# UNIVERSITY OF CAGLIARI

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**Research Doctorate in:**

*Design and Development of Antiviral Drugs*

XXIV Cycle

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# Identification and mode of action studies of new potent inhibitors of the RNA viruses HCV, BVDV and RSV


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2010/2011

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# ABSTRACT

Several of worldwide emerging infections are caused by RNA viruses. For this reason, research in the antiviral chemotherapy field is directed toward the development of compounds that target various steps of the virus life cycle. In this work the antiviral activity of 5-Acetyl-2-Arylbenzimidazoles and 2-[(benzotriazol-1/2 yl)methyl]benzimidazoles has been evaluated against representatives of several virus families, including HCV BVDV, YFV, REO-1, CVB-5, Sb-1 and RSV. Some of the new derivatives turned out to be very interesting for their potency and selectivity against BVDV and HCV (the former), and RSV (the latter), and could be promising candidates for the treatment of the related diseases.

# 1.0 Global burden of infectious diseases

Infectious diseases are a leading cause of death worldwide. About 17 million of 57 million annual deaths, in a world population of 6.2 billion people, are estimated to be related directly to infectious diseases.[1]

Actually, poverty, evolving human migration patterns, new infectious agents and changing environmental and development activities, all contribute to the expanding impact of infectious diseases. Remarkably, 95% of infection-related deaths occur in developing countries. This figure does not include the additional millions of deaths occurring as a consequence of complications associated with chronic infections.[2] In industrialized nations, over the past 100 years life expectancy has nearly doubled, and early childhood mortality has fallen 100-fold; notwithstanding, infectious disease mortality still disproportionately affects in those countries indigenous and disadvantaged minorities.[3]

Since the discovery of the deadly Ebola virus in 1976, numerous previously unknown pathogenic viruses have been discovered. Moreover, in recent years, several viruses have widely expanded their territory causing the death of an increasing number of people. Since the publication in 1992 of the Institute of Medicine (IOM) report, Emerging Infections – Microbial Threats to Health in the United States, and its update in 2003, Microbial Threats to Health – Emergence, Detection and Response, the concept of new and emerging diseases has captured the general attention.[4] Over the past decade there has been a renewed public and official concern about infectious diseases as a major health threat, and the terms “emerging” and “re-emerging” infectious diseases (EIDs) have entered the vocabulary of medical science. Newly emerging infections are those that have not previously been recognized in man. Re-emerging infections are those that existed in the past, but are now rapidly increasing either in incidence or in geographical / human host range. Infections similar to those that we now define as EIDs have for millennia threatened the survival of human societies who share ecosystems with rapidly evolving microbial organisms and their non-human hosts, vectors, and reservoirs. As far as viral EIDs are concerned, comparison of historical and modern emerging diseases suggests that their determinants are largely the same: i) viral (mutation, natural selection, virus evolution and adaptation), ii) human and iii) ecological.[5]

Several among the emerging infectious diseases are also classified as neglected tropical diseases (NTDs) which, according to WHO, are hidden diseases as they affect almost exclusively extremely poor populations living in remote areas of Africa, Asia and the Americas, beyond the reach of health services. Among the NTDs with the highest-burden there are 13 parasitic (helmintic and

protozoal) and bacterial tropical infections, and 20 ectoparasitic, fungal and viral infections. Each year NTDs kill approximately 534,000 people worldwide.

The European Parliament recognized that NTDs have not received the attention they deserve from EU actions. Neglected diseases are given low priority because they have low mortality, occur almost exclusively in poor developing countries, offer negligible marketable and profitable issues. Thus, for the pharmaceutical industry, which carries out the main research and development of new drugs, it is too costly and risky to invest in drugs for diseases essentially occurring in low-income countries where public spending on drugs is less than 6 USD, compared to ~ 240 USD spent in countries belonging to the organization for Economic Cooperation and Development (OECD). It is estimated that less than 10% of the world's biomedical research funds are dedicated to problems dealing with 90% of the world's burden of disease.

