, 128.7, 128.0, 126.7, 125.6, 125.0, 122.0, 119.8, 118.5. Anal. Calcd for $C_{17}H_{11}ClO_2$: C, 72.22; H, 3.92; Cl, 12.54. Found: C, 72.43; H, 3.82; Cl, 12.42.

4.1.1.5. 3-[(1*E*,3*E*)-4-Phenylbuta-1,3-dienyl]-4*H*-chromen-4-one (2a). Yield: 46%, mp = 139–140 °C from ethyl alcohol. IR (KBr): 1644 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.29 (dd, 1H, H5, J_{5-6} = 8.0 Hz, J_{5-7} = 1.7 Hz), 8.05 (s, 1H, H2), 7.67 (ddd, 1H, H7, J_{6-7} = 7.1 Hz, J_{7-8} = 8.6 Hz, J_{5-7} = 1.7 Hz), 7.53–7.40 (m, 5H, Hγ, H6, H8, H2′, H6′), 7.33 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.4 Hz), 7.24 (t, 1H, H4′, $J_{3'-4'}$ = 7.4 Hz), 6.92 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.4 Hz, $J_{\beta-\gamma}$ = 10.6 Hz), 6.71 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.4 Hz), 6.54 (d, 1H, Hδ, $J_{\gamma-\delta}$ = 15.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 176.4, 155.8, 152.9, 137.3, 133.6, 133.4, 132.5, 129.6 128.6, 127.7, 126.5, 126.3, 125.2, 124.1, 122.8, 121.9, 118.0. Anal. Calcd for $C_{19}H_{14}O_2$: C, 83.19; H, 5.14. Found: C, 83.32; H, 5.21.

4.1.1.6. 3-[(1*E*,3*E*)-4-(4-Chlorophenyl)buta-1,3-dienyl]-4*H*-chromen-4-one (2b). Yield: 48%, mp = 170–171 °C from ethyl alcohol. IR (KBr): 1643 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.29 (dd, 1H, H5, J_{5-6} = 7.9 Hz, J_{5-7} = 1.7 Hz), 8.04 (s, 1H, H2), 7.67 (ddd, 1H, H7, J_{6-7} = 7.2 Hz, J_{7-8} = 8.4 Hz, J_{5-7} = 1.7 Hz), 7.54–7.40 (m, 3H, Hγ, H6, H8), 7.36 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.2 Hz), 7.29 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.2 Hz), 6.87 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.4 Hz, $J_{\beta-\gamma}$ = 10.6 Hz), 6.65 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.4 Hz), 6.53 (d, 1H, Hδ, $J_{\gamma-\delta}$ = 15.9 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 176.5, 155.8, 153.1, 135.8, 133.5, 133.2, 132.3, 132.1, 130.2, 128.8, 127.6, 126.3, 125.3, 124.1, 123.5, 121.8, 118.0. Anal. Calcd for $C_{19}H_{13}ClO_2$: C, 73.91; H, 4.24; Cl, 11.48. Found: C, 74.10; H, 4.32; Cl, 11.56.

4.1.1.7. 6-Chloro-3-[(1*E*,3*E*)-4-(4-chlorophenyl)buta-1,3-dienyl]-4*H*-chromen-4-one (2c). Yield: 50%, mp = 176–178 °C from ethyl acetate. IR (KBr): 1639 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.25 (d, 1H, H5, J_{5-7} = 2.6 Hz), 8.04 (s, 1H, H2), 7.61 (dd, 1H, H7, J_{7-8} = 8.9 Hz, J_{5-7} = 2.6 Hz), 7.49 (m, 1H, Hγ, $J_{\beta-\gamma}$ = 10.6 Hz, $J_{\gamma-\delta}$ = 15.5 Hz), 7.43 (d, 1H, H8, J_{7-8} = 8.9 Hz), 7.37 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.5 Hz), 7.30 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 6.87 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.7 Hz, $J_{\beta-\gamma}$ = 10.5 Hz), 6.66 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.7 Hz), 6.50 (d, 1H, Hδ, $J_{\gamma-\delta}$ = 15.5 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 175.2, 154.1, 153.1, 135.7, 133.8, 133.4, 132.8, 132.5, 131.3, 130.0, 128.9, 127.6, 125.7, 125.0, 122.9, 121.8, 119.8. Anal. Calcd for $C_{19}H_{12}Cl_2O_2$: C, 66.49; H, 3.52; Cl, 20.66. Found: C, 66.73; H, 3.53; Cl, 20.58.

