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**SEARCHING FOR ANTIVIRALS AGAINST
NOROVIRUS:**

2-styrylchromones as potential anti-norovirus agents



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There is a tide in the affairs of men.
Which, taken at the flood, leads on to fortune;
Omitted, all the voyage of their life
Is bound in shallows and in miseries.
On such a full sea are we now afloat,
And we must take the current when it serves,
Or lose our ventures.

William Shakespeare, *Julius Caesar*

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Resumo

Os norovirus humanos (NoVs) são actualmente reconhecidos como a causa mais frequente de surtos epidémicos e casos esporádicos de gastroenterite aguda transmitida pelos alimentos. Afectam todos os grupos etários e surgem frequentemente em locais onde há uma concentração de indivíduos como os barcos de cruzeiro, lares de idosos, hospitais, escolas e restaurantes. Os norovírus são classificados como agentes biológicos da classe B devido à sua elevada infecciosidade e estabilidade no meio ambiente, à alta transmissibilidade pessoa-a-pessoa e à natureza debilitante da doença.

Apesar do elevado impacto económico da doença e da sua considerável morbilidade não existem actualmente medicamentos ou vacinas disponíveis no mercado para a tratar ou prevenir, logo a descoberta de fármacos anti-norovírus é fundamental.

Devido à inexistência de um modelo de cultura celular e de um pequeno modelo animal para o estudo dos NoVs humanos, muito permanece ainda para estudar e descobrir sobre estes vírus. O norovirus murino (MNV) replica eficazmente em cultura celular e tem sido proposto como modelo alternativo ao NoV humano. O MNV é geneticamente relacionado com o NoV humano e é provável que muitos dos mecanismos fundamentais de replicação e características bioquímicas sejam conservadas entre estes vírus.

Muitos flavonóides, incluindo as 2-estirilcromonas, têm-se mostrado capazes de inibir a replicação de vários picornavírus. Dado que muitas das características de replicação dos picornavírus são semelhantes às dos calicivirus, família à qual os norovirus pertencem, é previsível que estes compostos possuam actividade anti-norovírus.

Os objectivos deste trabalho foram: (i) pesquisar uma potencial actividade anti-norovírus de uma série de cromonas e 2-estirilcromonas e (ii) procurar estabelecer uma relação estrutura/ actividade para este grupo de compostos.

Os resultados mostram que, enquanto as cromonas testadas não apresentam actividade anti-norovírus, várias 2-estirilcromonas apresentam uma actividade de varia entre moderada ($IC_{50}=20-50\ \mu M$) a muito potente ($IC_{50}<10\ \mu M$). 4'-metoxi-2-estirilcromona e 5-hidroxi-2-estirilcromona mostraram ser os compostos mais potentes, com um IC_{50} de 7,0 μM .

Apesar de o presente estudo incluir um pequeno grupo de compostos, foi observada uma interessante relação estrutura/ actividade: (i) a introdução do grupo 2-estiril no núcleo da cromona foi responsável pelo aparecimento de actividade anti-norovírus; (ii) a natureza dos substituintes na posição 4' e 5 da 2-estirilcromona influenciaram a potência dos

compostos, sendo os grupos metoxi- e hidroxi- os melhores substituintes, respectivamente.

Concluindo, este trabalho põe em evidência as 2-estirilcromonas como candidatos interessantes para serem desenvolvidos como fármacos anti-norovírus. Para além disso, o seu efeito potente justifica a elucidação do seu mecanismo de acção.

Palavras-chave: norovirus, actividade antivirica, técnica de formação de placas, 2-estirilcromonas, MNV

Abstract

Human noroviruses (NoVs) are now recognized as the most frequent cause of outbreaks and sporadic cases of acute gastroenteritis. They affect people of all age groups and often occur in crowded locations, such as cruise ships, nursing homes, hospitals, schools and restaurants. Noroviruses are class B biological agents due to their high infectivity and stability, rapid person-to-person spread and debilitating nature of the disease.

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. Murine norovirus (MNV) is able to replicate in cell culture and has been proposed as a surrogate model for human NoV. 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.

Many flavonoids, including 2-styrylchromones have shown to inhibit the replication of picornavirus. Since the replication strategy of the picornavirus family shares many features with the calicivirus family, to which NoV belongs, this allows us to predict possible anti-norovirus activity for these compounds.

The aim of this work was therefore to: (i) search for potential anti-norovirus activity of a series of chromones and 2-styrylchromones and (ii) try to establish a structure/ activity relationship.

Results showed that the chromones studied have no anti-norovirus activity while 2-styrylchromones exhibited an antiviral effect that ranges from moderate (IC_{50} =20-50 μ M) to very potent (IC_{50} <10 μ M). 4'-methoxy-2-styrylchromone and 5-hydroxy-2-styrylchromone were the most potent compounds, showing an IC_{50} of 7,0 μ M.

Although this study included a small group of compounds, an interesting structure/ activity relationship was observed: (i) the introduction of the 2-styryl group in chromone moiety was responsible for the appearance of the anti-norovirus activity; (ii) the nature of the 4' and the 5 substituent in 2-styrylchromones influenced the potency of compounds, being the methoxy and the hydroxy group the best substituents, respectively.

In conclusion, the present work points out that 2-styrylchromones are attractive candidates to be developed as anti-norovirus drugs. Furthermore the potent anti-norovirus effect of some justifies the elucidation of their mechanism of action.

Keywords: Norovirus, antiviral activity, plaque assay, 2-styrylchromones, MNV

Abbreviations

CC ₅₀	50% cytotoxic concentration
CDC	Center for Disease Control and Prevention
CO ₂	carbon dioxide
CPE	cytopathic effect
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
ECACC	European Collection of Cell Cultures
EIA	enzyme immunoassays
E-MEM	Minimum Essential Medium - Eagle
FCV	feline calicivirus
FBS	fetal bovine serum
FUT	fucosyltransferase
G	genogroup
h	hours
HBGA	histo-blood group antigen
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IEM	immune electron microscopy
μL	microliters
μM	micromolar
min	minutes
MNTC	maximum non-toxic concentration
MNV	murine norovirus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEAA	non-essential amino acids
NoV	norovirus

nm	nanometers
NO	nitric oxide
ORF	open reading frame
PBS	phosphate buffered saline
RNA	ribonucleic acid
RdRp	RNA-dependent RNA polymerase
r.p.m.	revolutions per minute
SEM	standard error of the mean
SD	standard deviation
SI	selectivity index
TCID ₅₀	tissue culture 50% infectious dose
VP	viral protein
USA	United States of America

I.

INTRODUCTION

I. INTRODUCTION

Viral gastroenteritis and the importance of Norovirus

Gastroenteritis, both epidemic and sporadic, is a common cause of morbidity and mortality among people of all ages, accounting for over 1,8 million deaths in children under 5 years of age worldwide (Bryce *et al.*, 2005). The causes of gastroenteritis include a large variety of bacteria, parasites and viruses, yet in many settings the relative contribution of these agents is unknown (Patel *et al.*, 2009).

The establishment of a viral etiology for gastroenteritis was a decades-long process hampered by the fastidious nature of many of these viruses in cell culture (Green, 2007). In 1972, a 27-nm virus-like particle was discovered by use of immune electron microscopy (IEM) in an infectious stool filtrate derived from an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, USA. Kapikian *et al.* were able to demonstrate through serum antibody responses in key individuals infected under natural or experimental conditions along with other evidence that this virus-like particle was the etiologic agent of the Norwalk gastroenteritis outbreak, naming it Norwalk virus (Kapikian *et al.*, 1972; Kapikian, 2000). This virus is considered to be the prototype virus of the genus *Norovirus* (previously denoted as “*Norwalk-like viruses*”) that belongs to the *Caliciviridae* family. Three other genera are now described in the *Caliciviridae* family: *Sapovirus* (previously called “*Sapporolike viruses*”), *Lagovirus*, and *Vesivirus*, and a fifth genus (proposed *Nebovirus*) is pending. Only *Norovirus* and *Sapovirus* infect humans, causing acute gastroenteritis (Green, 2007).

Noroviruses (NoVs) are now recognized as the most common cause of epidemic gastroenteritis in all age groups, accounting for greater than 90% of viral gastroenteritis and ~50% of all-cause outbreaks worldwide (Buesa *et al.*, 2002; Lopman *et al.*, 2003b; Reuter *et al.*, 2005; van Duynhoven *et al.*, 2005; Ike *et al.*, 2006; Patel *et al.*, 2009). Noroviruses are also common causes of sporadic cases of gastroenteritis but the prevalence of NoV in non-outbreak settings has been poorly documented and underappreciated in most regions of the world (Patel *et al.*, 2009).

Characteristics

Norovirus are non-enveloped viruses with an icosahedral capsid of 27-32 nm, and a single (+)-strand RNA genome of 7,5-7,7 kb, with three open-reading frames (ORFs). ORF 1 encodes a large polyprotein which undergoes proteolytic cleavage to produce six non-structural viral proteins including a nucleoside triphosphatase, a “3C-like” protease and a RNA-dependent RNA polymerase. ORF 2 and ORF 3 encode respectively VP 1, the major structural protein, and VP 2, the minor capsid protein (Green, 2007).

The molecular cloning of the Norwalk virus genome in 1990 led to remarkable progress in understanding the molecular virology and epidemiology of NoV, development of molecular diagnostic assays, and defining the global burden of NoV disease (Xi *et al.*, 1990). Although NoVs have not been cultivated and thus direct serotyping using neutralizing antibodies is not possible, reverse transcription-polymerase chain reaction (RT-PCR) and genomic sequencing has demonstrated that NoVs are genetically and antigenically diverse (Zheng *et al.*, 2006).

NoVs are a diverse group of viruses that are today classified into five genogroups (GI-V) which can be divided into at least 28 genetic clusters or genotypes based on sequence diversity in the complete capsid protein VP 1 (Zheng *et al.*, 2006). Genogroups share >60% amino acid identity in VP 1 and each genetic cluster or genotype >80% identity in amino acid sequence of VP 1 (Green *et al.*, 2000; Zheng *et al.*, 2006). Human NoVs have been associated with GI, GII and GIV. NoVs of genetic cluster GII.4 have been predominant in outbreaks throughout the world whereas GIV viruses are very rare and only a few outbreaks with this virus have been reported (Lindesmith *et al.*, 2008).

Clinical and epidemiological features

Noroviruses infect all age groups and often occur in crowded everyday settings such as schools, hospitals, day care centers, nursing homes, hotels, cruise ships, restaurants, usually causing a short-term, self-limiting disease (Hutson *et al.*, 2004; Estes *et al.*, 2006). Clinical NoV infection generally has an incubation of 24–48 h and is characterized by acute onset of nausea, vomiting, abdominal cramps, myalgias, and non-bloody diarrhea (Green, 2007). NoV illness can present with relatively severe symptoms of vomiting and non-bloody diarrhea, which usually resolve in 2–3 days. Still, the median duration of illness can be longer (i.e., 4–6 days) and fever is also often reported (Patel *et al.*, 2009). Severe dehydration is more commonly observed in infants and the elderly because they are more sensitive to volume depletion (Hutson *et al.*, 2004).

Cases of chronic diarrhea in transplant recipients undergoing immunosuppressive therapy have been attributed to NoV infection (Hutson *et al.*, 2004). Deaths have been reported during outbreaks in nursing homes (Gotz *et al.*, 2002) and NoV has been associated with necrotizing enterocolitis (Turcios-Ruiz *et al.*, 2008). According to a recent review concerning hospitalized patients NoVs accounted for ~12% of severe gastroenteritis cases among children <5 years of age worldwide (Patel *et al.*, 2008). Consequently, NoVs appear to be an important cause of endemic diarrhea in children, second only to rotavirus as an important cause of severe gastroenteritis among children worldwide (Patel *et al.*, 2008; Patel *et al.*, 2009).

Although NoV disease outbreaks are reported year-round, they peak during months with cold weather in temperate climates (Green, 2007). However, recent studies have demonstrated Spring and Summer peaks in NoV outbreaks and diarrheal hospitalizations among children <5 years of age (Lopman *et al.*, 2003a).

Transmission

NoV are classified as class B biological agents due to their high infectivity, rapid person-to-person spread, the explosiveness of outbreaks and debilitating nature of the disease (Hutson *et al.*, 2004; Estes *et al.*, 2006). Several characteristics of NoVs facilitate their spread in epidemics including: (i) the low infectious dose of NoVs (<10 viral particles); (ii) prolonged duration of viral shedding, even after symptoms resolve, increases the risk of secondary spread; (iii) the stability of the virus in relatively high concentrations of chlorine and at a wide range of temperatures (from freezing to 60°C); and (iv) repeated infections can occur throughout life with re-exposure, likely because lack of complete cross-protection against the diverse NoV strains and inadequate long-term immunity (Duizer *et al.*, 2004a; Estes *et al.*, 2006; Teunis *et al.*, 2008; Patel *et al.*, 2009).