If NTDs are defined as a group of infectious tropical diseases in developing countries that are both poverty-promoting and long-lasting in their health impact, then arboviruses (arthropod-borne viruses) certainly qualify as NTDs. These viruses belong to taxonomically diverse groups representing at least 8 virus Families and 14 Genera. There are currently 534 viruses registered in the International Catalogue of Arboviruses, 134 of which are known to cause disease in humans and approximately 40 to infect livestock. The most significant arboviruses causing human illness belong to the Families Flaviviridae, Bunyaviridae and Togaviridae.[6]

Some zoonotic infectious agents have largely been in the hands of scientists and public officials associated with animal health and agriculture. These include, for example, agents that cause substantial morbidity and mortality in livestock or poultry, especially the agents that cause "foreign animal diseases". Other zoonotic infectious agents have been associated with public health. These include, for example: 1) the rabies virus; 2) many arthropod-borne viruses (as well as bacteria and protozoa); 3) several rodent-borne viruses (and bacteria); 4) primate-borne agents. Despite all this, it seems that the concept of new and emerging zoonotic diseases has not been fully exploited, especially given the overriding realization that nearly all of the emergent disease episodes that have caught the public attention in the past 15 years have involved zoonotic infectious agents.[4]

The ***Flaviviridae*** family includes single-stranded positive-sense RNA ($ssRNA^+$) viruses, which cause significant diseases in humans and animals. They are distributed into three genera: the *Hepacivirus* genus includes, as sole representative, the Hepatitis C virus (HCV) [agents such as GB virus-A and GB virus-A-like, GB virus-D and GBV-C or hepatitis G virus, although closely related to HCV, represent unassigned members of Flaviviridae]; the *Flavivirus* genus comprises Yellow Fever, West Nile, Dengue Fever, Japanese Encephalitis and Tick-Borne Encephalitis viruses; the

*Pestivirus* genus comprises Bovine Viral Diarrhoea (BVDV), Border Disease and Classical Swine Fever viruses.

**HCV** is a major cause of human hepatitis.[7] The WHO estimates that over 170 million people worldwide are presently infected by this virus.[8,9] Most infections become persistent, and about 60% progress towards chronic liver disease. Chronic HCV infection can lead to development of cirrhosis, hepatocellular carcinoma and liver failure.[10,11] Pegylated interferon, in combination with ribavirin, is used in the clinic for HCV infections. Unfortunately, this therapy has limited efficacy and is often associated with severe, adverse events.[12]

***Flaviviruses*** are human pathogens prevalent throughout the world and cause a range of acute febrile illness, encephalites and hemorrhagic diseases. Flaviviruses are small, spherical, enveloped viruses containing an approximately 11K-nucleotide, positive-sense RNA genome. The viral RNA contains a single open reading frame which, when translated, gives rise to a poly-protein that is co- and post-translationally processed by viral and cellular proteases into three structural proteins (capsid [C], pre-membrane [prM], the precursor form of [M], and envelope [E]) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The non-structural proteins are responsible for various enzymatic activities, including RNA-dependent RNA polymerase (NS5) (RdRp), helicase (NS3) and protease, and together form the replicase complex (RC) that primarily functions to carry out viral genome replication. The structural proteins, along with the host-derived lipid bilayer and a single copy of the RNA genome, assemble to form the flavivirus virion. The latter is 50 nm in diameter, with a relatively smooth outer surface that is constructed from 180 copies of both the major E protein and the small M protein.[13]

The vectorborne transmission of **Yellow Fever Virus** (YFV) occurs via the bite of an infected mosquito, primarily *Aedes* or *Haemagogus* spp. Nonhuman and human primates are the main reservoirs of the virus, with anthroponotic (human-to-vector-to-human) transmission occurring. There are three transmission cycles for YFV: sylvatic (jungle), intermediate (savannah), and urban. The sylvatic (jungle) transmission cycle involves transmission of the virus between nonhuman primates and mosquito species found in the forest canopy. The virus is transmitted via mosquitoes from monkeys to humans when the humans encroach into the jungle during occupational or recreational activities. In Africa, an intermediate (savannah) cycle involves transmission of YFV from tree hole-breeding *Aedes* spp. to humans living or working in jungle border areas. In this cycle, the virus may be transmitted from monkeys to humans or from human to human via these

mosquitoes. The urban transmission cycle involves transmission of the virus between humans and urban mosquitoes, primarily *Aedes aegypti*.

