Bioorganic & Medicinal Chemistry 18 (2010) 4195–4201

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

(*E*)-2-Styrylchromones as potential anti-norovirus agents

Joana Rocha-Pereira [a,b], Ricardo Cunha [a], Diana C. G. A. Pinto [c], Artur M. S. Silva [c], Maria São José Nascimento [a,b,*]

[a] *Serviço de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*
[b] *CEQUIMED-UP, Faculdade de Farmácia, Universidade do Porto, Portugal*
[c] *Departamento de Química & QOPNA, Universidade de Aveiro, Portugal*

ARTICLE INFO

Article history:
Received 8 January 2010
Revised 19 April 2010
Accepted 4 May 2010
Available online 7 May 2010

Keywords:
Norovirus
MNV
Antiviral activity
(*E*)-2-Styrylchromones

ABSTRACT

Human noroviruses (NoV) are now recognized as the most frequent cause of outbreaks and sporadic cases of acute gastroenteritis. Despite the significant economic impact and considerable morbidity of norovirus disease, no drug or vaccine is currently available to treat or prevent this disease, therefore the discovery of anti-norovirus drugs is urgent.

In the present work, a total of 12 structure related chromone and (*E*)-2-styrylchromones were evaluated for their potential anti-norovirus activity using the murine norovirus (MNV) as a surrogate model for human NoV.

From the 12 compounds studied, six (*E*)-2-styrylchromones were found to have with interesting anti-norovirus activity. The best compounds of the series were (*E*)-5-hydroxy-2-styrylchromone and (*E*)-4′-methoxy-2-styrylchromone with an $IC_{50} \approx 7$ μM. A first insight into the mechanism of action of these compounds was possible. An interesting relationship between the anti-norovirus activity and the chemical structure was observed. The present study points out that the (*E*)-2-styrylchromones skeleton is an important one which deserves to be developed and further explored as new antiviral drugs against NoV.

1. Introduction

Human noroviruses (NoV) are now recognized as the most frequent cause of outbreaks and sporadic cases of acute gastroenteritis.[1,2] They affect people of all age groups and often occur in crowded locations, usually causing a short-term, self-limiting disease. However, complications derived from severe dehydration may occur in infants and the elderly because they are more sensitive to volume depletion. Moreover norovirus infection has put healthy people in intensive care and has been associated with chronic diarrhea among transplant patients.[3] Despite the significant economic impact and considerable morbidity of norovirus disease, no drug or vaccine is currently available to treat or prevent this disease, therefore the discovery of anti-norovirus drugs is urgent.

Little is known about the biology of human NoV, which is mainly due to the absence of a cell culture system or small animal model.[4] Murine norovirus (MNV) is able to replicate in the murine macrophage cell line RAW 264.7 and this system is considered today the best surrogate model for human NoV.[5,6] MNV is genetically related to non-cultivable human NoV and it is likely that many fundamental mechanisms of replication and biochemical features are conserved between these viruses.[6] Therefore, screening chemical compounds against the MNV/RAW 264.7 surrogate system by classic infectivity assay (plaque reduction assay) constitutes a good strategy to discover active compounds against NoV and has been frequently used in disinfection and inactivation studies of NoV.[7–9]

The replication strategy of NoV is not well understood but its family, *Caliciviridae*, shares many characteristics with the family *Picornaviridae*.[4,10,11] Both include ssRNA(+) virus that encode a large polyprotein matured by proteolytic cleavage into a viral protease and a RNA-dependent RNA polymerase, among others. Thus, it is possible that some inhibitors of picornavirus replication may also have an effect on norovirus replication. Many flavonoids of natural or synthetic origin have been shown to inhibit the replication of picornavirus, including poliovirus, coxsackievirus A/B and rhinovirus.[11–15] Recently, 2-styrylchromones have emerged as a new class of flavonoid-type compounds with activity against rhinovirus, a picornavirus.[16–18] Therefore, the aim of the present work was to study a series of structure related chromones and (*E*)-2-styrylchromones (Fig. 1) for their potential anti-norovirus activity, establish a structure–activity relationship and get some insights into the mechanism of action of the most active compounds.

2. Results

2.1. Synthesis

Chromone (**2**) were obtained by a Claisen condensation of the appropriate 2′-hydroxyacetophenones and methyl formate[19] lead-

* Corresponding author.
E-mail address: saojose@ff.up.pt (M. S. J. Nascimento).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.05.006

ing to the 2-hydroxy-4-chromanone (**1**), which was then dehydrated by treatment with a catalytic amount of iodine in refluxing DMSO (Scheme 1). This dehydration method is currently used in the synthesis of 2-substituted chromones[20,21] and is now applied for the first time to 2-unsubstituted chromone.

(*E*)-2-Styrylchromones (**4–9**) have been prepared by aldol condensation/oxidative cyclization procedure[22] (Scheme 2). The base-catalysed aldol condensation of 2′-hydroxyacetophenones with cinnamaldehydes gave the corresponding 2′-hydroxycinnamylideneacetophenones (**3**) in good yields (85–93%). The oxidative cyclization of (**3**) with a catalytic amount of iodine in refluxing DMSO for half an hour yielded 2-styrylchromones (**4–6**) in good yields (85–93%).[23] However, the cyclodehydrogenation of cinnamylideneacetophenones bearing a 6′-benzyloxy group (R^1 = OBn, $R^2 = R^3$ = H) lead to 5-hydroxy-2-styrylchromone (**7**) with a longer reflux time (2 h). This means that it is possible to obtain the starting material oxidative cyclization and further debenzylation of the formed chromone (**6**) in one step, leading to 5-hydroxy-2-styrylchromone (**7**).[24] In the oxidative cyclization of 2′-hydroxycinnamylideneacetophenones unsubstituted in the vinylic moiety, only the isomer (*E*)-2-styrylchromones (**4–7**) were obtained, whereas in the case of 2′-hydroxycinnamylideneacetophenone bearing a γ-methyl group ($R^2 = CH_3$, $R^1 = R^3$ = H) both isomers (*E*)-2-α-methylstyrylchromone (**8**) and (*Z*)-2-α-methylstyrylchromone **(9)** were formed, the (*E*) being obtained in higher proportion.[23]