Fecal-oral spread is generally the most important mode of transmission of NoVs. Often, primary cases in an outbreak result from consumption of contaminated food (raw oysters, bakery products, fresh fruit and vegetables), after intake of water (from ice, well or bottled water and during swimming) or following direct person-to-person contact (Hutson *et al.*, 2004; Estes *et al.*, 2006; Patel *et al.*, 2009). Further propagation of the epidemic is commonly due to person-to-person spread among contacts of primary cases (Becker *et al.*, 2000). Transmission through infectious vomit, both by mechanical transmission from environmental surfaces (i.e., through hand/mouth contact) and aerosolization, might account for the rapid and extensive spread of disease outbreaks in closed settings, such as hospitals, hotels, cruise ships, and day-care centers (Widdowson *et al.*, 2005).

Evidence of contamination of environmental surfaces with NoVs has been documented during outbreaks in institutions suggesting that this may serve as a reservoir that sustains an outbreak (Anonymous, 2008).

Host susceptibility, immunology, and pathogenesis

Susceptibility to norovirus infection involves both acquired immunity and genetic resistance. Volunteer studies found that some individuals were repeatedly susceptible to NoV infection whereas others were repeatedly resistant (Parrino *et al.*, 1977). Although it was initially unclear why some subjects did not develop illness, recent research suggests that host genotype is a prominent factor in the development of NoV infection since it depends on the presence of specific human histo-blood group antigen (HBGA) receptors in the gut of susceptible hosts (Lindesmith *et al.*, 2003).

ABH histo-blood group antigens and Lewis antigens are carbohydrates present on gut epithelial cells. These antigens provide diversity within the human population and their biosynthesis is controlled by the enzyme products of alleles at the ABH, fucosyltransferase (FUT) 2, and FUT 3 loci (Hutson *et al.*, 2004). Norovirus binding specificities to histo-blood group antigens varies among different viral strains (Huang *et al.*, 2003; Hutson *et al.*, 2004). Further work is needed to continue to understand the complex interactions between specific virus strains and susceptibility to infection and to determine whether a common site for carbohydrate binding can be identified that might allow development of inhibitory reagents that block infectivity of a broad range of noroviruses (Estes *et al.*, 2006).

Strategies to develop antiviral drugs

To date, no specific antiviral drugs are available for treatment of human NoV illness. Severe cases of dehydration are treated by electrolyte replacement and bed rest. Therefore and considering the significant economic impact and considerable morbidity of norovirus gastroenteritis, the discovery of anti-norovirus drugs is urgent.

The understanding of norovirus replication is still in its infancy. However, the replication strategy elucidated thus far show that NoV shares many features with other (+)-strand RNA virus (Wobus *et al.*, 2006). Norovirus derive their mature non-structural proteins by proteolytic cleavage of a large polyprotein encoded by ORF 1. This cleavage is mediated by the protease itself, which also mediates cleavage of the capsid precursor protein VP 1

(Green, 2007). Given the importance of non-structural proteins and of VP 1, the major capsid protein responsible for the recognition of the host cell receptor, to target the protease seems to be one of the best approaches to interfere with the life cycle of NoV. The viral encoded RNA-dependent RNA polymerase (RdRp) was determined in 2004 (Ng *et al.*, 2004) and its structure has an overall architecture common to a wide range of viral RdRps, as well as a unique structure in which the carboxyl terminus of the protein lies in the active site cleft. It is thought that the location of this carboxyl-terminal segment may allow this part of the protein to play a role in the initiation of RNA synthesis, with slight variations in different viruses (Ng *et al.*, 2004). Understanding the role of this segment in viral replication may be important for the design of structure-based therapeutics against noroviruses and possibly other (+)-strand RNA viruses. Drug compounds that specifically disrupt interactions between the carboxyl-terminal segment and the active site cleft may interfere with viral replication and act as effective antiviral agents (Ng *et al.*, 2004). Despite the difficulty in cultivating NoVs, significant advances in understanding the genomic structure, individual viral proteins, RNA replication strategy, and virus-host interaction of the virus have been made. These advancements provide new strategies in the development of antiviral agents against NoV, which can aim the inhibition of viral attachment to host cells through carbohydrate receptors, inhibition of viral protease and polymerase functions, and interfering with viral replication (Tan and Jiang, 2008).

Searching for a cell culture system to study human NoV

Little is known about biology of human NoV, which is mainly due to the absence of a cell culture system or small animal model. So far, all attempts to propagate human NoV in routine laboratory cell culture or primary tissue cultures have been unsuccessful (Duizer *et al.*, 2004b). However, a recent report using a 3-D cell culture system demonstrated for the first time successful passage of both GI and GII NoV *in vitro* (Straub *et al.*, 2007). While this method is an exciting new avenue for research, it will take some time before a routine cell culture assay will be available.

For this reason, most information on basic virology questions regarding replication and immune correlates of protection has come from human challenge studies and studies using model or surrogate viruses. The most frequently used surrogate models for NoV infectivity have included viruses from other genera within the family *Caliciviridae*, namely porcine enteric calicivirus, a *Sapovirus*, canine calicivirus and the feline calicivirus (FCV), both *Vesivirus* (Doultree *et al.*, 1999; Duizer *et al.*, 2004a). Until recently, FCV has been the most frequently used surrogate model for survival, persistence and inactivation studies of human NoVs (Thurston-Enriquez *et al.*, 2003a; Thurston-Enriquez *et al.*, 2003b;

Cannon *et al.*, 2006; Bae and Schwab, 2008). However, use of FCV as model for human NoV has been criticized because FCV: (i) does not belong to the genus *Norovirus* (ii) is a respiratory virus and (iii) cannot survive at low pH, a necessary characteristic of enteric viruses that must survive passage through the stomach (Duizer *et al.*, 2004a; Cannon *et al.*, 2006; Wobus *et al.*, 2006; Bae and Schwab, 2008) .

Recently, murine norovirus (MNV) was discovered and this virus is to date the only *Norovirus* able to replicate both in cell culture and in a small animal and has been accepted as the best surrogate model for human NoV (Wobus *et al.*, 2004; Wobus *et al.*, 2006). MNV is a genogroup V NoV and can grow routinely in the murine macrophage cell line RAW 264.7.

Murine norovirus, a surrogate model for human NoV

The MNV model system provides nowadays the best opportunity to understand the relationship between basic mechanisms of norovirus replication in tissue culture and pathogenesis in a natural host, since this virus shares biological and molecular properties with human NoV (Wobus *et al.*, 2004; Wobus *et al.*, 2006). MNV can be reverse engineered via an infectious clone and represents the first norovirus for which the immune response can be fully explored in an animal model and to which neutralizing antibodies have been isolated (Katpally *et al.*, 2008).

MNV has the size, shape, and buoyant density characteristic of human NoV (Karst *et al.*, 2003; Green, 2007). MNV genome has also the three ORFs characteristic of noroviruses. The conserved molecular features of MNV and human NoV genomes suggest that many fundamental mechanisms of replication are conserved between murine and human noroviruses (Wobus *et al.*, 2006). From a pathogenic point of view, MNV also shares with human NoV the capacity to spread through the fecal-oral route and MNV RNA is shed at high levels in the feces (Karst *et al.*, 2003; Wobus *et al.*, 2004). However, MNV does not cause gastroenteritis in mice but hepatitis, pneumonia, or inflammation of the nervous system and is for that reason very different from the clinical presentation of the human NoVs (Karst *et al.*, 2003). Hence, the use of MNV has been criticized and does not constitute yet the ideal model for studying human NoV.

MNV allows the use of immunodeficient mice to identify the immune mechanisms required to control and eliminate mucosal, lymphatic, and systemic norovirus infection (Karst *et al.*, 2003; Wobus *et al.*, 2004; Katpally *et al.*, 2008). It is important to note that the relationship between the pathogenesis of MNV infection and human norovirus infection is not clear (Wobus *et al.*, 2006). However, it is believed that mechanistic studies of MNV immunity in

well-defined mouse systems may provide valuable insights into conserved aspects of norovirus immunity while a better surrogate model hasn't been discovered (Chachu *et al.*, 2008).

Screening for anti-norovirus activity in chemical compounds

Traditionally, antiviral drugs are discovered by testing chemicals for their ability to inhibit virus replication in labor- and time-consuming infectivity assays, such as cytopathic effect and plaque assays (Coen *et al.*, 2007). These chemicals had usually been synthesized for some other purpose, and discovery of their antiviral activities has been often serendipitous. Examples of drugs that were discovered this way are amantadine and acyclovir (Davies *et al.*, 1964; Schaeffer *et al.*, 1978). When particular chemicals were identified as having antiviral activity, a variety of other chemicals with related structures were then tested for activity. A more modern and rational approach of high-throughput screening of large libraries of chemicals has been used to discover some newer drugs (Coen *et al.*, 2007). These can be *target*- or *cell*- based and start with the identification of a viral gene product that would make a good drug target (Coen *et al.*, 2007). It is important that the target protein can be expressed at high levels and purified, and that it can be assayed rapidly using robots. Most non-nucleoside reverse transcriptase inhibitors, which are active against HIV-1, were discovered using this approach (for example, nevirapine) (Seifer *et al.*, 1998). A still more rational approach is to exploit detailed knowledge about a viral protein to design drugs that will inhibit its activity (Coen *et al.*, 2007). In particular, knowledge of the three-dimensional structure of the protein, which can enable visualization of its active site and understanding of its mechanism of action, can also be used to design small molecules that can block the active site and the protein's action. An example of a drug discovered using this approach is the neuraminidase inhibitor, zanamivir, which is active against influenza A and B virus (von Itzstein *et al.*, 1993).

Currently, there are no anti-norovirus drugs commercially available for treatment of NoV illness. Additionally, and to our knowledge, there are no proposed molecules undergoing phase II or III clinical trials. Therefore the discovery of chemical compounds with anti-norovirus activity is urgent.

Regarding molecular targets, it was recently reported that hippuristanol, a small molecule inhibitor of eukaryotic translation initiation factor 4A (eIF4A), inhibits calicivirus replication in cells, highlighting this novel paradigm of translation initiation as a good target for antiviral intervention (Chaudhry *et al.*, 2006; Goodfellow and Roberts, 2008).

Nevertheless, this work constitutes, to our knowledge, the only paper reporting chemical compounds studied for anti-norovirus activity in a virus/ cell culture system.

The classical strategy based on the identification of active chemical compounds belonging to various chemical families still remains an interesting approach of drug discovery because it provides novel chemical structures with new mechanisms of action. Therefore, screening structure relative derivatives against the surrogate model MNV /RAW 264.7 by the means of a classic infectivity assay (the plaque reduction assay) apparently constitutes a good strategy to identify active compounds against NoV.

Flavonoids are a large group of phenolic compounds found in fruits, vegetables, nuts, seeds, herbs, spices, tea and red wine. These compounds display a remarkable spectrum of biological activities affecting various cellular systems (Middleton *et al.*, 2000). Many flavonoids have been shown to inhibit the replication of multiple picornavirus including poliovirus, coxsackievirus A/B and rhinovirus (Bauer *et al.*, 1981; Conti *et al.*, 1998; Robin *et al.*, 2001; Tait *et al.*, 2006; De Palma *et al.*, 2008). Particularly, synthetic 2-styrylchromones have been previously tested for antiviral activity and led to the identification of some analogues active against rhinoviruses (Desideri *et al.*, 2000; Desideri *et al.*, 2003). On the basis of these data, 2-styrylchromones were considered an interesting new class of antipicornavirus flavonoids (Desideri *et al.*, 2003). Picornavirus share many characteristics with the calicivirus family, to which NoV belongs (Ng *et al.*, 2004; Green, 2007; De Palma *et al.*, 2008). Both are small, non-enveloped icosahedral (+)-strand RNA virus of approximately 30 nm and encode a large polyprotein matured by proteolytic cleavage with a viral protease and a RNA-dependent RNA polymerase. Since their replication strategy shares many features, this allows us to predict possible anti-norovirus activity for 2-styrylchromones.

When studying the antiviral activity of compounds, the exact concentration of compound required to inhibit the replication of a virus will depend on the infectivity input of the virus, the cell type used, the duration of the experiment, the kinetic of infection in that particular cell type, and the sensitivity of the assay being used (Johnson and Byington, 1990). The plaque reduction assay is an infectivity assay that still remains the 'gold standard' for determining the antiviral activity of compounds by which other tests are evaluated, despite being laborious and time-consuming (Storch, 2007). Alternative susceptibility assays include the reduction in cytopathic effect (CPE), which is either microscopically evaluated or colorimetrically detected (measuring cell viability), or the quantification of specific enzymes or viral antigens (Al-Jabri *et al.*, 1996). These methods have been described in the literature against picornavirus, herpesvirus, influenza virus and others, but they are

often also laborious, poorly standardized, and the 50% inhibitory concentrations often do not well correspond with that of plaque reduction assays (Schmidtke *et al.*, 2001).