4.1.1.8. 6-Chloro-3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-4*H*-chromen-4-one (2d). Yield: 69%, mp = 172–173 °C from ethyl alcohol. IR (KBr): 1644 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.24 (d, 1H, H5, J_{5-7} = 2.6 Hz), 8.04 (s, 1H, H2), 7.60 (dd, 1H, H7, J_{7-8} = 8.9 Hz, J_{5-7} = 2.6 Hz), 7.52–7.41 (m, 4H, Hγ, H8, H2′, H6′), 7.34 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.5 Hz), 7.24 (t, 1H, H4′, $J_{3'-4'}$ = 7.5 Hz), 6.91 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.6 Hz, $J_{\beta-\gamma}$ = 10.6 Hz), 6.72 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.6 Hz), 6.51 (d, 1H, Hδ, $J_{\gamma-\delta}$ = 15.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 175.2, 154.1, 152.9, 137.2, 134.0, 133.7,133.0, 131.2, 129.4, 128.7, 127.8, 126.5, 125.7, 125.0, 122.2, 122.0, 119.8. Anal. Calcd for $C_{19}H_{13}ClO_2$: C, 73.91; H, 4.24; Cl, 11.48. Found: C, 73.77; H, 4.44; Cl, 11.62.

4.1.2. General procedure for the synthesis of 2*H*-chromene-3-carbaldehydes (5a,d)

Acrolein (75 mmol) was added dropwise to a suspension of the appropriate 2-hydroxybenzaldehyde (50 mmol) and K_2CO_3 (79 mmol) in dry dioxane (115 mL). The mixture was refluxed for 4 h. After cooling, the solvent was removed under reduced pressure and the residue was distributed between dichloromethane and 2 N NaOH. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with a 1:1 mixture of dichloromethane and light petroleum (1:1).

4.1.2.1. 2*H* -Chromene-3-carbaldehyde (5a). Yield: 70%, mp = 41–42 °C (lit. 42–43 °C).[26] The compound exhibited spectroscopic data identical to those previously reported.[26]

4.1.2.2. 6-Chloro-2*H*-chromene-3-carbaldehyde (5d). Yield: 81%, mp = 92–93 °C (lit. 93–95 °C).[25] The compound exhibited spectroscopic data identical to those previously reported.[25]

4.1.3. General procedure for the synthesis of the (*E*)-3-styryl-2*H*-chromenes (3a–d) and 3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromenes (4a,d)

A solution of the appropriate diethyl benzylphosphonate (11 mmol) or diethyl *trans*-cinnamylphosphonate (11 mmol) in dry THF (30 mL) was added dropwise to a stirred suspension of sodium hydride (11 mmol) in dry THF (15 mL) under nitrogen. The mixture was stirred for 10 min at room temperature, then a solution of the appropriate 2*H*-chromene-3-carbaldehyde (**5a,d**) (10 mmol) in dry THF (15 mL) was added dropwise. After stirring for 20 h at room temperature, water was added to the mixture and THF was removed under reduced pressure. The solid collected by filtration was washed with water, and purified by column chromatography on silica gel eluting with a mixture of dichloromethane and light petroleum (1:1 for **3a–d**, and 1:3 for **4a,d**).