(*E*)-4′-Methyl-2-styrylchromone (**12**) and (*E*)-4′-methoxy-2-styrylchromone (**13**) have been prepared by the Baker–Venkataraman approach starting from 2′-hydroxyacetophenone and cinnamic acid derivatives (Scheme 3). 2′-Cinnamoyloxyacetophenones (**10**) were obtained from the reaction of the 2′-hydroxyacetophenone with the appropriate cinnamoyl chlorides, prepared in situ from cinnamic acids and phosphorous oxychloride. The Baker–Venkataraman rearrangement of (**10**) into the corresponding 1,3-diketones, which exists in solution as their enolic form (**11**), was performed upon treatment with sodium hydroxide in DMSO at room temperature. The treatment (**11**) with a mixture of DMSO and *p*-toluenesulfonic acid led to their cyclodehydration yielding the expected (*E*)-2-styrylchromone (**12**, **13**).[25] (*E*)-4′-Hydroxy-2-styrylchromone (**14**) was obtained by demethylation of (*E*)-4′-methoxy-2-styrylchromone (**13**) with boron tribromide (see Section 5).

The (*E*)-3-hydroxy-2-styrylchromones (**15**, **16**) were obtained by the Algar–Flynn–Oyamada type-reaction,[26] which consists in the oxidative cyclisation of the appropriate 2′-hydroxycinnamylideneacetophenones with hydrogen peroxide (Scheme 4). (*E*)-3-Methoxy-2-styrylchromones (**17**) was obtained by methylation of (*E*)-3-hydroxy-2-styrylchromone (**15**) with methyl sulfate in alkaline medium.[18]

2.2. Cytotoxicity of chromones and (*E*)-2-styrylchromones

Prior to evaluating the anti-norovirus activity of compounds it was first necessary to establish which was their highest concentration which did not cause significant toxicity to RAW cells (MNTC). Their potential cytotoxicity was determined by measuring the MTT reduction capacity of RAW cells. Concentrations of compounds associated with a toxicity <10% ($MNTC_{90}$) were considered ideal but those concentrations associated with a MTT reduction capacity $\geqslant$70% ($MNTC_{70}$) were still considered tolerable.[27,28] Hence, the $MNTC_{90}$ and $MNTC_{70}$ values of all the compounds were determined together with their CC_{50} (concentration of compounds that causes 50% cytotoxicity) and are summarized in Table 1.

Results showed that compounds **8** and **12** had no significant toxicity for RAW cells even at the highest concentration tested ($MNTC_{90}$ >150 μM), while compounds **2**, **4**, **5**, **6**, **7**, **13** and **14** exhibited a tolerable toxicity ($MNTC_{70}$ >150 μM). Hence, all these compounds presented a relatively large margin of security that enabled further testing for anti-norovirus activity at concentrations of 150 μM. On the contrary, RAW cells treated with 150 μM of compounds **15**, **16** and **17** showed a different toxicity profile. For these compounds, an acceptable percentage of viable cells (>70%) was only observed with concentrations $\leqslant$75 μM for compound **17** and $\leqslant$18.75 μM for compounds **15** and **16**.

Taken together, these results led us to consider 50 μM as the maximum concentration of all compounds to be tested for the screening of antiviral activity, except for compounds **15** and **16** for which only concentrations $\leqslant$10 μM were considered.

2.3. Anti-norovirus activity of chromones and (*E*)-2-styrylchromones

The anti-norovirus activity of chromone (**2**) and (*E*)-2-styrylchromones (**4–8**, **12–17**) was evaluated by plaque reduction assay. The IC_{50} of each compound studied together with their selectivity index (SI) are summarized in Table 1.

Chromone **2** and (*E*)-2-styrylchromones **5**, **6**, and **14** showed no anti-norovirus activity even when tested at the highest concentration of 50 μM. Compounds **15** and **16** also showed no antiviral

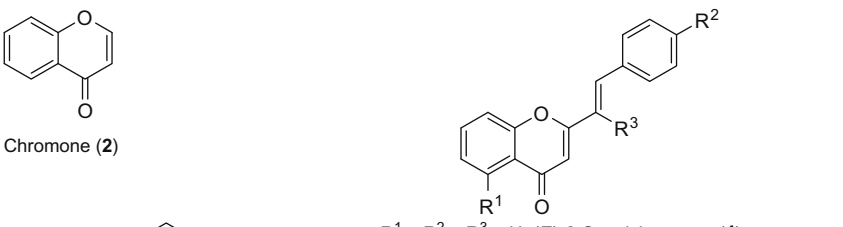
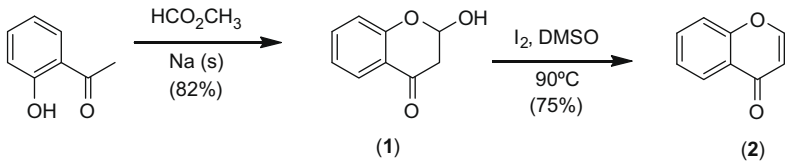
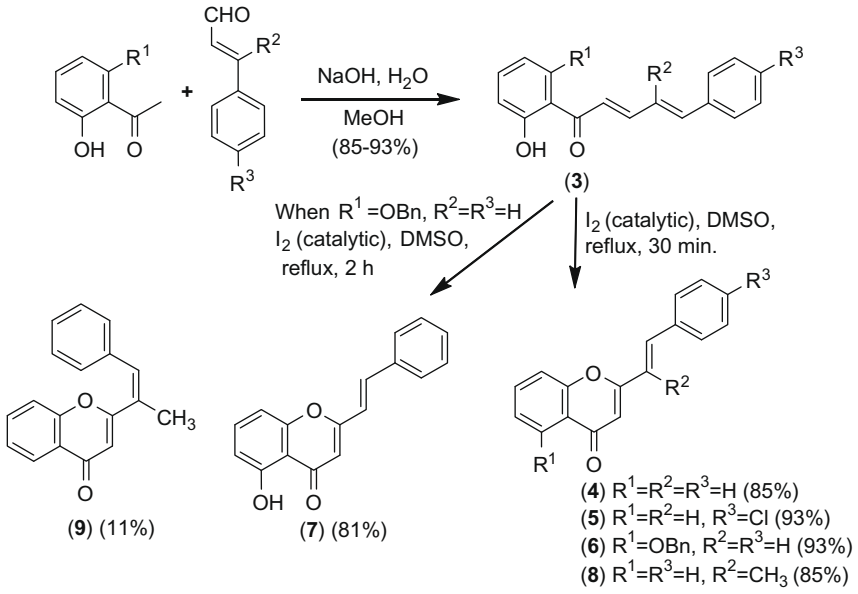