As far as norovirus are concerned, the plaque reduction assay remains also the most sensitive and suitable method to test antiviral activity against norovirus. This method has been described in the literature in disinfection and inactivation studies with MNV as a surrogate model for human NoV (Cannon *et al.*, 2006; Bae and Schwab, 2008; Baert *et al.*, 2008a; Baert *et al.*, 2008b).

In order to screen chemical compounds for antiviral activity it is necessary to determine primarily if they are toxic to cells and at which concentrations, because it is essential to ensure that a potential reduction of the virus titer is due to a specific effect of the compound on the virus and not to an effect on cell viability that impairs virus replication. Normally the cytotoxicity of compounds is studied by the MTT assay, a colorimetric assay based on the reduction of the yellow tetrazolium salt into blue formazan crystals by metabolically active cells (Mosmann, 1983). It is also important that this assay is performed in the exact same conditions as the antiviral screening that followed.

Before proceeding with the antiviral tests it is necessary to establish criteria regarding which degree (if any) of cytotoxicity of compounds is acceptable, therefore a maximum concentration to test must be defined. In this work we defined as acceptable a concentration in which the MTT reduction capacity of treated cells was 90% or more when compared to untreated control cells, designated by maximum non-toxic concentration (MNTC).

Regarding the virus, the first step consists of determining a virus titer by infectivity assays, in order to subsequently measure a reduction of such titer by a potentially active compound. There are two kinds of infectivity assays, quantal or quantitative assays (Payment and Trudel, 1993; Hierholzer and Killington, 1996). Quantal assays obtain a value for the virus titer based on an “all or none” approach such as the presence or absence of virus-induced cytopathic effect, while quantitative assays allow the detection and counting of foci of cytopathic effect (plaques) and so the number of infectious virus particles present in a suspension can be quantified (Hierholzer and Killington, 1996). The TCID₅₀, defined as that dilution of virus required to infect 50% of cells, is a quantal assay that relies on the presence of cytotoxic virus particles, thus is adequate to virus capable of causing this kind of CPE such as MNV. Most virologists, however, consider the plaque assay to be the easiest, most accurate and sensitive form of assaying virus infectivity (Hierholzer and Killington, 1996).

The plaque assay is an infectivity assay in which theoretically one plaque is initiated by infection from a single infectious virus particle and the number of plaques, and thus the numbers of virions, can be quantified. An overlay medium insures that the spread of progeny virus is restricted to the surrounding cells so that a *plaque* – a localized area of virus-induced cytopathology - can develop (Burleson *et al.*, 1992). However, since virus samples are inevitably heterogeneous populations, infectivity is not an all-or-nothing phenomenon and is dependent on the assay conditions. At high dilutions sometimes virus particles may clump (Burleson *et al.*, 1992). Thus, in practice, the plaque titer obtained may not always accurately represent the number of infectious particles present. Therefore it is essential while establishing a plaque assay protocol to determine the most sensitive and suitable conditions for the virus/cell system in question as the methods vary in relation to detail (Hierholzer and Killington, 1996) (Burleson *et al.*, 1992). Such details include:

- i. the sensitivity of the cell to virus infection, as well as the health status of the cells and their ability to form a confluent monolayer;
- ii. the time required for virus adsorption to cells;
- iii. the ability of the virus to cause a detectable CPE in tissue culture;
- iv. the type of agar used in the overlay medium;
- v. the type of dye selected, its concentration and the staining procedure selected;
- vi. the time of incubation necessary for the development of visible but discrete plaques;
- vii. the time of incubation necessary to allow the dye to stain the monolayer effectively and allow plaques to be visible and easily countable.

II.

OBJECTIVES

II.OBJECTIVES

Our research center has a large library of compounds from natural and synthetic sources, including flavonoids and in particular chromones and 2-styrylchromones. Many flavonoids and namely 2-styrylchromones have shown to inhibit the replication of multiple picornaviruses. Since picornavirus share many characteristics with the calicivirus family, to which NoV belongs, this allows us to predict possible anti-norovirus activity for 2-styrylchromones.

The aim of this work was to:

- i. search for potential anti-norovirus activity of a series of chromones (two) and 2-styrylchromones (eleven);
- ii. try to elucidate the structure/ activity relationship of the structure related compounds.

With this purpose it was necessary to:

- i. evaluate the cytotoxicity of the compounds studied;
- ii. propagate and titrate MNV on murine macrophage cell line RAW 264.7 that was used as a surrogate model for human NoV;
- iii. optimize the viral plaque assay for the MNV/ RAW 264.7 system.

III.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

1. REAGENTS AND SOLUTIONS

The following **reagents** were purchased from:

Gibco Invitrogen Co., Scotland, UK:

- Dulbecco's Modified Eagle Medium (DMEM), liquid, high glucose;
- Fetal Bovine Serum (FBS) EU approved origin;
- L-glutamine 200mM;
- Non-essential amino acids (NEAA) 10 mM;
- Phosphate buffer saline (PBS) tablets;
- Penicillin-Streptomycin 100X;
- Sodium pyruvate 100 mM.

Lonza Group Ltd., Basel, Switzerland:

- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Buffer 1M;
- Agarose SeaKem LE;
- Minimum Essential Medium – Eagle (E-MEM) 2X, liquid, without phenol red.

Sigma Chemical Co., Saint Louis, USA:

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT);
- Dimethyl sulfoxide (DMSO);
- Neutral red solution 3,3 g/L;
- Sodium bicarbonate 7,5%;
- Trypan blue solution 0,4%.

The following **work solutions** were prepared subsequently:

- Agarose solution – 2,5 g of agarose in 250 mL of distilled water, autoclaved for 15 min at 121 °C and stored at 4 °C.
- MTT solution – 5 mg/mL in PBS solution, stored at -20 °C. At time of use it was further diluted to a final concentration of 0,5 mg/mL in maintenance medium.
- PBS solution – 1 tablet in 500 mL of distilled water, autoclaved for 15 min at 121 °C and stored at room temperature.
- Trypan blue solution – 0,2% in PBS solution, stored at 4 °C.

2. EQUIPMENT

The following equipment was used:

- Cell incubator LEEC Limited, Cardiff, UK;
- Centrifuge Sigma 3k-2, Sigma Laborzentrifugen GmbH, Germany;
- Inverted microscope Diaphot-TMD, Nikon Instruments, Japan;
- Laminar flow cabinet SterilGard III Advance, The Baker Company, Sanford, USA;
- Microwave oven, Becken;
- Multiwell spectrophotometer Bio-tek Instruments Inc., PowerWave XS, Winooski, USA;
- Upright microscope Eclipse E400, Nikon Instruments, Japan;
- Ultrasonic bath SONOREX RK100, Bandelin Electronic, Berlin, Germany;
- Water bath Memmert U350, Memmert GmbH + Co. KG, Schwabach, Germany.

3. CHEMICAL COMPOUNDS

A total of 13 synthetic compounds, which included two chromones (**1** and **2**) and eleven 2-styrylchromones (**3-13**) were studied (table 1). They were all gently provided by Prof. Doutor Artur Silva's group of Departamento de Química, Universidade de Aveiro.

Stock solutions of 60 mM in DMSO were prepared for each compound and stored at -20°C. Before each assay stock solutions were defrosted and sonicated for 5 minutes. Appropriate dilutions were freshly prepared just prior to every assay.

Table 1 – Compounds tested

Compounds tested	
chromones	Chromone (1)
	5,7-Dimethoxychromone (2)
2-Styrylchromones	2-Styrylchromone (3)
	4'-Methyl-2-styrylchromone (4)
	4'-Methoxy-2-styrylchromone (5)
	4'-Chloro-2-styrylchromone (6)
	α -Methyl-2-styrylchromone (7)
	5-Hydroxy-2-styrylchromone (8)
	5-Benzyloxy-2-styrylchromone (9)
	3-Hydroxy-2-styrylchromone (10)
	3-Methoxy-2-styrylchromone (11)
	3-Hydroxy- α -methyl-2-styrylchromone (12)
	4'-Hydroxy-2-styrylchromone (13)

4. VIRUS

The murine norovirus (MNV) was used as the surrogate model for human NoV to study anti-norovirus activity. It was used the virus strain MNV-1.CW1, gently provided by Herbert Virgin IV (Washington University, St. Louis, USA).

5. CELL CULTURE

Cell culture

The murine macrophage cell line RAW 264.7 was used to propagate MNV.

Cell culture conditions

RAW cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U penicillin/mL /100 µg/mL streptomycin and 10% (for growth medium) or 2% (for maintenance medium) of inactivated fetal bovine serum (FBS), at 37°C in a humidified atmosphere of 5% CO₂. FBS was inactivated by heating at 56°C for 1 h in a water bath. All manipulations of cultured cells were carried out in a vertical laminar flow cabinet (Biosafety Level 2).

RAW cells were grown as an adherent monolayer in 25 or 75 cm² tissue culture flasks containing respectively 5 or 15 mL of culture medium. Cells were subcultivated at ~80% confluence once or twice a week. For this purpose, culture media was removed and replaced with fresh growth media after which cells were detached with a plastic cell scraper and split at a 1:3 to 1:5 ratio.

Confluent RAW cells used in all assays were obtained by plating 5,0x10⁵ viable cells/mL in growth media (see Assessment of cell viability by the trypan blue exclusion assay) followed by a 24 h incubation. Maintenance media was used from this step on in all assays to maintain confluent cells with little or no growth due to its low percentage of FBS.

The overlay media for viral plaque assay was prepared with Minimum Essential Medium – Eagle (E-MEM) 2x without phenol red. This medium was 2x concentrated because it

would be mixed with agarose solution in equal proportions later on. Supplements were equally used in doubled concentrations 4 mM L-glutamine, 0,2 mM NEAA, 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 used in viral plaque assay contained in addition neutral red at a final concentration of 0,6%.

Assessment of cell viability by the trypan blue exclusion assay

RAW cells viability was evaluated by staining with trypan blue, a vital stain that selectively dyes dead cells blue while live cells, with intact membranes, are excluded from staining, remaining uncolored. For that, RAW cells were scraped and centrifuged for 5 min at 1200 rpm. The total number of viable cells per mL was determined in a Neubauer counting chamber after a 1:10 dilution in trypan blue 0,2% solution (50 µL of cell suspension in 450 µL of trypan blue).

6. VIRUS PROPAGATION AND STOCK PREPARATION

Confluent RAW cells in 75 cm² tissue culture flasks were used to propagate MNV. Briefly, cells were infected for 1h at 37°C to promote virus attachment and penetration, after which fresh maintenance media was added and cells were further incubated until virus-induced cytopathic effect stopped progressing.

MNV stocks were obtained by submitting infected cells to three consecutive freeze-thaw cycles in order to release the viruses from infected cells. Virus were partially purified from these cell lysates by centrifugation at 2000 rpm for 10 min and the supernatant was stored in aliquots (virus stock) and frozen at -80°C.

7. VIRUS TITRATION

Virus stock titration was performed by two infectivity assays, namely the TCID₅₀ and the plaque assay.

TCID₅₀ assay

To determine the infectious titer of MNV by the TCID₅₀ assay, confluent RAW cells in 96-well plates were infected with a series of ten-fold virus dilutions in maintenance media ranging from pure virus to 10⁻¹⁰ dilution (eight wells per dilution; 20 µL/well). Viruses were allowed to attach and penetrate during 1 h at 37°C after which fresh maintenance media was added to cells (180 µL/well). Cells were incubated for further 48 h. Uninfected control cells were treated in the same manner.

Untreated control cells and infected cells were monitored microscopically every day during the assay to register the progression of CPE. After 48 h each well was observed and scored as being positive, if CPE was present, or negative, if CPE was absent. The proportion of positive and negative wells was recorded and two intermediate dilutions, one where CPE is present in over 50% of wells and other where CPE is present below 50%, were used to calculate MNV infectivity titer using the Reed and Muench method. According to the Reed and Muench formula, a proportionate distance between those intermediate dilutions is calculated by $TCID_{50} = A + [(B-50\%)/(B-C)] \times \log_{10}10$, where A corresponds to the dilution where CPE>50%, B to the % positive above 50% and C to the % positive below 50%.

Plaque Assay

To determine the infectious titer of MNV by the plaque assay, confluent RAW cells in 6-well plates were infected with a series of ten-fold virus dilutions in maintenance media ranging from 10⁻² to 10⁻⁶ (3 mL/well in duplicate). Viruses were allowed to attach and penetrate during 1 h at 37°C. Uninfected control cells were treated in the same manner.

Meanwhile the overlay media without phenol red was prepared and heated in a 37°C water bath, while agarose solution was melted in the microwave oven and put in a 45°C water bath. At the end of the hour of viral infection culture media was removed again and both overlay media and agarose were mixed in equal proportions and then added to each well (3 mL/well in duplicate). Plates were then allowed to cool and solidify at room temperature before being incubated for 24 h at 37°C.