4.1.3.1. (*E*)-3-Styryl-2*H*-chromene (3a). Yield: 47%, mp = 122-124 °C from *n*-hexane. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.44 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.6 Hz), 7.34 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.6 Hz), 7.25 (t, 1H, H4′, $J_{3'-4'}$ = 7.6 Hz), 7.11 (dt, 1H, H7, J_{6-7} = J_{7-8} = 7.5 Hz, J_{5-7} = 1.5 Hz), 7.03 (dd, 1H, H5, J_{5-6} = 7.5 Hz, J_{5-7} = 1.5 Hz), 6.91–6.81 (m, 3H, H6, H8, Hβ), 6.52 (s, 1H, H4), 6.44 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.5 Hz), 4.83 (d, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.8, 137.0, 130.7, 129.1, 128.8, 128.0,

127.9, 127.0, 126.6, 126.4, 124.0, 122.8, 121.5, 115.5, 65.6. Anal. Calcd for $C_{17}H_{14}O$: C, 87.15; H, 6.02. Found: C, 86.01; H, 6.22.

4.1.3.2. (*E*)-3-(4-Chlorostyryl)-2*H*-chromene (3b). Yield: 42%, mp = 167–170 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.36 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.5 Hz), 7.30 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.11 (ddd, 1H, H7, J_{6-7} = 7.5 Hz, J_{7-8} = 8.0 Hz, J_{5-7} = 1.7 Hz), 7.03 (dd, 1H, H5, J_{5-6} = 7.5 Hz, J_{5-7} = 1.7 Hz), 6.88 (dt, 1H, H6, J_{5-6} = J_{6-7} = 7.5 Hz, J_{6-8} = 1.1 Hz), 6.84–6.79 (m, 2H, H8, Hβ), 6.52 (s, 1H, H4), 6.37 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.5 Hz), 5.07 (d, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.8, 135.5, 133.4, 130.4, 129.3, 128.9, 127.6, 127.2, 127.0, 126.6, 124.6, 122.6, 121.6, 115.5, 65.5. Anal. Calcd for $C_{17}H_{13}ClO$: C, 75.98; H, 4.88; Cl, 13.19. Found: C, 76.37; H, 4.99; Cl, 13.02.

4.1.3.3. (*E*)-6-Chloro-3-(4-chlorostyryl)-2*H*-chromene (3c). Yield: 64%, mp = 186–190 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.37 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.4 Hz), 7.30 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.05 (dd, 1H, H7, J_{7-8} = 8.5 Hz, J_{5-7} = 2.3 Hz), 7.00 (d, 1H, H5, J_{5-7} = 2.3 Hz), 6.81 (d, 1H, Hβ, $J_{\alpha-\beta}$= 16.5 Hz), 6.75 (d, 1H, H8, J_{7-8} = 8.5 Hz), 6.45 (s, 1H, H4), 6.40 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.5 Hz), 5.06 (d, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.2, 135.2, 133.8, 131.6, 129.0, 128.7, 127.7, 127.6, 126.7, 126.3, 124.2, 123.2, 116.8, 115.1, 65.7. Anal. Calcd for $C_{17}H_{12}Cl_2O$: C, 67.35; H, 3.99; Cl, 23.39. Found: C, 67.52; H, 4.08; Cl, 23.47.

4.1.3.4. (*E*)-6-Chloro-3-styryl-2*H*-chromene (3d). Yield: 90%, mp = 154–157 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.44 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.6 Hz), 7.35 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.6 Hz), 7.26 (t, 1H, H4′, $J_{3'-4'}$ = 7.6 Hz), 7.04 (dd, 1H, H7, J_{7-8} = 8.5 Hz, J_{5-7} = 2.5 Hz), 6.99 (d, 1H, H5, J_{5-7} = 2.5 Hz), 6.84 (d, 1H, Hβ $J_{\alpha-\beta}$ = 16.5 Hz), 6.75 (d, 1H, H8, J_{7-8} = 8.5 Hz), 6.47 (d, 1H, Hα, $J_{\alpha-\beta}$ = 16.5 Hz), 6.43 (s, 1H, H4), 5.08 (d, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.2, 136.7, 131.8, 128.9, 128.8, 128.5, 128.1, 126.5, 126.2, 126.1, 124.1, 122.7, 116.7, 65.7. Anal. Calcd for $C_{17}H_{13}ClO$: C, 75.98; H, 4.88; Cl, 13.19. Found: C, 76.25; H, 5.01; Cl, 13.29.