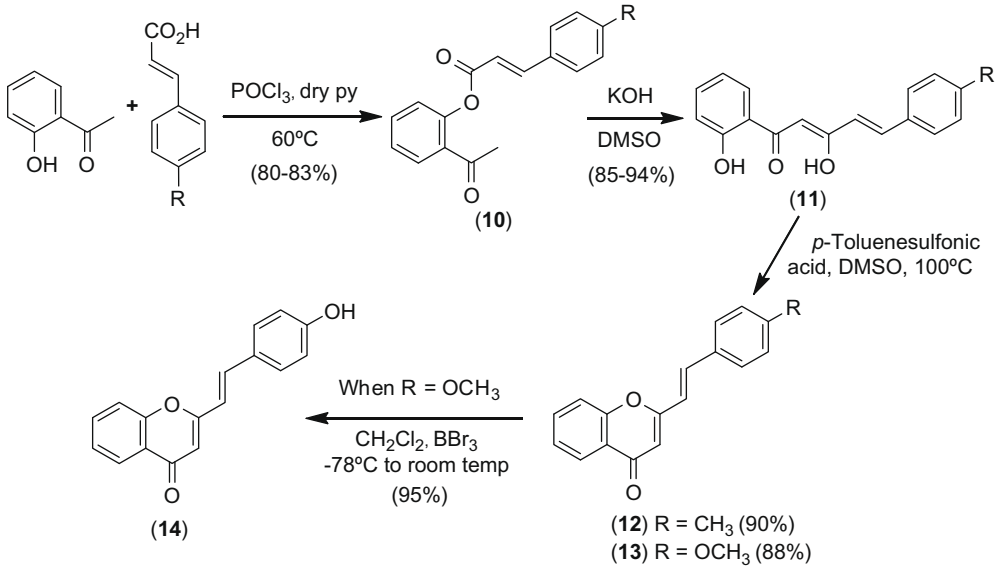
Figure 1. Chemical structures of chromones (**2**) and (*E*)-2-styrylchromones (**4–8**, **12–17**).



Scheme 1. Synthesis of chromone (**2**).



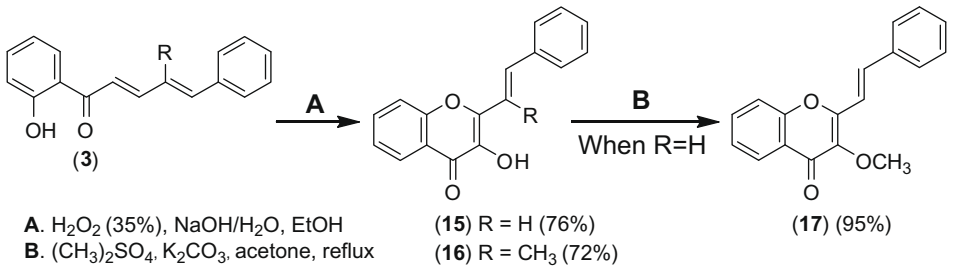
Scheme 2. Synthesis of (*E*)-2-styrylchromones (**4**–**9**).



Scheme 3. Synthesis of (*E*)-2-styrylchromones (**12**–**14**).

effect, but the maximum concentration tested was only 10 μM due to their toxicity to RAW cells.

However, an anti-norovirus activity was observed for (*E*)-2-styrylchromones **4**, **7**, **8**, **12**, **13** and **17**. Their antiviral effect ranged from moderate (IC_{50} = 20–50 μM) to potent (IC_{50} <10 μM), being (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**) the most active compounds with IC_{50} values of 7.0 μM and 7.4 μM, respectively. These two compounds (**7** and **13**) exerted their anti-norovirus activity in a dose-dependent way, presenting no significant cytotoxicity for effective concentrations (Fig. 2). Consequently, they presented high selectivity index (SI) values (>21.6 and >13.5, respectively).



Scheme 4. Synthesis of (*E*)-3-substituted-2-styrylchromones (**15**–**17**).