After this incubation period a second overlay containing a similar mixture of agarose and overlay media, but with neutral red at a final concentration of 0,6%, was added to each well and allowed to cool and solidify at room temperature. Plates were further incubated for 24 h at 37°C, to allow neutral red to stain the cell monolayer.

The small round clear spots visible 24 h after staining are the *plaques* or *plaque forming units* (PFU) and represent the foci of infection. These PFU were counted and the titer of virus was defined as the number of PFU/mL of virus suspension.

8. EVALUATION OF CYTOTOXICITY OF COMPOUNDS BY THE MTT ASSAY

The cytotoxicity of compounds was evaluated in confluent cells after a continuous exposure of 48 h. For that confluent RAW cells in 96-well plates were exposed to serial two-fold concentrations of each compound in maintenance medium, ranging from 2,34 to 150 μ M (200 μ L/well in triplicate) for 48 h. Untreated control cells were exposed to the maximum concentration (0,25%) of drug vehicle (DMSO) and incubation time.

After 48 h treatment, MTT solution (0,5 mg/mL) was added (20 μ L/well) and plates were incubated for further 4h to allow the reduction of MTT. Then, medium was removed and DMSO was added (100 μ L/well) to solubilize the formazan crystals. Absorbance was measured at 550 nm on a multiwell spectrophotometer. The percentage of MTT reduction was calculated comparing the absorbance of compound treated cells to the untreated control cells (corresponding to 100% MTT reduction).

The CC₅₀ value, defined as the concentration that reduced the absorbance of treated cells by 50% when compared to untreated control cells, was determined for each compound.

Non-toxic concentrations of compounds were defined as those in which the MTT reduction capacity of treated cells was at least 90% or more when compared to untreated control cells. Only non-toxic concentrations of compounds were further tested for antiviral activity.

9. SCREENING FOR ANTI-NOROVIRUS ACTIVITY BY PLAQUE REDUCTION ASSAY

The anti-norovirus activity of compounds was evaluated in confluent cells after a continuous exposure of 48 h. Briefly, confluent RAW cells in 6-well plates were exposed to serial five-fold non-toxic concentrations of each compound in maintenance medium (2, 10

and 50 μ M) and to a virus dilution that would ultimately result in a number of 20-80 plaques per well for 48 h. Plates were incubated for 1 h at 37°C.

Meanwhile a new series of the same non-toxic concentrations of compounds in overlay media without phenol red were heated in a 37°C water bath. Agarose solution was melted in the microwave oven and put in a 45°C water bath. At the end of the hour of infection culture media was removed and both overlay media (with the different compound dilutions) and agarose were mixed in equal proportions and then added to each well (3 mL/well in duplicate). Plates were then allowed to cool and solidify at room temperature before being incubated for 24 h at 37°C.

Untreated control cells were present in all the experiments and were exposed to the same virus dilution used in all the other wells and to the maximum concentration (0,084%) of drug vehicle (DMSO) and incubation time.

After this period a second overlay containing a similar mixture of agarose and overlay media, but with neutral red at a final concentration of 0,6%, was added to each well and allowed to cool and solidify at room temperature. These plates were further incubated for 24 h at 37°C to allow neutral red to stain the cell monolayer. The number of PFU per well was then counted in untreated control cells and treated cells.

The IC_{50} value, defined as the concentration of compound that reduced the plaque number by 50% when compared to untreated control cells, was determined for each control.

IV.

RESULTS AND DISCUSSION

IV. RESULTS AND DISCUSSION

1. COMPOUNDS STUDIED

In this work a total of 13 synthetic compounds were studied which include two chromones (**1** and **2**) and eleven 2-styrylchromones (**3-13**). The chemical structures of all these compounds are represented in Fig 1.

These compounds were selected for this work because 2-styrylchromones have been previously identified as having antiviral activity against rhinoviruses (Desideri *et al.*, 2000; Desideri *et al.*, 2003) and were therefore considered an interesting new class of antipicornavirus flavonoids (Desideri *et al.*, 2003). Since picornavirus share many characteristics with the calicivirus family, to which NoV belongs, and their replication strategy shares many features, this allows us to predict possible anti-norovirus activity for 2-styrylchromones.

Prior to the evaluation of their potential anti-norovirus activity, it was necessary to study the toxicity of these compounds to RAW cells in order to establish which non-toxic concentrations could be further tested for antiviral activity.

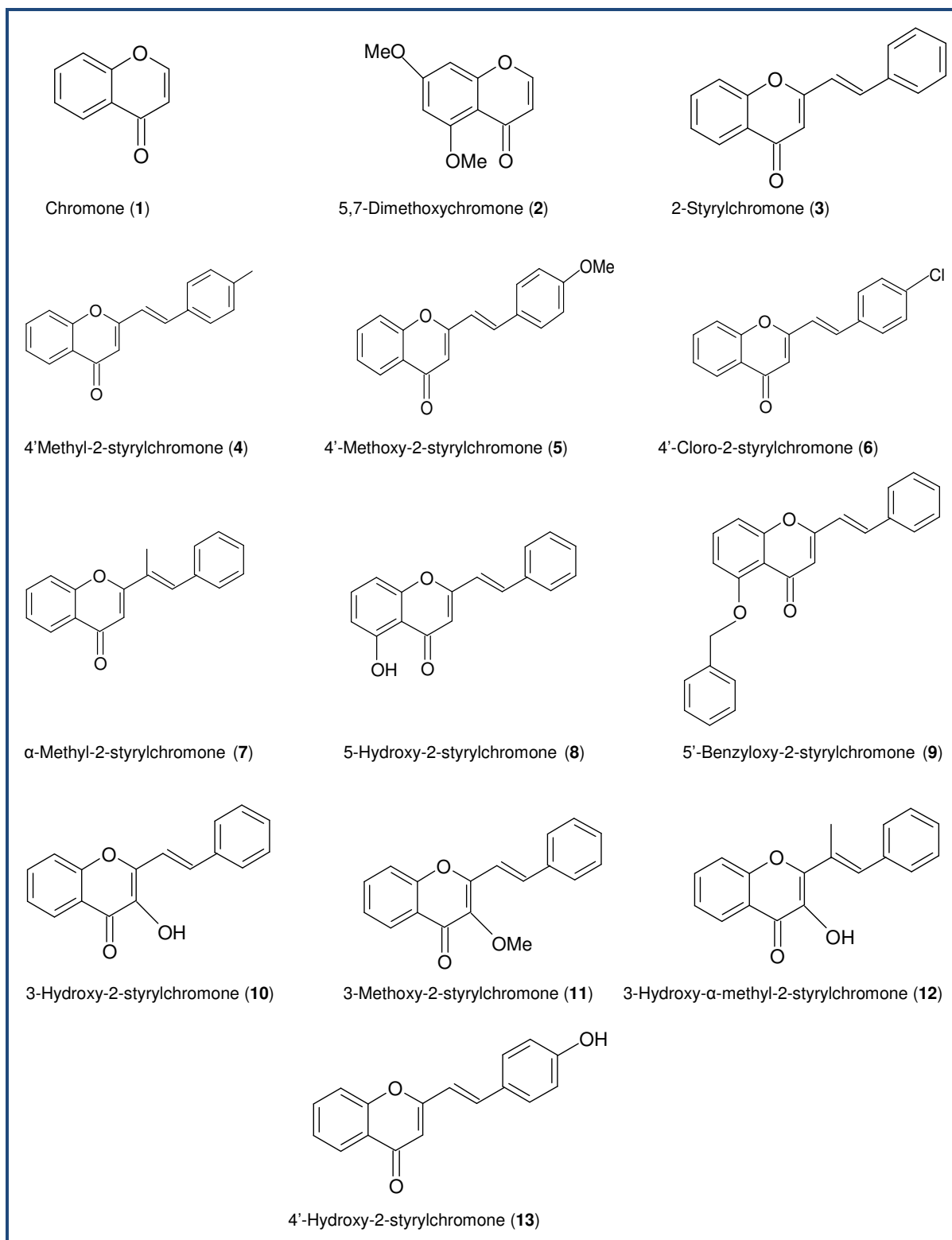


Fig. 1 - Chemical structures of chromones (1 and 2) and 2-styrylchromones (3-13)

2. CYTOTOXICITY OF CHROMONES AND 2-STYRYLCHROMONES IN RAW CELLS

The toxicity of chromones (**1** and **2**) and 2-styrylchromones (**3-13**) was evaluated in confluent RAW cells after a continuous exposure of 48 h. RAW cells were exposed to serial concentrations of compounds, starting in 150 μ M (or 100 μ M for compound **5**) and the viability of cells was measured by their capacity to reduce MTT. The results of this evaluation are presented in Fig. 2.

Results show that RAW cells treated with compounds **2**, **4** and **7** present a MTT reduction capacity $\geq 90\%$ even at the highest concentration of 150 μ M, demonstrating that these compounds have no toxicity for these cells. On the contrary, some minor toxicity was observed for compounds **1**, **3**, **5**, **6**, **8**, **9** and **13**. Cells treated with these compounds maintain, however, a MTT reduction capacity $\geq 80\%$ for the majority of concentrations tested, which leads us to consider this slight loss of viability tolerable for testing antiviral activity.

Regarding compound **11** only concentrations ≤ 75 μ M are safe to RAW cells since a significant decrease of the MTT reduction capacity (68,6%) was observed when cells were treated with 150 μ M.

Finally, major toxicity was observed with compounds **10** and **12**. Their toxicity showed to be dose-dependent and only the lowest concentration tested (2,34 μ M) had no toxic effects. Cells treated with this concentration of compounds **10** and **12** still retain a MTT reduction capacity of RAW cells $\geq 90\%$ and $\geq 80\%$, respectively.

It was interesting to observe that compounds **3**, **4** and **6** induced an increase of MTT reduction capacity in treated RAW cells when compared to untreated control cells. Maximum MTT reduction of 113%, 108% and 109% were obtained, respectively. This enhanced MTT reduction could be due to the presence of a higher number of viable cells or to an increased metabolic activity of RAW cells. These findings should be further explored starting with assessing the number of viable cells in both treated and untreated control cells by trypan blue exclusion assay.

Meanwhile we found that compound **10**, 3-hydroxy-2-styrylchromone, induced an alteration in the morphology of RAW cells, which exhibit a fusiform shape that resembles the morphology of activated macrophages. Further studies were developed to evaluate