4.1.3.5. 3-[(1*E*,3*E*)-4-Phenylbuta-1,3-dienyl]-2*H*-chromene (4a). Yield: 35%, mp = 162–167 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.42 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.7 Hz), 7.33 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.7 Hz), 7.23 (t, 1H, H4′, $J_{3'-4'}$ = 7.7 Hz), 7.10 (dt, 1H, H7, J_{6-7} = J_{7-8} = 7.9 Hz, J_{5-7} = 1.6 Hz), 7.01 (dd, 1H, H5, J_{5-6} = 7.4 Hz, J_{5-7} = 1.6 Hz), 6.90–6.80 (m, 3H, H6, H8, Hβ), 6.63 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.5 Hz), 6.43 (s, 1H, H4), 6.42 (d, 1H, Hδ, $J_{\delta-\gamma}$ = 15.7 Hz), 6.31 (dd, 1H, Hγ, $J_{\delta-\gamma}$ = 15.7 Hz, $J_{\beta-\gamma}$ = 10.2 Hz), 5.02 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.7, 137.2, 133.4, 130.9, 130.6, 129.1, 129.0, 128.8, 128.7, 127.7, 126.9, 126.4, 123.6, 122.9, 121.5, 115.5, 65.5. Anal. Calcd for $C_{19}H_{16}O$: C, 87.66; H, 6.19. Found: C, 87.90; H, 6.22.

4.1.3.6. 6-Chloro-3-[(1*E*,3*E*)-4-phenylbuta-1,3-dienyl]-2*H*-chromene (4d). Yield: 43%, mp = 152–158 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.42 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 7.7 Hz), 7.33 (t, 2H, H3′, H5′, $J_{2'-3'}$ = $J_{3'-4'}$ = 7.7 Hz), 7.24 (t, 1H, H4′, $J_{3'-4'}$ = 7.7 Hz), 7.03 (dd, 1H, H7, J_{7-8} = 8.5 Hz, J_{5-7} = 2.5 Hz), 6.97 (d, 1H, H5, J_{5-7} = 2.5 Hz), 6.86 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.6 Hz, $J_{\beta-\gamma}$ = 9.5 Hz), 6.74 (d, 1H, H8, J_{7-8} = 8.5 Hz), 6.65 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.6 Hz), 6.41 (d, 1H, Hδ, $J_{\delta-\gamma}$ = 15.8 Hz), 6.36 (s, 1H, H4), 6.34 (dd, 1H, Hγ, $J_{\delta-\gamma}$ = 15.8 Hz, $J_{\beta-\gamma}$ = 9.5 Hz), 5.01 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.2, 137.0, 134.1, 132.0, 130.0, 129.8, 128.8, 128.7, 128.5, 127.9, 126.5, 126.3, 126.2, 124.3, 122.3, 116.7, 65.6. Anal. Calcd for $C_{19}H_{14}Cl_2O$: C, 69.32; H, 4.29; Cl, 21.54. Found: C, 69.10; H, 4.42; Cl, 21.65.

4.1.4. General procedure for the synthesis of (*E*)-3-(2*H*-chromen-3-yl)acrylaldehydes (6a,d)

tert-BuOK (13 mmol) was added to a cooled (0 °C) and magnetically stirred suspension of [(1,3-dioxolan-2-yl)methyl]triphenylphosphonium bromide (13 mmol) in dry THF (100 mL). After 30 min, a solution of the appropriate 2*H*-chromene-3-carbaldehyde (**5a,d**) (10 mmol) in dry THF (25 mL) was added dropwise. The mixture was stirred at room temperature for 2 h and refluxed for 6 h. After cooling, a solution of oxalic acid (10 g) in water (100 mL) was added and the mixture stirred overnight at room temperature. THF was removed under reduced pressure and the mixture extracted with Et_2O. The combined organic phases were washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with dichloromethane/light petroleum (1:1) (**6b**) or dichloromethane (**6c**).