Table 1
Cytotoxicity and anti-norovirus activity of chromones (**2**) and (*E*)-2-styrylchromones (**4**–**8**, **12**–**17**)

Compounds	CC_{50}[a] (μM)	$MNTC_{70}$[b] (μM)	$MNTC_{90}$[b] (μM)	IC_{50}[c] (μM)	SI[d]
Chromone (**2**)	>150*	>150*	37.5	>50* (29.9%)	—
(*E*)-2-Styrylchromone (**4**)	>150	>150	75	17.7 ± 6.5	>8.5
(*E*)-4′-Chloro-2-styrylchromone (**5**)	>150	>150	75	>50 (7.6%)	—
(*E*)-5-Benzyloxy-2-styrylchromone (**6**)	>150	>150	75	>50 (39.4%)	—
(*E*)-5-Hydroxy-2-styrylchromone (**7**)	>150	>150	4.69	7.0 ± 0.7	>21.6
(*E*)-α-Methyl-2-styrylchromone (**8**)	>150	>150	>150	18.0 ± 5.2	>8.3
(*E*)-4′-Methyl-2-styrylchromone (**12**)	>150	>150	>150	23.7 ± 7.5	>6.3
(*E*)-4′-Methoxy-2-styrylchromone (**13**)	>100	>100	50	7.4 ± 1.3	>13.5
(*E*)-4′-Hydroxy-2-styrylchromone (**14**)	>150*	>150*	<2.34* (86%)	>50* (21.1%)	—
(*E*)-3-Hydroxy-2-styrylchromone (**15**)	52.1 ± 10.6	18.75	2.34	>10 (6.8%)	—
(*E*)-3-Hydroxy-α-methyl-2-styrylchromone (**16**)	46.7 ± 8.7	18.75	<2.34 (81%)	>10 (40.8%)	—
(*E*)-3-Methoxy-2-styrylchromone (**17**)	>150	75	37.5	37.9 ± 6.3	>4.0

[a] Results of CC_{50} (concentrations of compounds that cause 50% cytotoxicity when compared to control cells) show means ± SEM of 3–4 independent experiments performed in duplicate.

[b] $MNTC_{70}$ and $MNTC_{90}$ are maximum non-toxic concentrations (the highest concentration tested in which the MTT reduction capacity of RAW cells was at least 70% or 90% when compared to control cells). When a >90% reduction was not achieved, results at the minimum concentration tested were reported in parentheses.

[c] Results of IC_{50} (concentrations of compounds that reduced the plaque number by 50% when compared to control cells) show means ± SEM of 3–7 independent experiments performed in duplicate. When a 50% reduction was not achieved, the results obtained at the maximum concentration tested (10 or 50 μM) were reported in parentheses.

[d] The selectivity index (SI) was the CC_{50}/IC_{50}.

* Results from two independent experiments performed in duplicate.

2.4. Effect of time of addition of compounds in the anti-norovirus activity

In an attempt to understand if compounds **7** and **13** interfere with an early or a late step of norovirus replication cycle, two time-points of compound addition were tested and compared with standard conditions (Table 2). The anti-norovirus activity of compounds significantly decreased (P <0.0001) when they were present only during the 1 h of viral infection, according to our protocol. However, the potent effect of these compounds remained when they were added after the viral infection and for a continuous period of 48 h. These results indicate that the studied compounds interfere more with steps of viral replication that occur during the 48 h, rather than those occurring during the 1 h of infection.

2.5. Anti-norovirus activity versus anti-rhinovirus activity of (*E*)-2-styrylchromones

A review of published studies concerning the antiviral activity of (*E*)-2-styrylchromones[17,18] common to the present study was

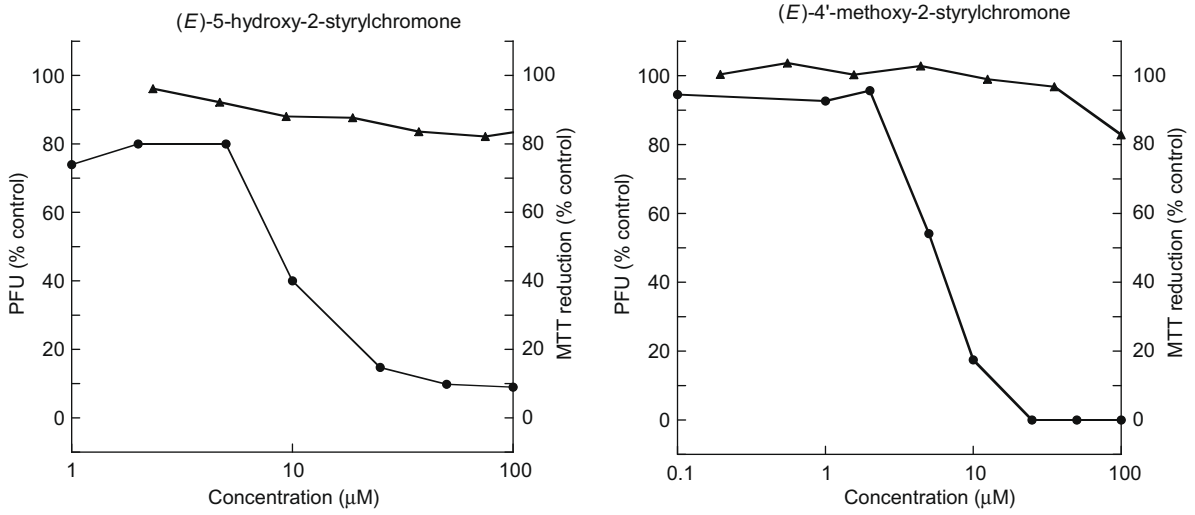


Figure 2. Cytotoxicity (—▲—) and anti-norovirus activity (—●—) of (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**).

Table 2
Effect of time of addition of (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**)

Compounds	IC_{50} (μM) 1 h + 48 h[a]	IC_{50} (μM) 1 h only[b]	IC_{50} (μM) 48 h[c]
(*E*)-5-Hydroxy-2-stryrylchromone (**7**)	6.9 ± 0.4	39.0 ± 3.8[†]	8.2 ± 1.3
(*E*)-4′-Methoxy-2-stryrylchromone (**13**)	6.9[*]	47.7[*,†]	8.4[*]

Results show means ± SEM of three independent experiments performed in duplicate.
[a] Effect in MNV replication of different times of exposure to compounds **7** and **13**: during the hour of viral infection plus 48 h in the overlay (standard conditions).
[b] Only during the hour of viral infection.
[c] Only for 48 h in the overlay.
[*] Results from two independent experiments performed in duplicate.
[†] A significant difference (P <0.0001) was observed relatively to the standard conditions.

conducted and a comparison of the antiviral activities was attempted (Table 3). As observed, some of these compounds showed to be active against both norovirus and rhinovirus, while others were only effective against one of these viruses. It was noticeable that the most potent compound for rhinovirus (**17**) presented only a modest anti-norovirus activity whereas one of the most active compounds against NoV, 4′-methoxy-2-stryrylchromone (**13**), was considered by the authors to be inactive for HRV 14, although it presented a slight antiviral effect against HRV 1B.[17] These different profiles of antiviral effect of 2-styrylchromones against these two viruses probably reflect a different interaction of the compounds with norovirus and rhinovirus.