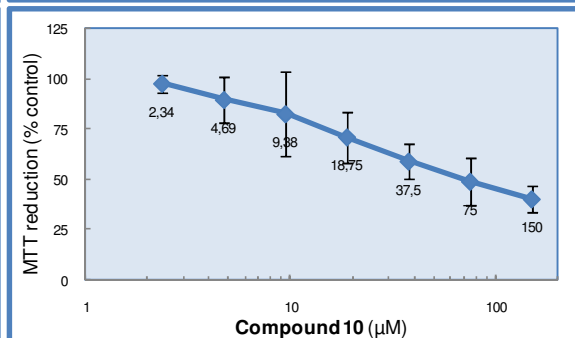
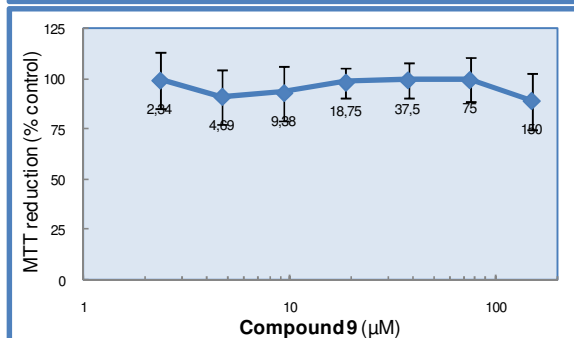
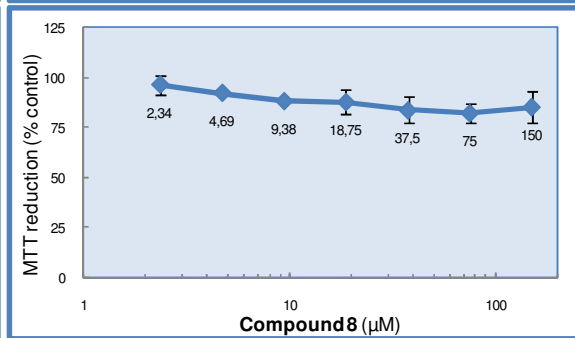
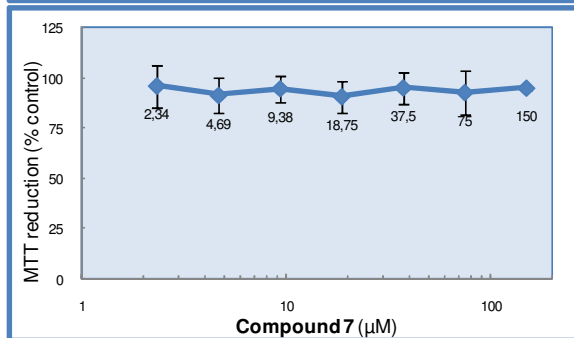
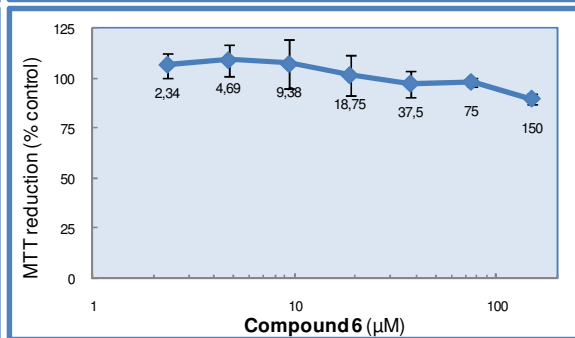
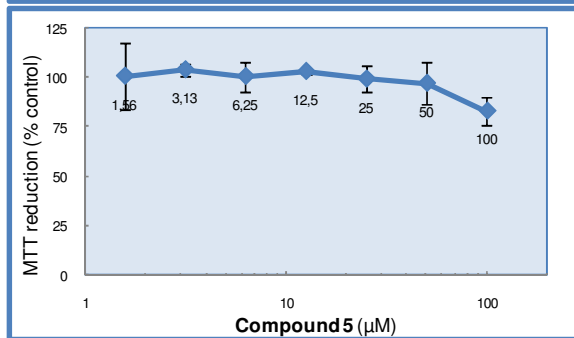
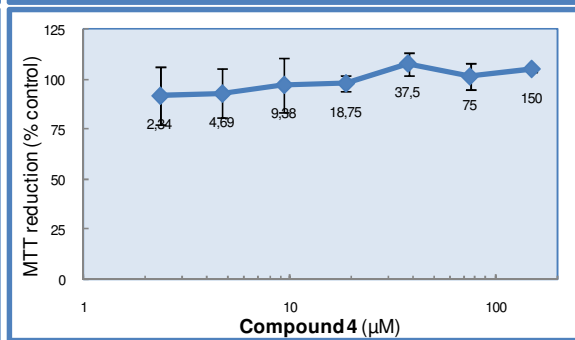
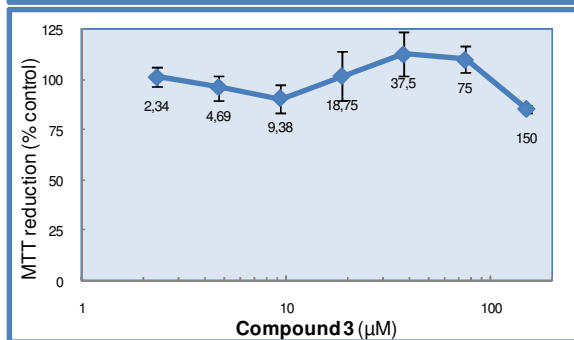
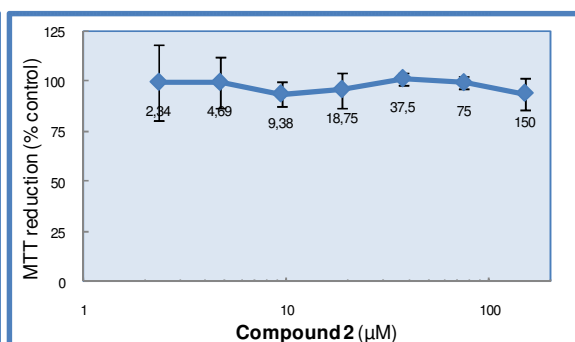
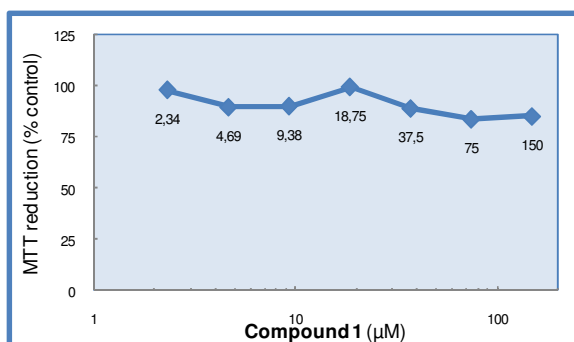
this alteration but it escapes the aims of this work and are therefore presented as attachments 1 and 2 (section VII. of this report).

In order to evaluate the anti-norovirus activity of compounds it was necessary to establish which concentrations of compounds do not show significant toxicity to RAW cells. The highest concentration to fit this requisite was therefore defined as the maximum non-toxic concentration (MNTC). Only MNTC or lower concentrations could be further tested for anti-norovirus activity. With this purpose we established in the present work a MNTC₉₀ for each compound as the highest concentration that exhibited 90% MTT reduction capacity (loss of viability under 10%) when compared to untreated control cells. Although the ideal would be 0% of toxicity, we considered tolerable this slight loss of viability (10%). Since some authors still consider acceptable as non-toxic a MTT reduction capacity $\geq 70\%$ (Cerqueira *et al.*, 2003; Cerqueira *et al.*, 2008), we therefore defined, besides MNTC₉₀, another maximum non-toxic concentration, MNTC₇₀ (the highest concentration that exhibited 70% MTT reduction capacity when compared to untreated control cells). These MNTC values plus the CC₅₀, the concentration of compounds that causes 50% cytotoxicity are presented in table 2.

From the results of table 2 it can be observed that only compounds **2**, **4** and **7** present a MNTC₉₀ >150 μM . While compounds **1**, **3**, **5**, **6**, **8**, **9** and **13** did not present this profile, they still present a MNTC₇₀ > 150 μM . Therefore, all these compounds have a relatively large margin of security that enables testing for anti-norovirus activity even at concentrations of 150 μM . This concentration showed however not to be acceptable for compound **11** because its MNTC₇₀ is only 75 μM . Still, this was not a limitation because we chose, for all these compounds, 50 μM as the maximum concentration to be studied for antiviral activity. Antiviral effects found at higher concentrations would not be interesting enough to explore compounds as antiviral drugs.

The high toxicity of compounds **10** and **12** result in very low MNTC₉₀ (< 2,34 μM). However, their MNTC₇₀ values of 18,75 μM allow us to consider concentrations ≤ 10 μM as having tolerable toxicity to be further evaluated for antiviral activity.

Nevertheless, when looking at the CC₅₀ of all the compounds studied we can conclude that they offer a good margin of security to be further evaluated for antiviral activity since their CC₅₀ values are mostly > 150 μM and even the two more toxic compounds (**10** and **12**) show a CC₅₀ ≥ 50 μM .



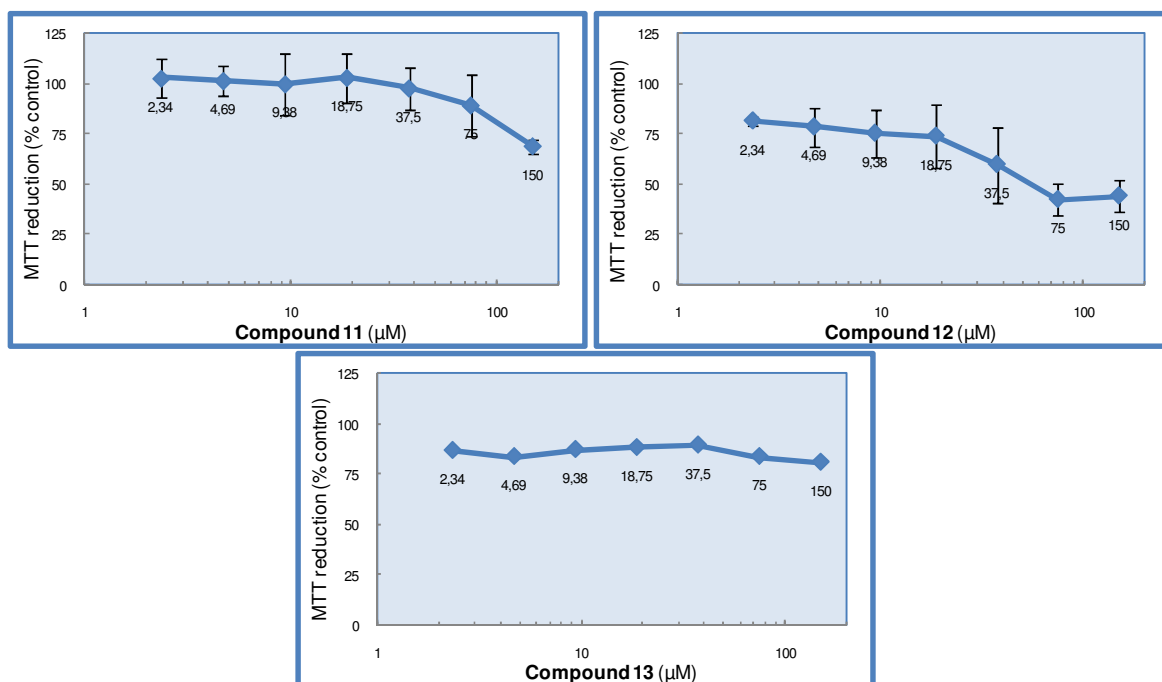
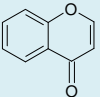
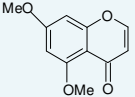
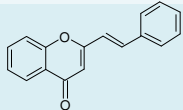
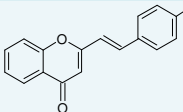
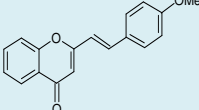
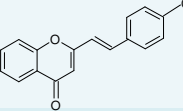
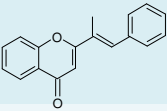
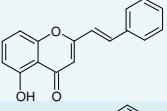
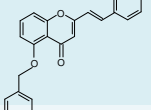
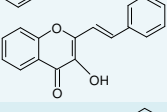
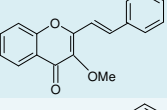
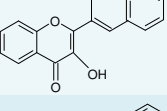
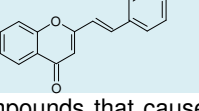


Fig. 2 - Evaluation of cytotoxicity of chromones and 2-styrylchromones by the MTT assay.

Graphics show log of concentrations of compounds *versus* % of MTT reduction compared to untreated control cells (means $\pm$ standard deviation of 3-4 independent experiments performed in duplicate, except for compounds **1** and **13** which show results from 2 independent experiments)

Table 2 - Evaluation of cytotoxicity of chromones and 2-styrylchromones by the MTT assay

Compounds		CC ₅₀ ^a μM	MNTC ₇₀ ^b μM	MNTC ₉₀ ^b μM
Chromone (1)		>150*	>150*	37,5*
5,7-Dimethoxychromone (2)		>150	>150	>150
2-Styrylchromone (3)		>150	>150	75
4'-Methyl-2-styrylchromone (4)		>150	>150	>150
4'-Methoxy-2-styrylchromone (5)		>100	>100	50
4'-Chloro-2-styrylchromone (6)		>150	>150	75
α-Methyl-2-styrylchromone (7)		>150	>150	>150
5-Hydroxy-2-styrylchromone (8)		>150	>150	4,69
5-Benzyloxy-2-styrylchromone (9)		>150	>150	75
3-Hydroxy-2-styrylchromone (10)		52,1 ± 10,6	18,75	2,34
3-Methoxy-2-styrylchromone (11)		>150	75	37,5
3-Hydroxy-α-methyl-2-styrylchromone (12)		46,7 ± 8,7	18,75	<2,34 (81%)
4'-Hydroxy-2-styrylchromone (13)		>150*	>150*	<2,34* (86%)

^aCC₅₀ is the concentration of compounds that causes 50% cytotoxicity when compared to untreated control cells as determined by the MTT assay. Results show means ± SEM of 3-4 independent experiments performed in duplicate.

*Results from 2 independent experiments performed in duplicate.

^bMNTC₇₀ and MNTC₉₀ (maximum non-toxic concentration) are the highest concentration tested in which the MTT reduction capacity of RAW cells was at least 70% or 90% when compared to untreated control cells. When a >90% reduction was not achieved, the MTT reduction capacity obtained at the minimum concentration tested was reported in parentheses.

3. VIRUS TITRATION BY TCID₅₀ ASSAY AND PLAQUE ASSAY

It became necessary before screening compounds for their anti-norovirus activity to titrate the virus stock of MNV. Only knowing the quantity of virus present it is possible to subsequently measure a reduction of such titer by a potentially active compound. This titration was performed by the infectivity assay TCID₅₀ at a first stage and later by plaque assay.

The TCID₅₀, defined as that dilution of virus required to infect 50% of cells, is a quantal assay that relies on the presence of cultivable virus that causes CPE, such as MNV in RAW cells. The plaque assay is a quantitative infectivity assay that allows the detection and counting of foci of cytopathic effect (plaques) and so the number of infectious virus particles present can be quantified.