Yellow fever occurs in sub-Saharan Africa and tropical South America, where it is endemic and intermittently epidemic. Most yellow fever disease in humans is due to sylvatic or intermediate transmission cycles. However, urban yellow fever occurs periodically in Africa and sporadically in the Americas. In Africa, natural immunity accumulates with age, and thus, infants and children are at highest risk for disease. In South America, yellow fever occurs most frequently in unimmunized young men who are exposed to mosquito vectors through their work in forested or transitional areas.

Humans infected with YFV experience the highest levels of viremia and can transmit the virus to mosquitoes shortly before onset of fever and for the first 3–5 days of illness. Given the high level of viremia, bloodborne transmission theoretically can occur via transfusion or needlesticks.

There is no cure for yellow fever. Treatment is symptomatic, aimed at reducing the symptoms for the comfort of the patient. Vaccination, available since the late 1930s, is the most important preventive measure against yellow fever. The vaccine is safe, affordable and highly effective, and appears to provide protection for 30–35 years or more. The vaccine provides effective immunity within one week for 95% of persons vaccinated; nevertheless, its use is not systematic in many areas.[14] Therefore, YFV continues to be responsible for a significant disease burden. WHO estimates that up to 200,000 cases of yellow fever (YF) still occur each year, resulting in approximately 30,000 deaths. The vast majority (~ 90%) of cases and deaths takes place in sub-Saharan Africa, where YF is a major public health problem occurring in epidemic patterns. Africa also experiences periodic, yet unpredictable outbreaks of urban YF. Thirty-two African countries are now considered at risk, with a total population of 610 million people, among which more than 219 million live in urban settings. YF is endemic in ten South and Central American countries and in several Caribbean islands. Although the disease usually causes only sporadic cases and small outbreaks, nearly all major urban centres in the American tropics have been re-infested with Aedes aegypti and most urban dwellers are vulnerable because of low immunization coverage. Latin America is now at greater risk of urban epidemics than at any time in the past 50 years. Also the risk of international spread is greater than before.[15-17]

**Dengue Virus** (DENV) infections may occur asymptomatically, produce a mild self-limiting acute febrile illness, dengue fever (DF) (50 to 100 million cases yearly), or a life-threatening illness, dengue haemorrhagic fever (DHF) (250,000 to 500,000 yearly, case fatality ratio = 1-5%)[15] DENV infections are a serious cause of morbidity and mortality in most tropical and sub-tropical countries, mainly Southeast and South Asia, Central and South America, and the Caribbean. According to

WHO estimates, there are approximately 2.5 billion people at risk of infection.[18] DENV infections are currently endemic in over 100 tropical and non-tropical countries, and imported cases have been reported in several non-endemic countries. The rise in incidence has been marked by an expanding geographical distribution of DENV and of its mosquito vector Aedes aegypti, which is found worldwide between latitudes 35ºN and 35ºS.[19]

**West Nile Virus** (WNV) is maintained and transmitted through an enzootic cycle involving birds as main amplifying hosts, and ornithophilic mosquitoes of the genus Culex as main vectors. Human and horses are only incidental hosts in the transmission cycle of WNV. Both are dead-end hosts for the virus since the intensity and duration of viraemia are insufficient to allow infection of biting mosquitoes.[20] Despite that, infections by WNV may have dramatic consequences in both human and equine populations, and the recent emergence of WNV in North America has clearly illustrated its possible impact for human public health. An estimated 16,000 people in the United States have become ill from West Nile virus since 1999, and more than 600 have died. A far greater number of people have probably been infected without knowing it because they did not have any symptoms. Outside North America, regular outbreaks also occur in Africa, Europe, and Asia. In 1996-97, for example, an outbreak near Bucharest, Romania, infected more than 500 people and killed about 50. There are no valid statistics on the number of people infected worldwide. What is known is that, since its first appearance, WNV caused over 18,000 cases, 800 human deaths, and a large but indeterminate number of equine deaths. The huge extension of WNV in the Americas has revealed other unexpected modes of transmission of the virus in humans, mainly through blood donation or organ transplantation, but also through intra-uterine contamination or sporadic transmission by breast-feeding.[21]