4.1.4.1. (*E*)-3-(2*H*-Chromen-3-yl)acrylaldehyde (6a). Yield: 46%, mp = 173–176 °C. IR (KBr): 2840, 2720, 1700 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 9.63 (d, 1H, CHO, $J_{\beta-CHO}$ = 7.6 Hz), 7.25–7.15 (m, 2H, H7, Hα), 7.14 (d, 1H, H5, J_{5-6} = 7.8 Hz), 6.95–6.84 (m, 3H, H4, H6, H8), 6.05 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 16.2 Hz, $J_{\beta-CHO}$ = 7.6 Hz), 5.00 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 193.1, 155.0, 147.6, 131.2, 131.0, 129.8, 128.5, 127.9, 126.8, 121.9, 117.5, 65.0. Anal. Calcd for $C_{12}H_{10}O_2$: C, 77.40; H, 5.41. Found: C, 77.63; H, 5.24.

4.1.4.2. (*E*)-3-(6-Chloro-2*H*-chromen-3-yl)acrylaldehyde (6d). Yield: 49%, mp = 155–156 °C. IR (KBr): 2820, 2730, 1685 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 9.63 (d, 1H, CHO, $J_{\beta-CHO}$ = 7.5 Hz), 7.19–7.14 (m, 2H, H7, Hα), 7.08 (d, 1H, H5, J_{5-7} = 2.3 Hz), 6.80–6.77 (m, 2H, H4, H8), 6.08 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.9 Hz, $J_{\beta-CHO}$ = 7.5 Hz), 4.99 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 193.0, 153.1, 147.8, 131.1, 131.0, 129.6, 127.9, 127.6, 126.7, 122.8, 117.4, 65.1. Anal. Calcd for $C_{12}H_9ClO_2$: C, 65.32; H, 4.11; Cl, 16.07. Found: C, 65.11; H, 4.23; Cl, 16.37.

4.1.5. General procedure for the synthesis of 3-[(1*E*,3E)-4-(4-chlorophenyl)buta-1,3-dienyl]-2*H*-chromenes (4b,c)

A solution of diethyl 4-chlorobenzylphosphonate (11 mmol) in dry THF (30 mL) was added dropwise to a stirred suspension of sodium hydride (11 mmol) in dry THF (15 mL) under nitrogen. The mixture was stirred 10 min at room temperature, then a solution of the appropriate (*E*)-3-(2*H*-chromen-3-yl)acrylaldehyde (**6a,d**) (10 mmol) in dry THF (15 mL) was added dropwise. After stirring for 22 h at room temperature, water was added to the mixture and THF removed under reduced pressure. The solid obtained was removed by filtration, and crystallized from ethyl acetate (**4b**) or purified by column chromatography on silica gel eluting with a 1:4 mixture of ethyl acetate and light petroleum (**4c**).

4.1.5.1. 3-[(1*E*,3*E*)-4-(4-Chlorophenyl)buta-1,3-dienyl]-2*H*-chromene (4b). Yield: 60%, mp = 203–204 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.33 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.5 Hz), 7.28 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.5 Hz), 7.10 (dt, 1H, H7, J_{6-7} = J_{7-8} = 7.8 Hz, J_{5-7} = 1.6 Hz), 7.01 (dd, 1H, H5, J_{5-6} = 7.4 Hz, J_{5-7} = 1.6 Hz), 6.90–6.78 (m, 3H, H6, H8, Hβ), 6.57 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.6 Hz), 6.46 (s, 1H, H4), 6.43 (d, 1H, Hδ, $J_{\delta-\gamma}$ = 15.6 Hz), 6.31 (dd, 1H, Hγ, $J_{\delta-\gamma}$ = 15.6 Hz, $J_{\beta-\gamma}$ = 10.2 Hz), 5.01 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 153.8, 135.7, 133.3, 131.9, 131.1, 130.8, 129.6, 129.2, 128.9, 128.5, 127.6, 126.9, 124.0,

122.8, 121.6, 115.5, 65.5. Anal. Calcd for $C_{19}H_{15}ClO$: C, 77.42; H, 5.13; Cl, 12.03. Found: C, 77.29; H, 5.24; Cl, 12.22.