In Fig. 3 there is an example of a schematic representation of MNV titration by TCID₅₀ assay. RAW cells were monitored microscopically after 48 h of viral infection and the eight wells of each virus dilution (from pure to 10⁻¹⁰) were scored as positive or negative for the presence of CPE. The obtained data was then compiled and the titer calculated by the Reed and Muench formula as demonstrated in table 3.

An example of the microscopic observation of four wells of the microplate used in MNV titration is given in Fig. 4. It is easily observed the different extension of CPE caused by three different dilutions of MNV (10⁻³, 10⁻⁴, 10⁻⁵) in RAW cells and the absence of CPE in uninfected control cells.

The results of this titration method gives us an indication of the amount of virus present in the virus stock but these results are largely dependent on the experience of the observer, who must score all wells of the plate for positive or negative CPE. Besides, it is extremely time-consuming and also does not reflect the extension of CPE observed. As illustrated in Fig. 5, while in the first dilutions CPE is evident and destroys the cell monolayer completely, in the subsequent dilutions there is usually a single and very discrete focus of infection and often becomes quite difficult to decide whether to score the well as positive or negative. This aspect is particularly relevant because these intermediate dilutions are the ones that enter the Reed and Muench formula and so are the most relevant for the

final titer obtained. Moreover, the extension of CPE presented in each well is not contemplated by the formula and so this TCID₅₀ titer reflects very little information.

The establishment of a protocol for titration of MNV by plaque assay and its optimization is described in detail in the following point of this report (4. Optimization of the viral plaque assay).

The titration of MNV by plaque assay was performed several times during the optimization process. However, the number of PFUs per well showed to be more relevant than the virus titer to establish an effective assessment of antiviral activity by plaque reduction assay. Such data is also presented in the following point of this report. (4. Optimization of the viral plaque assay).

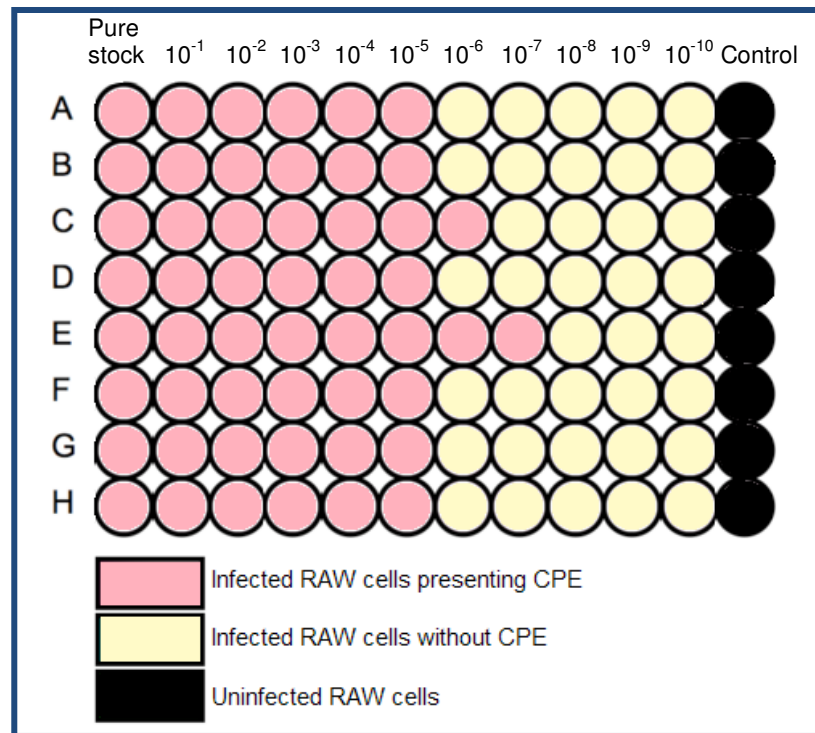


Fig. 3 – Schematic representation of MNV titration by TCID₅₀ assay.

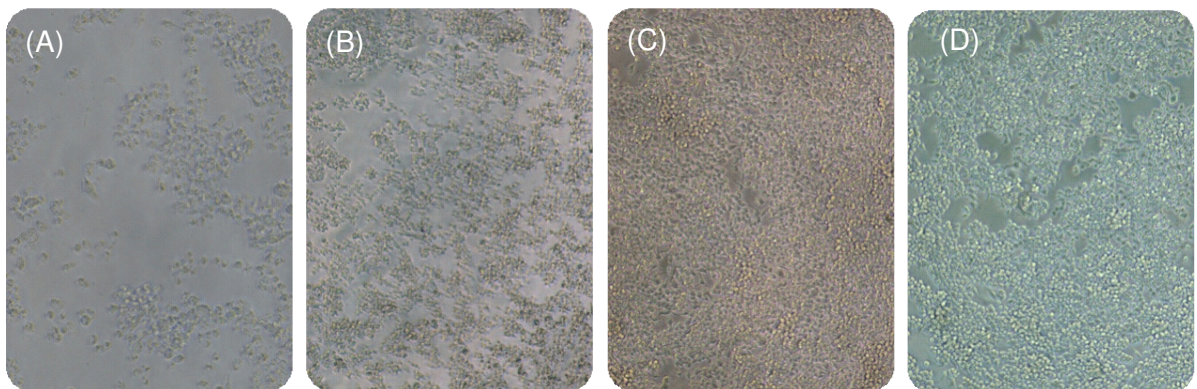


Fig. 4 - Light microscopy (100x) of TCID₅₀ titration. RAW cells infected with 10⁻³ (A), 10⁻⁴ (B) and 10⁻⁵ (C) dilution of MNV and uninfected control cells (D)

Table 3 – Results of MNV titration by TCID₅₀ assay.

Log virus dilution	N. ^o infected wells	Σ wells CPE positive (A)	Σ wells CPE negative (B)	A/(A+B)	% wells CPE positive	Virus Titer*
5	8/8	11	0	11/11	100%	3,0x10 ⁷ TCID ₅₀ /mL
6	2/8	3	6	3/9	33,3%	
7	1/8	1	13	1/14	7,1%	

Calculations necessary to determine the percentage of wells where CPE is positive in order to apply the Reed and Muench formula. Results are from a representative experiment of two carried out independently.

*Result of the virus titer is the mean of two independent experiments

4. OPTIMIZATION OF THE VIRAL PLAQUE ASSAY

In the present work, the plaque assay was the infectivity assay used to screen compounds for anti-norovirus activity since it remains the most sensitive and suitable method for determining the antiviral activity of compounds. This method has been used intensively in disinfection and inactivation studies using the MNV/RAW cells system (Cannon *et al.*, 2006; Bae and Schwab, 2008; Baert *et al.*, 2008a; Baert *et al.*, 2008b).

Nevertheless, it is essential while establishing a plaque assay protocol to determine the most sensitive and suitable conditions for the virus/ cells system, as the methods vary in relation to detail (Burleson *et al.*, 1992; Hierholzer and Killington , 1996).

Regarding RAW cell culture we used the previously described conditions to obtain a confluent monolayer (5×10^5 viable cells/mL, 24 h incubation). Also for MNV the standard time of viral infection (1 h at 37°C) was used. But concerning the type of agar of the overlay medium, the type/concentration of the dye and the staining procedure of PFU, it became necessary to optimize some of these conditions because for the MNV/RAW cells system there is still no consensus concerning the plaque assay. For that several attempts were made.

Our first approach was to follow the standard operating procedure (SOP 3 WP 5.2) for adenoviruses/A549 system used in VIROBATHE project¹ that used carboxymethylcellulose as the overlay medium, a fixation of cells with formalin and a staining with crystal violet after 48 h. However, this procedure was inadequate for MNV/RAW cells system since macrophage cells do not attach firmly to the plastic as do A549 and much of the cell monolayer was lost during the fixation and staining procedures. Besides, the carboxymethylcellulose in the overlay was difficult to manipulate making the protocol particularly hard to execute.

Secondly, we tried a double-layer method described for the quantification of enteroviruses (Moce-Llivina *et al.*, 2004) which used a combination of the traditional monolayer plaque assay with a suspended-cell plaque assay, therefore significantly increasing the number of plaques counted. In this method the overlay containing agar is removed with a spatula after the incubation period and the cell monolayer stained with crystal violet. Once again we found that this method did not work with the RAW macrophages which are semi-adherent and were removed together with the agar.

¹ <http://www.virobathe.org/>

Finally, we tried the procedure used in the National Calicivirus Laboratory of the CDC, Atlanta, USA (gently provided by Doctor Jan Vinjé) that uses a monolayer plaque assay with agarose in the overlay and neutral red as dye in a second overlay added above the first one that is not removed. This was the procedure selected for this work because it was the most adequate for the MNV/RAW cells system (described in detail in section III. of this report).

It was also necessary to establish the endpoint of the plaque assay, corresponding to the time when the PFUs were scored. The optimal time would be the one that allowed us to obtain visible, discrete and easily countable PFUs. Still, countable PFUs also depend on the virus dilution used and the neutral red staining conditions. For statistical reasons 20-100 PFUs per well is an ideal number to count, although the number is limited by the size of the well in which the plaque assay is performed and by the PFU size (Burleson *et al.*, 1992; Hierholzer and Killington, 1996).

In this work, since the plaque assay was performed in 6-well plates, it was ideal to obtain only small PFUs, clearly individualized and in a short period of time to avoid PFUs to fuse together. We first tested ten-fold dilutions of the virus stock but it proved not to be a good approach since countable plaques were only obtained in one dilution. Thus, we tested two-fold virus dilutions, which proved to be adequate since we obtained countable plaques in different dilutions, allowing us to choose the viral dilution with the ideal number of PFUs per well. In our case, 60-80 PFUs seemed to be the maximum number to count since 100 PFUs per well was too much to count with certainty given the size of the well. Therefore, we predetermined 20-80 PFUs per well as the ideal number for our work conditions.

As neutral red takes at least 12 h to properly stain the monolayer it became clear that counting could not take place before 12-24 h after staining. Therefore, we evaluated the addition of neutral red after 24 h or 48 h of viral infection, counting PFUs 24 h after that, which resulted in two endpoints of the plaque assay of 48 h or 72 h, respectively.

The optimization of the plaque assay conditions was performed with the MNV titration. As shown in Fig.5, plates A and B differ from plates C and D on the time of addition of the overlay with neutral red after MNV infection, which was 24 h or 48 h, respectively. It can be easily seen that PFU were considerably smaller and more individualized on plates A and B in comparison with those in plates C and D where plates are larger and some have fused together. This has led us to conclude that addition of neutral red after 24 h gives the best scoring of PFUs.

From the results of table 4 we can observe that the ideal number of PFUs (20-80) was obtained with a 1:2500 virus dilution for an endpoint of 48 h as well as with a 1:5000 and 1:10000 for an endpoint of 72 h. However, PFUs scored after 72 h were larger, probably as a result of a fusion of plaques, when compared to PFUs scored after 48 h (data not shown). Therefore we selected for all the plaque assays performed in the course of this work the 1:2500 virus dilution and the endpoint of 48 h.

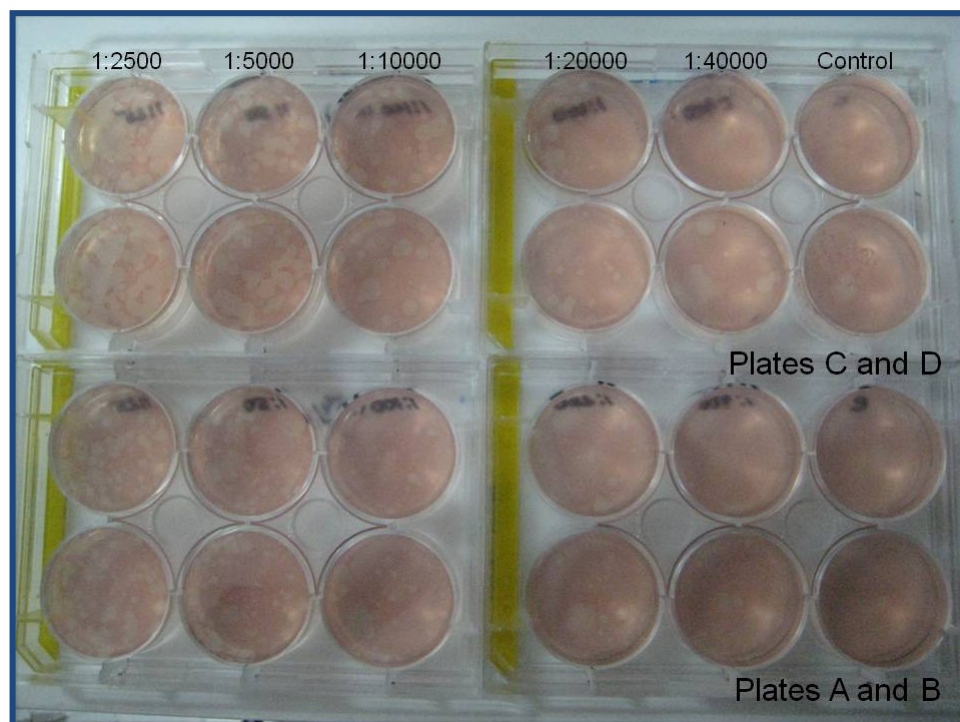


Fig. 5 – Titration of MNV by plaque assay under different conditions. Plates A and B differ from plates C and D on the time of addition of the overlay with neutral red (NR) after MNV infection, which was 24 h or 48 h, respectively. It can be easily seen that PFU are considerably smaller and more individualized on plates A and B in comparison with those in plates C and D where plates are larger and some have fused together.