3. Discussion

In the present work, a total of twelve structure related chromone (**2**) and (*E*)-2-styrylchromones (**4–8**, **12–17**) were evaluated for their potential anti-norovirus activity using the human NoV surrogate model MNV/RAW 264.7 cells. The choice of this class of flavonoid-type compounds was based on the previously described anti-rhinovirus activity of 2-styrylchromones[16–18] and the similarity of physical properties and replication strategy between picornavirus and calicivirus families.

From the 12 compounds studied, six (*E*)-2-styrylchromones exhibited interesting anti-norovirus activity, being (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**) the best compounds of the series, with an $IC_{50} \approx 7$ μM. Moreover, the high SI values presented by these compounds (>21.6 and >13.5, respectively) confers them an apparently good toxicological profile to be developed as antiviral drugs.

Although it may not be accurate to draw a structure–activity relationship from this small group of compounds, an interesting relationship between the anti-norovirus activity and the chemical structure was observed. Hence, when comparing the IC_{50} of chromone **2** (>50 μM) with (*E*)-2-styrylchromone **4** (17.7 μM), it is remarkable to note that the introduction of the (*E*)-2-styryl group in the chromone moiety was responsible for the appearance of the anti-norovirus effect. Moreover, the nature of the 4′-substituent in the 2-styrylchromone moiety influenced the potency of the anti-norovirus effect as demonstrated when the activities of 2-styrylchromones **12** and **13** were compared (IC_{50} 23.7 μM vs 7.4 μM, respectively). The 4′-methoxy group showed to be the best substituent, conferring to compound **13** an activity three times superior to the 4′-methyl substituted compound **12**. On the contrary, the 4′-chloro and 4′-hydroxy substituents of compounds **5** and **14** were responsible for a loss of antiviral activity. It was also observed that the nature of the 5-substituent of (*E*)-2-styrylchromones **6** and **7** was relevant for the anti-norovirus activity. When comparing their IC_{50} with that of (*E*)-2-styrylchromone **4** (17.7 μM) we can observe that the 5-hydroxy substituent of compound **7** was responsible for the appearance of one of the most potent anti-norovirus effects (7.0 μM) while the 5-benzyloxy group in compound **6** was responsible for a loss of antiviral activity (>50 μM). Additionally, it was curious to note that the 3-hydroxy group of (*E*)-2-styrylchromones appears to confer cytotoxicity to compounds **15** and **16**, the only ones that present this substituent and exhibit high toxicity ($CC_{50} \approx 50$ μM).

The potent activity presented by (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**) justifies further investigation of the mechanism of action underlying their anti-norovirus effect. A first insight into the mechanism of action of these compounds was reached by studying the effect of their addition at different time-points, which revealed that compounds **7** and **13** interfere more with steps of virus life cycle that follow the entrance of virus in cells. This may include either the blocking of viral enzymes involved in RNA replication (e.g., RNA-dependent RNA polymerase) or in proteolytic maturation, or an interference with virus release. Inhibitors of viral polymerases have shown to be related to antiproliferative drugs targeting cellular polymerase in highly replicating tumor cells.[29] In a previous study, our team demonstrated that (*E*)-4′-methoxy-2-styrylchromone was a potent inhibitor of the growth of a variety of human tumor cell lines, being much less efficient in inhibiting non-tumor human cells.[30] This ability of preferentially suppressing highly replicating tumor cells and the capacity of (*E*)-4′-methoxy-2-styrylchromone (**13**) to inhibit a late step of norovirus life cycle raise the possibility of this compound targeting the polymerase of norovirus. However, the present experimental data allows only this initial hypothesis for the mechanism of action of these compounds. Additional studies are needed to unveil the specific viral target of these (*E*)-2-styrychromones, which will include the generation of drug-resistant virus and their molecular characterization.

Table 3
Anti-norovirus versus anti-rhinovirus activity of (*E*)-2-styrylchromones (**4**, **5**, **12**–**15**, **17**)

Compounds	MNV IC_{50}[a] (μM)	HRV 1B IC_{50}[b] (μM)	HRV 14 IC_{50}[b] (μM)
(*E*)-2-Styrylchromone (**4**)	17.7 ± 6.5	6.19	>25
(*E*)-4′-Chloro-2-stryrylchromone (**5**)	>50	> 12.5	6.19
(*E*)-4′-Methyl-2-stryrylchromone (**12**)	23.7 ± 7.5	>6.25	>6.25
(*E*)-4′-Methoxy-2-stryrylchromone (**13**)	7.4 ± 1.3	>3.12	Inactive
(*E*)-4′-Hydroxy-2-stryrylchromone (**14**)	>50	9.29	6.41
(*E*)-3-Hydroxy-2-stryrylchromone (**15**)	>10	5.13	9.76
(*E*)-3-Methoxy-2-stryrylchromone (**17**)	37.9 ± 6.3	1.1	1.6

MNV = murine norovirus; HRV = human rhinovirus (strains 1B and 14).
[a] Results of anti-norovirus activity obtained in the present study.
[b] Results of anti-rhinovirus activity published by other authors (Desideri et al., 2000; Desideri et al., 2003).