4.1.5.2. 6-Chloro-3-[(1*E*,3*E*)-4-(4-chlorophenyl)buta-1,3-dienyl]-2*H*-chromene (4c). Yield: 67%, mp = 193–194 °C from ethyl acetate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.34 (d, 2H, H2′, H6′, $J_{2'-3'}$ = 8.6 Hz), 7.29 (d, 2H, H3′, H5′, $J_{2'-3'}$ = 8.6 Hz), 7.03 (dd, 1H, H7, J_{7-8} = 8.5 Hz, J_{5-7} = 2.5 Hz), 6.98 (d, 1H, H5, J_{5-7} = 2.5 Hz), 6.83 (dd, 1H, Hβ, $J_{\alpha-\beta}$ = 15.6 Hz, $J_{\beta-\gamma}$ = 10.1 Hz), 6.74 (d, 1H, H8, J_{7-8} = 8.5 Hz), 6.59 (d, 1H, Hα, $J_{\alpha-\beta}$ = 15.6 Hz), 6.42 (d, 1H, Hδ, $J_{\delta-\gamma}$ = 15.7 Hz), 6.37 (s, 1H, H4), 6.32 (dd, 1H, Hγ, $J_{\delta-\gamma}$ = 15.7 Hz, $J_{\beta-\gamma}$ = 10.1 Hz), 5.01 (s, 2H, H2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 152.2, 135.6, 133.5, 132.6, 131.9, 130.6, 129.4, 129.3, 128.9, 128.6, 127.6, 126.3, 126.2, 124.2, 122.7, 116.7, 65.6. Anal. Calcd for $C_{19}H_{14}Cl_2O$: C, 69.32; H, 4.29; Cl, 21.54. Found: C, 69.10; H, 4.42; Cl, 21.65.

4.2. Virology

4.2.1. Cells

HeLa (Ohio) cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 using Eagle's minimum essential medium (MEM) supplemented with 100 μg/mL of streptomycin, 100 U/mL of penicillin G and 8% heat-inactivated foetal calf serum (FCS) (growth medium). Hep-2 (human epithelioma) cells were cultured as above using Dulbecco's modified Eagle's medium (high glucose). The serum concentration was reduced to 2% for cell maintenance (maintenance medium).

4.2.2. Compounds

Stock solutions of compounds were prepared in ethanol (1, 0.5 or 0.1 mg/mL) and further diluted in tissue culture medium shortly before use.

4.2.3. Viruses

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) for 1 h at 33 °C. Infected cells were then incubated at 33 °C in maintenance medium. When the viral-induced cytopathic effect involved most of the cells, the cultures were freeze-thawed three times and the clarified supernatants stored in aliquots at −80 °C until use. The virus was titrated by plaque assay, essentially the same as described by Fiala and Kenny.[35]

EV71 reference strain (ATCC, VR-784) was propagated and titrated by plaque assay in Hep-2 cells at 37 °C as previously reported.[15]

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.[27] Cells were seeded in 96-well tissue culture plates at 2×10^3 cells for each 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) 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})

Confluent monolayers of HeLa or Hep-2 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 for HRV or 1 h at 37 °C for EV 71, the virus inoculum was removed and the cells were overlaid with medium for plaques, in the presence or absence of fourfold dilution of drugs. After three days of incubation at 33 °C (HRV) or two days at 37 °C (EV 71), the cells were stained with a neutral red solution at 0.2 mg/mL in pH 7.4 phosphate buffered saline (PBS) and the plaques were counted. The IC_{50} value was 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 **3a** (20 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 **3a** (20 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 five 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 five in the presence or absence of **3a** (20 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 (20 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 **3a**, 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.

Acknowledgement

We gratefully acknowledge the financial support from 'Istituto Pasteur-Fondazione Cenci Bolognetti', Università degli Studi di Roma 'La Sapienza'.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.103. These data include MOL files and InChiKeys of the most important compounds described in this article.

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