Table 4 – Titration of MNV by plaque assay under different conditions

Dilution of virus stock	Number of plaques per well (in duplicate)		
	Plates A and B NR after 24 h		Plates C and D NR after 48 h
	Endpoint 48 h	Endpoint 72 h	
1:2500	28 32	91 78	>100 >100
1:5000	21 16	60 66	53 51
1:10000	3 9	22 27	27 30
1:20000	2 4	18 18	19 15
1:40000	0 1	5 2	5 15

Plates A and B differ from plates C and D on the time of addition of the overlay with neutral red (NR) after MNV infection, which was 24 h or 48 h, respectively. The endpoint represent the ending time of the plaque assay when the PFU were scored.

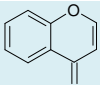
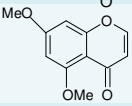
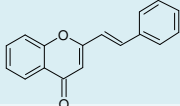
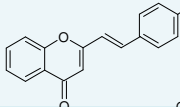
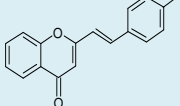
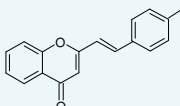
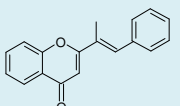
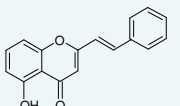
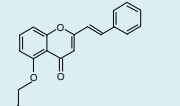
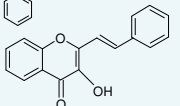
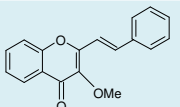
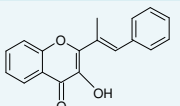
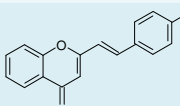
5. ANTI-NOROVIRUS ACTIVITY OF CHROMONES AND 2-STYRYLCHROMONES BY PLAQUE REDUCTION ASSAY

The inhibitory activity of chromones (**1** and **2**) and 2-styrylchromones (**3-13**) on MNV replication was evaluated in confluent RAW cells after a continuous exposure of 48 h by plaque reduction assay.

RAW cells infected with MNV were exposed to serial concentrations of compounds starting with the highest concentration of 50 μ M for compounds **1-9**, **11** and **13** and only with 10 μ M for compounds **10** and **12**. These concentrations proved to be safe for screening for antiviral activity as demonstrated by the results of the cytotoxicity assays.

The anti-norovirus activity of the compounds studied, as given by IC_{50} value, is summarized in table 3 as well as values of cytotoxicity and selectivity index (SI).

Table 5 – Cytotoxicity and anti-norovirus activity of chromones and 2-styrylchromones

Compounds		CC ₅₀ ^a μM	IC ₅₀ ^b μM	SI ^c
Chromone (1)		>150	>50* (29,9%)	-
5,7-Dimethoxy chromone (2)		>150	>50 (23,8%)	-
2-Styrylchromone (3)		>150	17,7 ± 6,5	>8,5
4'-Methyl-2-styrylchromone (4)		>150	23,7 ± 7,5	>6,3
4'-Methoxy-2-styrylchromone (5)		>100	7,4 ± 1,3	>13,5
4'-Chloro-2-styrylchromone (6)		>150	>50 (7,6%)	-
α-Methyl-2-styrylchromone (7)		>150	18,0 ± 5,2	>8,3
5-Hydroxy-2-styrylchromone (8)		>150	7,0 ± 0,7	>21,6
5-Benzoyloxy-2-styrylchromone (9)		>150	>50 (39,4%)	-
3-Hydroxy-2-styrylchromone (10)		52,1 ± 10,6	>10 (6,8%)	-
3-Methoxy-2-styrylchromone (11)		>150	37,9 ± 6,3	>4,0
3-Hydroxy-α-methyl-2-styrylchromone (12)		46,7 ± 8,7	>10 (40,8%)	-
4'-Hydroxy-2-styrylchromone (13)		>150	>50* (21,1%)	-

^aResults are expressed as CC₅₀, concentrations of compounds that cause 50% cytotoxicity when compared to untreated control cells by the MTT assay, and show means ± SEM of 3-4 independent experiments performed in duplicate.

^bResults show means ± SEM of one experiment performed in duplicate.

^cResults are expressed as IC₅₀, concentrations of compounds that reduced the plaque number by 50% when compared to untreated control cells, and show means ± SEM of 3-7 independent experiments performed in duplicate. When a 50% reduction was not achieved, the percent of inhibition obtained at the maximum concentration tested (10 or 50 μM) was reported in parentheses.

^cThe selectivity index (SI) was the CC₅₀/IC₅₀.

Results show that chromones **1** and **2** and 2-styrylchromones **6**, **9**, and **13** have no anti-norovirus activity even when tested at the highest concentrations of 50 μ M.

However, 2-styrylchromones **3**, **4**, **5**, **7**, **8** and **11** exhibited an antiviral effect that ranges from moderate (IC_{50} = 20-50 μ M) to very potent (IC_{50} < 10 μ M). The most potent compounds were 4'-methoxy-2-styrylchromone (**5**) and 5-hydroxy-2-styrylchromone (**8**) which exhibit an IC_{50} of 7,4 μ M and 7,0 μ M respectively.

Concerning compounds **10** and **12**, which were only tested at a maximum concentration of 10 μ M due to their toxicity to RAW cells, no anti-norovirus activity was detected. However, it was interesting to notice that compound **12** was able to reduce in 40,8% the number of viral plaques at this concentration. This could indicate that higher concentrations could have anti-norovirus activity but its toxicity limited further testing.

Although it is difficult to draw a structure activity relationship from this small group of compounds, an interesting relationship between the anti-norovirus effect and the chemical structure was, however, observed. When we compare the IC_{50} of chromone **1** (> 50 μ M) and 2-styrylchromone **3** (17,7 μ M) it is curious to note that the introduction of the 2-styryl group in chromone moiety was responsible for the appearance of the antiviral 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 **4** and **5** were compared. The 4'-methoxy group showed to be the best substituent giving to compound **5** an anti-norovirus activity three times higher than the 4'-methyl group of compound **4** (IC_{50} 7,4 μ M *versus* 23,7 μ M, respectively). On the contrary, the 4'-chloride and 4'-hydroxy groups of compounds **6** and **13** were responsible for a loss of antiviral activity.

It was also observed that the nature of the 5 substituent of 2-styrylchromones **8** and **9** was relevant for the anti-norovirus activity. When comparing their IC_{50} with that of 2-styrylchromone **3** (17,7 μ M) we can observe that the hydroxy group was responsible for the appearance of one of the most potent anti-norovirus effects (7,0 μ M) while the benzyloxy group was responsible for a loss of activity (> 50 μ M).

It was curious to note that the hydroxy group in position 3 of 2-styrylchromones appears to confer cytotoxicity to compounds **10** and **12**, the only ones who present this group and which exhibit a CC_{50} of 52,1 μ M and 46,7 μ M respectively.

Selectivity is crucial to better evaluate the toxicological profile of the molecules and for the clinical use of antiviral drugs (Coen *et al.*, 2007). Biologically, selectivity is the difference between the dose of the compound that exerts its antiviral effect and the dose that exerts its cytotoxic effect (Coen *et al.*, 2007). Selectivity index (SI) is usually expressed as a CC_{50} versus IC_{50} ratio and some authors consider acceptable a value $>1,5$ (da Silva *et al.*, 2006; Carriel-Gomes *et al.*, 2007).

Since some of the 2-styrylchromones studied exhibited interesting antiviral activities we considered worthwhile determining their SI values. All the active compounds present a SI $>1,5$ but the most potent compounds **5** and **8** show much higher SI values of $>13,5$ and $>21,6$, respectively. This indicates that these two compounds have a good toxicological profile for being further developed as antiviral drugs. Furthermore, their potent anti-norovirus effect justifies the elucidation of their mechanism of action.

V.

CONCLUSIONS

V. CONCLUSIONS

In this work, a total of 13 synthetic compounds were studied, including two chromones (**1** and **2**) and eleven 2-styrylchromones (**3-13**). These compounds were selected for this study because 2-styrylchromones have been described as inhibitors of the replication of picornavirus and since the replication strategy of this viruses shares many features with the calicivirus family, to which NoV belongs, this allowed us to predict potential anti-norovirus activity.

Prior to the evaluation of anti-norovirus activity, it was mandatory to study the toxicity of these compounds to RAW cells in order to establish which non-toxic concentrations could be further tested for antiviral activity. Most of the compounds showed no relevant cell toxicity allowing their evaluation of anti-norovirus activity using maximum concentrations of 50 μM .

The screening of anti-norovirus activity of compounds was performed by plaque reduction assay, an infectivity assay that still remains the most sensitive and suitable method for determining the antiviral activity of compounds.

Results showed that chromones have no anti-norovirus activity while 2-styrylchromones exhibited an antiviral effect that ranges from moderate ($\text{IC}_{50} = 20\text{-}50\ \mu\text{M}$) to very potent ($\text{IC}_{50} < 10\ \mu\text{M}$). 4'-methoxy-2-styrylchromone and 5-hydroxy-2-styrylchromone were the most potent compounds, showing an IC_{50} of 7,0 μM .

Although this study included a small group of compounds, an interesting structure/ activity relationship was observed: (i) the introduction of the 2-styryl group in chromone moiety was responsible for the appearance of the anti-norovirus activity; (ii) the nature of the 4' and the 5 substituent in 2-styrylchromones influenced the potency of compounds, being the methoxy and the hydroxy group the best substituents, respectively.

The most potent 4'-methoxy-2-styrylchromone and 5-hydroxy-2-styrylchromone showed very high Selectivity Index (SI) values indicating that these compounds have a good toxicological profile for being further developed as antiviral drugs. Furthermore, their potent anti-norovirus effect justifies the elucidation of their mechanism of action.

In summary, we have identified a series of compounds with low cytotoxicity and a good antiviral activity against norovirus which might prove useful as new antiviral drugs against this virus.

VI.

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VI. REFERENCES

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VII.

ATTACHMENTS

Attachment I.

Abstract in the book of congress/ Poster presentation

J. Pereira, Diana C.G.A. Pinto, Artur M.S. Silva, M.S.J. Nascimento, M. Pinto. *Search for anti-Norovirus activity in chromones and 2-stryrylchromones. 1st National Meeting in Medicinal Chemistry.* Porto, Portugal, 13-15 Novembro, **2008** (P-043)

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1.º Encontro Nacional de Química Terapêutica
1st National Meeting on Medicinal Chemistry

13-15 Novembro '08
PORTO, PORTUGAL

**LIVRO DE RESUMOS
BOOK OF ABSTRACTS**



The background of the poster is a grayscale image. The upper portion shows a complex molecular model with various sized spheres (black, white, grey) connected by lines, representing atoms and bonds. The lower portion features a stylized, black silhouette of a bridge with multiple arches, resembling the Dom Luís I Bridge in Porto. The text is overlaid on these images.

1^o Encontro Nacional de Química Terapêutica

13 a 15 NOV '08

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SEARCH FOR ANTI-NOROVIRUS ACTIVITY IN CHROMONES AND 2-STYRYLCHROMONES

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Abstract

Human norovirus (NoV) are the most frequent cause of epidemic outbreaks of viral gastroenteritis. They affect all age groups and occur often in crowded locations, such as cruise ships, hospitals, schools and restaurants. NoV are class B biological agents due to their high infectivity and stability, rapid person-to-person spread and debilitating nature of the disease.

Despite the considerable morbidity of NoV disease no drug or vaccine is currently available, therefore the discovery of anti-norovirus drugs is urgent. Little is known about biology of human NoV, which is due to the absence of a cell culture system or small animal model. Recently, murine norovirus (MNV) was discovered and this virus is able to replicate in cell culture and has been proposed as a surrogate model for human NoV. MNV is genetically related to noncultivable human NoV and it is likely that many fundamental mechanisms of replication and biochemical features are conserved between murine and human NoV.