4. Conclusion

To our knowledge, this is the first study reporting anti-norovirus activity of 2-styrylchromones. The interesting activity of (*E*)-5-hydroxy-2-styrylchromone and (*E*)-4′-methoxy-2-styrylchromone against norovirus led us to conclude that the (*E*)-2-styrylchromones skeleton is an important one, which deserves to be developed and explored as novel antiviral drugs against this important human pathogen.

5. Experimental

5.1. General methods

Melting points were determined on a *BUCHI Melting point* apparatus and are uncorrected. NMR spectra were recorded on Bruker

Avance 300 (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C) and Bruker Avance 500 (500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C) spectrometers. Chemical shifts (δ) are reported in ppm values (δ) and coupling constants (J) in hertz. The internal standard was TMS. ^{1}H assignments were made using two-dimensional gradient selected correlation spectroscopy (*g*COSY) and nuclear Overhauser effect spectroscopy (NOESY; 800 ms mixing time), experiments, while ^{13}C assignments were made using two-dimensional gradient selected heteronuclear single quantum coherence (*g*HSQC) and two-dimensional gradient selected heteronuclear multiple quantum coherence (*g*HMBC) (delays for one bond and long-range J C/H couplings were optimized for 145 and 7 Hz, respectively) experiments. Mass spectra (EI, 70 eV) were measured on a VG Autospec Q and M mass spectrometers.

5.2. Chemical compounds

Chromone (**2**) was obtained by condensation of the 2′-hydroxyacetophenone and methyl formate and their structural characterisation is as follow.

***Chromone* (2):** Mp 58–60 °C; ^{1}H NMR (300.13 Hz, $CDCl_3$): δ (ppm) 6.18 (d, 1 H, J = 6.0 Hz, H-3), 7.22 (ddd, 1 H, J = 0.9, 7.7 and 7.8 Hz, H-6), 7.26 (dd, 1 H, J = 0.9 and 8.2 Hz, H-8), 7.50 (ddd, 1 H, J = 1.7, 7.7 and 8.2 Hz H-7), 7.79 (d, 1 H, J = 6.0 Hz, H-2), 7.98 (dd, 1 H, J = 1.7 and 8.2 Hz H-7). ^{13}C NMR (75.47 Hz, $CDCl_3$): δ (ppm) 112.0 (C-3), 117.4 (C-8), 123.9 (C-10), 124.4 (C-6), 124.6 (C-5), 133.0 (C-7), 154.9 (C-2), 155.6 (C-9), 176.6 (C-4). EI-MS: *m*/*z* 146 (100) $M^{+\cdot}$, 120 (17), 118 (45), 92 (34), 90 (20), 74 (12), 63 (24), 53 (10). Anal. Calcd for $C_9H_6O_2$ (146.04): C, 73.97; H, 4.14. Found: C, 74.21; H, 4.10.

(E)-2-Styrylchromones (**4–8**, **12**, **13**) have been prepared by the Baker–Venkataraman method or the aldol condensation/oxidative cyclization procedure[22] and have shown spectroscopic and analytical data identical to those previously reported: ***(E)-2-styrylchromone* (4), *(E)-4′-chloro-2-styrylchromone* (5), *a-methyl-2-styrylchromone* (8)**;[23] ***(E)-4′-methyl-2-styrylchromone* (12) *and (E)-4′-methoxy-2-styrylchromone* (13)**;[25] ***(E)-5-benzyloxy-2-styrylchromone* (6)** and ***(E)-5-hydroxy-2-styrylchromone* (7)**.[24]

The (*E*)-3-alkoxy-2-styrylchromones (**15**–**17**) were obtained by the Algar–Flynn–Oyamada type-reaction.[26] Compounds (**15**, **17**) have shown spectroscopic and analytical data identical to those previously reported,[18] while ***(E)-3-hydroxy-α-methyl-2-styrylchromone*** (**16**) is a new compound: Mp 123–124 °C. ^{1}H NMR (500.13 MHz, DMSO-d_6): δ (ppm) 2.32 (d, 3 H, J = 1.3 Hz, α-C*H*$_3$), 7.34–7.37 (m, 1 H, H-4′), 7.44–7.49 (m, 5 H, H-6, H-2′,6′, H-3′,5′), 7.59 (s broad, 1 H, H-β), 7.70 (d, 1 H, J = 7.7 Hz, H-8), 7.93 (ddd, 1 H, J = 1.6, 7.0 and 7.7 Hz, H-7), 8.10 (dd, 1 H, J = 1.6 and 8.0 Hz, H-5). (125.77 MHz, DMSO-d_6): δ (ppm) 15.6 (α-*C*H$_3$), 118.3 (C-8), 121.2 (C-10), 124.7 (C-5), 124.5 (C-6), 127.7 (C-4′), 128.5 (C-2′,6′), 128.9 (C-α), 129.4 (C-3′,5′), 133.6 (C-7), 134.2 (C-β), 136.3 (C-1′), 138.9 (C-3), 148.3 (C-2), 154.3 (C-9), 172.7 (C-4). ESI$^+$-MS: 301 (35) $(M+Na)^+$, 279 (100) $(M+H)^+$. Anal. Calcd for $C_{18}H_{14}O_3$ (278.30): C, 77.68; H, 5.07. Found: C, 77.68; H, 5.04.