The classical strategy to identify active chemical compounds, belonging to various chemical families is an interesting approach of drug discovery by providing novel chemical structures with new mechanisms of action. Our research center has a large library of compounds from natural as well as synthetic sources belonging to different chemical families which have shown diverse biological activities, including antioxidative, antiinflammatory, antitumoral and immunomodulatory. Some of these compounds such as chromones and 2-styrylchromones have been shown to inhibit the replication of rhinovirus, (+)-strand RNA viruses of the picornavirus family. Since the replication strategy of picornavirus family shares many features with the calicivirus family which includes NoV, this allows to predict possible anti-norovirus activity for these compounds.

The aim of the present study was to search for potential anti-norovirus activity of a series of chromones and 2-styrylchromones. The cultivable murine norovirus MNV propagated in the murine macrophage cell line RAW 264.7 was used as a surrogate model. Compounds were considered active when they showed capacity to protect cells from viral infection through the reduction of virus-induced cytopathic effect. The cytotoxicity of compounds on the RAW 264.7 cells was evaluated by the MTT assay. Preliminary results showed these chromones and 2-styrylchromones have little or no toxicity for RAW 264.7 cells when tested at the highest concentration of 150µM. Therefore, these compounds could be tested at high concentrations for antiviral activity.

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The classical strategy to identify active chemical compounds, belonging to various chemical families is an interesting approach of drug discovery by providing novel chemical structures with new mechanisms of action. Chromones and 2-styrylchromones have been shown to inhibit the replication of rhinovirus, (+)-strand RNA viruses of the picornavirus family. Since the replication strategy of picornavirus family shares many features with the calicivirus family, to which NoV belongs to, this allows us to expect a possible anti-norovirus activity for these compounds.

The aim of the present study was to search for anti-norovirus activity of a series of chromones and 2-styrylchromones. The cultivable murine norovirus MNV propagated in the murine macrophage cell line RAW 264.7 was used as a surrogate model. Compounds were considered active when they showed capacity to protect cells from viral infection through the reduction of virus-induced cytopathic effect (CPE). The cytotoxicity of compounds on the RAW 264.7 cells was also evaluated to ensure that only non-toxic concentrations of compounds were tested for antiviral activity.

Materials and methods

Compounds

Chromone (1) and 2-styrylchromones (2-11) were synthesized as previously described [3]. A stock solution of each compound was prepared in DMSO and kept at -20°C. Appropriate dilutions were freshly prepared just prior to every assay.

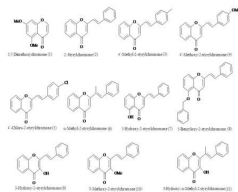


Fig. 1. Chemical structures of the compounds under study.

Cell culture, virus propagation and titration

MNV was propagated by infecting 80% confluent monolayers of murine macrophage RAW 264.7 cells cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 2% fetal bovine serum, 2mM L-glutamine, 10mM HEPES, 1 mM sodium pyruvate, 100 U penicillin/mL and 100 µg/mL streptomycin maintained at 37°C in a humidified 5% CO₂ atmosphere. Viruses were harvested when cytopathic effect stopped progressing, by consecutive freeze-thaw cycles followed by centrifugation and storage of the supernatants at -80°C.

Virus stocks were titrated by the TCID₅₀ infectivity assay (dilution of virus required to infect 50% of a given batch of inoculated cell culture). Briefly, a series of 10-fold virus dilutions were added to 96-well plates containing 80% confluent RAW 264.7 cells and incubated for 48h. The TCID₅₀/mL was then determined according to the Reed and Muench formula.

Cytotoxicity of compounds

The effect of compounds on the viability of RAW 264.7 cells (cytotoxicity) was evaluated by the MTT assay. A range of concentrations of compounds (2,34-150 µM) was added to 96-well plates containing 80% confluent RAW 264.7 cells and incubated for 48h. An MTT solution (0,5 mg/mL) was added and plates were incubated for further 4h. Then, medium was removed and DMSO was added to dissolve the formazan crystals. Absorbances were read at 550 nm. The percentages of cytotoxicity (CC₅₀) were calculated as [(A-B)/Ax100], where A and B are the absorbances of control and treated cells, respectively. For each compound, the obtained CC₅₀ value was defined as the concentration that reduced the absorbance of treated cells by 50% when compared to cell control. Only non-toxic concentrations of compounds were further tested for antiviral activity.

Anti-norovirus activity

The anti-norovirus activity of compounds was evaluated by measuring their capacity to protect RAW 264.7 cells from MNV viral infection through the reduction of virus-induced cytopathic effect. Non-toxic concentrations of compounds and a series of 10-fold virus dilutions (5, 50 and 500 TCID₅₀) were added simultaneously to confluent RAW 264.7 monolayers on 96-well plates. Toxicity controls, cell controls and virus controls were run simultaneously. Cells were visually scored for the inhibition of CPE 48h after infection.

Results and discussion

4'-Chloro-2-styrylchromone presents anti-norovirus activity

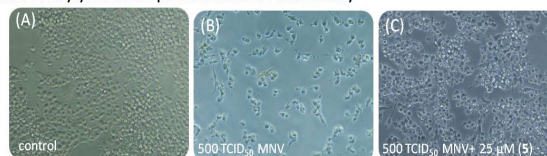


Fig. 2. Effect of 4'-chloro-2-styrylchromone (5) on RAW 264.7 cells infected with MNV. Light microscopy (200x) of uninfected control cells (A), infected cells with 500 TCID₅₀ of MNV untreated (B) and treated with 25 µM of compound (5) (C) 48h after inoculation. Uninfected control cells exposed to 25 µM of compound 5 showed no morphological changes (images not shown). RAW cells infected with 500 TCID₅₀ MNV (B) show an advanced stage of CPE, 4+, that is, almost 100% of the cells are infected. In cells treated with 25 µM of compound 5 a reduction of the CPE, 1+, is observed (C) where a large number of cells are preserved from MNV infection and are morphologically similar to control cells (A).

RAW cells infected with 500 TCID₅₀ of MNV and treated with 25 µM of 4'-chloro-2-styrylchromone (5) (fig. 2C) show a reduction of CPE (1+) when compared with that of untreated infected cells (4+) (fig. 2B). It can be observed that a large number of cells were protected from MNV infection and are morphologically similar to control cells (fig. 2A). Since this apparent anti-norovirus activity was evident at 25 µM and against a high concentration of virus (500 TCID₅₀) this promising compound deserves further studies to confirm and elucidate its mechanism of action.

3-Hydroxy-2-styrylchromone increases the infectivity of MNV in RAW cells

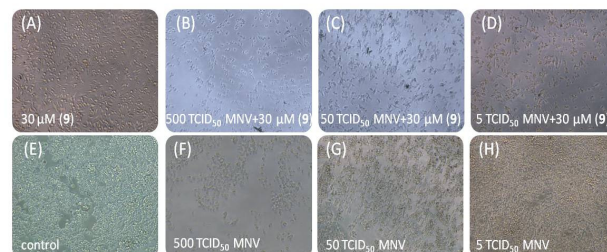


Fig. 3. Effect of 3-hydroxy-2-styrylchromone (9) on RAW 264.7 cells infected with MNV. Light microscopy (100x) of treated (A-H) and untreated (E-H) cells with 30 µM of 9 for 48h. Uninfected control cells (A) and cells infected with 500 (B) and 50 (C) and 5 (D) TCID₅₀ of MNV. Uninfected cells treated with 30 µM of 9 (A) exhibit a fusiform shape, that resembles activated macrophages. This effect was not observed in untreated control cells (E). Comparing infected cells treated with 9 present a much stronger CPE than untreated cells in all virus dilutions tested.

When searching for anti-norovirus activity of compound 3-hydroxy-2-styrylchromone (9) it was observed that treated but uninfected murine macrophage RAW cells exhibit a fusiform shape that resembles activated macrophages. Moreover, the infectivity of MNV seemed to increase in the presence of 3-hydroxy-2-styrylchromone, as demonstrated by the extensive CPE detected in treated cells infected with 50 (C) and 5 TCID₅₀ (B) of MNV when compared to those untreated (G and H). This increase of MNV infectivity could be due either to the activation of macrophages, that would allow an enhanced propagation of MNV in cell culture or to a direct effect of compound 9 in the virus replication cycle. More attempts and also different approaches are needed to clarify which hypothesis could explain this observable fact.

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Acknowledgements

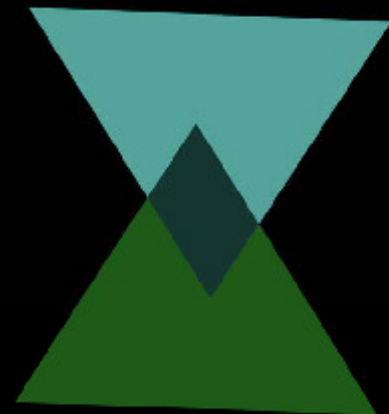
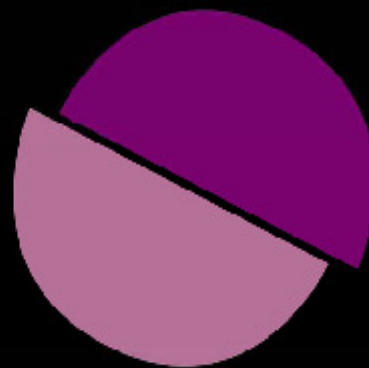
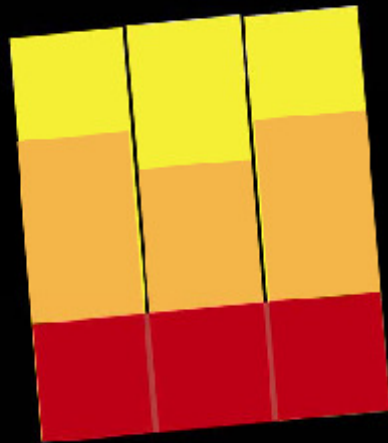
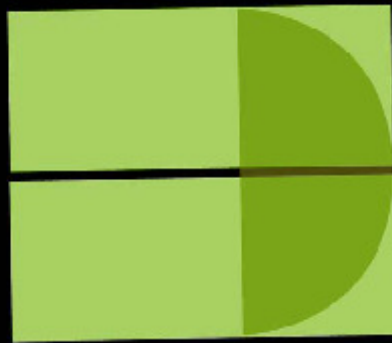
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Attachment II.

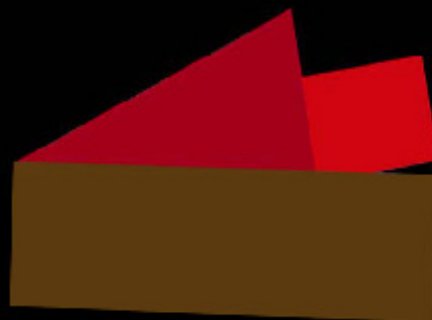
Abstract in the book of congress/ Poster presentation

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U. PORTO



3-hydroxy-2-styrylchromone increases the infectivity of MNV in murine macrophage RAW cells and changes cell morphology

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Human noroviruses (NoV) are the most frequent cause of foodborne epidemic outbreaks of viral gastroenteritis. They affect all age groups and occur often in crowded locations, such as cruise ships, hospitals, nursing homes and schools. NoV are class B biological agents due to their high infectivity and stability, rapid person-to-person spread and debilitating nature of the disease. Despite the considerable morbidity of NoV disease no drug or vaccine is currently available, therefore the discovery of anti-norovirus drugs is urgent. Human NoV is not cultivable in cell culture, so the cultivable murine norovirus (MNV), propagated in the murine macrophage cell line RAW 264.7, is now being used as a surrogate model for the search of anti-norovirus drugs.

When searching for anti-norovirus activity we found a compound, 3-hydroxy-2-styrylchromone, which induced an alteration in the morphology of RAW cells, that exhibit a fusiform shape that resembles the morphology of activated macrophages. Moreover, cells treated with 3-hydroxy-2-styrylchromone and simultaneously infected with MNV produce more viruses as determined by TCID₅₀ (an increase of two log) when compared to untreated infected cells.

The aim of the present study was to determine whether 3-hydroxy-2-styrylchromone is an inducer of activation of macrophages, therefore the production of its interleukins (IFN-gama, TNF, TGF) and of nitric oxide (NO) was evaluated. For this purpose cells were treated for 48h with and without 3-hydroxy-2-styrylchromone and cell culture supernatants were tested for IFN-gama, TNF and TGF-beta by EIA (enzyme immunoassays) and for NO production (Griess assay).

Preliminary results do not show an increase but, unexpectedly, an inhibition of the production of the tested interleukins and NO, indicating that macrophages are not activated. Further studies are under course to explore if 3-hydroxy-2-styrylchromone is inducing cell differentiation instead, which would explain the morphological alterations observed. We are also evaluating how this alteration of RAW morphology is related to the increase of infectivity of MNV.

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Acknowledgments

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FCT (I&D, n°226/2003), FEDER, POCI, U. Porto.