***(E)-4′-Hydroxy-2-styrylchromone* (14):** A solution of (*E*)-4′-methoxy-2-styrylchromone (**13**) (0.6 mmol) in dry CH_2Cl_2 was cooled in a propan-2-ol bath at −78 °C and then a solution of BBr_3 in CH_2Cl_2 (1 M, 1 mL) was added drop wise. After the addition, the bath was removed and the reaction mixture was stirred under nitrogen, at room temperature, for 24 h. After that period, the solution was poured into ice and water. The solid obtained was filtered off and washed with water and light petroleum. Mp 284–285 °C; ^{1}H NMR (500.13 MHz, DMSO-d_6): δ (ppm) 6.40 (s, 1 H, H-3), 6.85 (d, 2 H, J = 8.6 Hz, H-3′,5′), 6.99 (d, 1 H, J = 16.1 Hz, H-α), 7.47 (t, 1 H, J = 7.5 Hz, H-6), 7.59 (d, 2 H, J = 8.6 Hz, H-2′,6′), 7.63 (d, 1 H, J = 16.1 Hz, H-β), 7.71 (d, 1 H, J = 7.7 Hz, H-8), 7.81 (ddd, 1 H, J = 1.6, 7.5 and 7.7 Hz, H-7), 8.01 (dd, 1 H, J = 1.6 and 7.5 Hz, H-5), 10.05 (s, 1 H, 4′-OH). ^{13}C NMR (125.77 MHz, DMSO-d_6): δ 109.0 (C-3), 116.0 (C-3′,5′), 116.9 (C-α), 118.2 (C-8), 123.6 (C-10), 124.8 (C-5), 125.2 (C-6), 126.1 (C-1′), 129.8 (C-2′,6′), 134.2 (C-7), 136.9 (C-β), 155.5 (C-9), 159.4 (C-4′), 162.5 (C-2), 176.9 (C-4). ESI+-MS: *m*/*z* 287 (20) $(M+Na)^+$, 265 (100) $(M+H)^+$. HRMS-EI: Calcd for $C_{17}H_{13}O_3$ 264.0786; found: 264.0796.

5.3. Samples

Stock solutions (60 mM) of each compound **2**, **4–8** and **12–17** were prepared in DMSO (drug vehicle) and kept at −20 °C. Appropriate dilutions were freshly prepared just prior to every assay.

5.4. Cell culture

The murine macrophage cell line RAW 264.7 was used to propagate MNV. Cells were grown in DMEM supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U penicillin/mL, 100 μg/mL streptomycin and 10% (growth medium) or 2% (maintenance medium) of inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Confluent RAW cells were used in all assays and were obtained by plating 5.0×10^5 viable cells/mL in growth media followed by a 24 h incubation period. Culture media was then removed and replaced by maintenance medium.

The first overlay media for viral plaque assay was prepared in E-MEM 2 × without phenol red and supplemented with 4 mM L-glutamine, 0.2 mM non essential amino acids, 30 mM HEPES, 0.23% sodium bicarbonate, 2 mM sodium pyruvate, 200 U penicillin/mL, 200 μg/mL streptomycin and 10% FBS. The second overlay media contained in addition neutral red at a final concentration of 0.6%.

5.5. Virus

MNV (virus strain MNV-1.CW1) was provided by Herbert W. Virgin (Washington University, St. Louis, USA). MNV was propagated by infecting confluent RAW cells cultured in maintenance medium and harvested when cytopathic effect stopped progressing. MNV stocks were obtained by submitting infected cells to three consecutive freeze–thaw cycles followed by centrifugation to partially purify virus from cell lysates being the supernatants stored at −80 °C.

5.6. Cytotoxicity by the MTT assay

The toxicity of compounds was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. RAW cells in 96-well plates were exposed to serial concentrations of each compound (in duplicate) for 48 h, being 150 μM (or 100 μM for compound **13**) the highest concentration tested. Control cells were exposed to the maximum concentration of DMSO (0.25%) and the same incubation time. After treatment, MTT solution (0.5 mg/mL) was added and plates were incubated for further 4 h to allow the reduction of MTT. Then, medium was removed and DMSO was added to solubilize the formazan crystals. Absorbance was measured at 550 nm. The percentage of MTT reduction was calculated comparing the absorbance of compound treated cells to the control cells and the CC_{50} value of each compound (concentration that reduced the absorbance of treated cells by 50% when compared to control cells) was determined. Maximum non-toxic concentration of compounds (MNTC) was defined as those in which the MTT reduction capacity of treated cells was at least 90% ($MNTC_{90}$) or 70% ($MNTC_{70}$) when compared to control cells. Only non-toxic concentrations of compounds were further tested for antiviral activity.

5.7. Anti-norovirus activity by plaque reduction assay

The anti-norovirus activity of compounds was evaluated by plaque reduction assay. RAW cells in 6-well plates were infected with MNV for 1 h at 37 °C and simultaneously exposed to serial concentrations of each compound (in duplicate) for a continuous period of 48 h. These conditions will be from here on referred as standard. The highest concentration tested was 50 μM for all the compounds but 10 μM for compounds **15** and **16**. A MNV suspension producing 20–80 plaque forming units (PFU) per well was used. After viral infection period, culture media was removed and the first agarose overlay (containing equal concentrations of compounds) was added and incubated for 24 h. After this incubation period the second overlay with neutral red was added. Plates were incubated for more 24 h and then PFU were counted. Control cells were infected with the same MNV suspension and exposed to the maximum concentration of DMSO (0.084%) and the same incubation time. The IC_{50} value of each compound (concentration of compound that reduced the plaque number by 50% when compared to control cells) was determined. The SI value of each compound was calculated as CC_{50}/IC_{50}.

For the dose–response studies of (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**), standard conditions were used but compounds were tested starting at the highest concentration of 100 μM to allow a more detailed study.

The anti-norovirus effect of (*E*)-5-hydroxy-2-styrylchromone (**7**) and (*E*)-4′-methoxy-2-styrylchromone (**13**) was further evaluated by addition of compounds at two different time-points. Briefly, RAW cells were exposed to serial concentrations of compounds **7** and **13** only during the hour of viral infection (1 h at 37 °C) or only for 48 h in the overlay after viral infection. An experiment with the standard conditions was run simultaneously. The IC_{50} values for the different time-points were determined as described above.

5.8. Statistics

Results are expressed as mean values SEM (standard error of the mean). Statistical comparisons were made using one-way analysis of variance followed by the Bonferroni's multiple comparison post hoc test.

Acknowledgements

Thanks are due to *Fundação para a Ciência e a Tecnologia* (FCT), I&D units 4040/2007 (CEQUIMED-UP) and 62/94 (QOPNA), FEDER, POCI for financial support. The authors thank also FCT for the Ph.D. grant of Joana Rocha-Pereira (SFRH/BD/48156/2008). Authors are also grateful to Herbert W. Virgin for the generous provision of the MNV.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.006.

References and notes

1. Glass, R. I.; Parashar, U. D.; Estes, M. K. *N. Eng. J. Med.* **2009**, *361*, 1776.
2. Patel, M. M.; Hall, A. J.; Vinje, J.; Parashar, U. D. *J. Clin. Virol.* **2009**, *44*, 1.
3. Widdowson, M. A.; Monroe, S. S.; Glass, R. I. *Emerg. Infect. Dis.* **2005**, *11*, 735.
4. Green, K. Y. In *Fields Virology, Vol. 2*; Knipe, D. M., Howley, P. M., Eds.; Lippincott Williams & Wilkins: Philadelphia, 2007; pp 949–979.
5. Wobus, C. E.; Karst, S. M.; Thackray, L. B.; Chang, K. O. *Sosnovtsev S.V.; Belliot, G.; Krug, A.; Mackenzie, J.M.; Green, K.Y.; Virgin, H.W. PLoS Biol* **2004**, *2*, 432.
6. Wobus, C. E.; Thackray, L. B.; Virgin, H. W. *J. Virol.* **2006**, *80*, 5104.
7. Cannon, J. L.; Papafragkou, E.; Park, G. W.; Osborne, J.; Jaykus, L. A.; Vinje, J. *J. Food Prot.* **2006**, *69*, 2761.
8. Baert, L.; Uyttendaele, M.; Vermeersch, M.; Van Coillie, E.; Debevere, J. *J. Food Prot.* **2008**, *71*, 1590.
9. Baert, L.; Wobus, C. E.; Van Coillie, E.; Thackray, L. B.; Debevere, J.; Uyttendaele, M. *Appl. Environ. Microbiol.* **2008**, *74*, 543.
10. Ng, K. K.; Pendas-Franco, N.; Rojo, J.; Boga, J. A.; Machin, A.; Alonso, J. M.; Parra, F. *J. Biol. Chem.* **2004**, *279*, 16638.
11. De Palma, A. M.; Vliegen, I.; De Clercq, E.; Neyts, J. *Med. Res. Rev.* **2008**, *28*, 823.
12. Bauer, D. J.; Selway, J. W.; Batchelor, J. F.; Tisdale, M.; Caldwell, I. C.; Young, D. A. *Nature* **1981**, *292*, 369.
13. Conti, C.; Mastromarino, P.; Sgro, R.; Desideri, N. *Antiviral Chem. Chemother.* **1998**, *9*, 511.
14. Robin, V.; Irurzun, A.; Amoros, M.; Boustie, J.; Carrasco, L. *Antiviral Chem. Chemother.* **2001**, *12*, 283.
15. Tait, S.; Salvati, A. L.; Desideri, N.; Fiore, L. *Antiviral Res.* **2006**, *72*, 252.
16. Conti, C.; Mastromarino, P.; Goldoni, P.; Portalone, G.; Desideri, N. *Antiviral Chem. Chemother.* **2005**, *16*, 267.
17. Desideri, N.; Conti, C.; Mastromarino, P.; Mastropaolo, F. *Antiviral Chem. Chemother.* **2000**, *11*, 373.
18. Desideri, N.; Mastromarino, P.; Conti, C. *Antiviral Chem. Chemother.* **2003**, *14*, 195.
19. G.P. Ellis, in: G.P. Ellis (Ed.), General methods of preparing chromones in Chromenes, Chromanones and Chromones, John Wiley & Sons, New York, 1977, pp. 495-555, Chapter IX.
20. Makrandi, J. K.; Kumari, V. *Chem. Ind.* **1988**, 630.
21. Santos, C. M. M.; Silva, A. M. S.; Cavaleiro, J. A. S. *Eur. J. Org. Chem.* **2003**, 4575.
22. Price, W. A.; Silva, A. M. S.; Cavaleiro, J. A. S. *Heterocycles* **1993**, *36*, 2601.
23. Silva, A. M. S.; Pinto, D. C. G. A.; Tavares, H. R.; Cavaleiro, J. A. S.; Jimeno, M. L.; Elguero, J. *Eur. J. Org. Chem.* **1998**, 2031.
24. Pinto, D. C. G. A.; Silva, A. M. S.; Cavaleiro, J. A. S. *Heterocycl. Commun.* **1996**, *2*, 145.
25. Pinto, D. C. G. A.; Silva, A. M. S.; Cavaleiro, J. A. S.; Levai, A.; Patonay, T. *J. Heterocycl. Chem.* **1998**, *35*, 217.
26. Gormley, T. R.; O'Sullivan, W. I. *Tetrahedron* **1973**, *29*, 369.
27. Cerqueira, F.; Cidade, H.; van Ufford, L.; Beukelman, C.; Kijjoa, A.; Nascimento, M. S. *Int. Immunopharmacol.* **2008**, *8*, 597.
28. Cerqueira, F.; Cordeiro-da-Silva, A.; Araujo, N.; Cidade, H.; Kijjoa, A.; Nascimento, M. S. *Life Sci.* **2003**, *73*, 2321.
29. Müller, B. H.-G. Kraüsslich. In *Handbook of Experimental Pharmacology, Vol. 189*; Krausslich, H.-G., Bartenschlager, R., Eds.; Springer-Verlag: Berlin, 2009; pp 2–24.
30. Marinho, J.; Pedro, M.; Pinto, D. C. G. A.; Silva, A. M. S.; Cavaleiro, J. A. S.; Sunkel, C. E.; Nascimento, M. S. J. *Biochem. Pharmacol.* **2008**, *75*, 826.