***Pestivirus*** infections of domesticated livestock cause significant economic losses worldwide. They cause a range of clinical manifestations, including abortion, teratogenesis, respiratory problems, chronic wasting disease, immune system dysfunction and predisposition to secondary viral and bacterial infections. BVDV can also establish a persistent infection (PI) in animals, that succumb or remain viremic throughout life and serve as continuous virus reservoirs. Furthermore, BVDV also shows the ability to cross the placenta of susceptible animals causing a variety of fetal infections.[22,23]

With the exception of YFV and BVDV, no vaccines exist against Flaviviridae pathogens, as well as no selective antiviral drugs are yet available in the clinic to prevent and/or treat their infections. The only exceptions are two protease inhibitors, recently approved by FDA for the treatment of HCV

infections.[24-27] Hence the need to continue studies aimed at identifying new lead compounds targeted at virus-specifc steps of the Flaviviridae replication cycle.

**Human respiratory syncytial virus** (RSV) was first isolated in 1956 from a laboratory chimpanzee with upper respiratory tract disease.[28] Respiratory syncytial virus (RSV) is a cause of acute respiratory tract infections in persons of all ages and is the most common cause of lower respiratory tract infection in children aged <1 year. By the age of 2 years, almost all children have been infected, and re-infection is a common occurrence. RSV transmission occurs by inoculation of nasopharyngeal or ocular mucous membranes after contact with fomites or secretions containing RSV. The most common route of transmission is direct contact, but droplets have also been implicated. RSV causes seasonal outbreaks worldwide. Patients at risk for serious RSV infection include infants aged <6 months, infants born before 35 weeks of gestation and infants and children with underlying lung disease or congenital heart disease.[29]

The World Health Organization estimates that RSV causes 64 million infections and 160,000 deaths annually.[28]

RSV is classified in the genus Pneumovirus of the family Paramyxoviridae.[30] Two strains, subtypes A and B, have been identified and often circulate concurrently in annual epidemics.[31] RSV is an enveloped virus with a single-stranded negative-sense RNA genome of 15.2 kb. There are animal versions of RSV, including bovine RSV (BRSV) and pneumonia virus of mice (PVM), suggesting that species jumping occurred during the evolution of these viruses. However, there is no animal reservoir for human RSV. Viral gene expression and RNA replication occur in the cytoplasm, and virions acquire a lipid envelope by budding through the plasmid membrane. Virions are pleomorphic and include spheres and long, fragile filaments. The negative-sense RNA genome contains a short 3'-extragenic leader region, 10 viral genes in a linear array and a 5'-trailer region. Each gene is transcribed into a separate, capped, polyadenylated mRNA encoding a single viral protein, except in the case of the M2 mRNA, which contains two overlapping open reading frames that are expressed by a ribosomal stop-restart mechanism into two distinct proteins, M2-1 and M2-2.

Five RSV proteins are involved in nucleocapsid structure and/or RNA synthesis. The nucleocapsid N protein tightly encapsidates genomic RNA as well as its positive-sense replicative intermediate, called the antigenome. This provides protected, flexible templates and probably reduces detection of these viral RNAs by host cell toll-like receptors (TLRs) and intracellular RNA recognition helicases that initiate innate immune responses through interferon (IFN) regulatory factors and nuclear factor κB (NF- κB). The large L protein is the major polymerase subunit and contains the catalytic

domains. The P phosphoprotein is an essential cofactor in RNA synthesis and also is thought to associate with free N and L to maintain them in soluble form for assembly of and interaction with nucleocapsids. The M2-1 and M2-2 proteins are factors involved, respectively, in transcription and in modulating the balance between transcription and RNA replication.

Four other RSV proteins associate with the lipid bilayer to form the viral envelope. The matrix M protein lines the inner envelope surface and is important in virion morphogenesis. The heavily glycosylated G, fusion F, and small hydrophobic SH proteins are transmembrane surface glycoproteins. G and F are the only virus neutralization antigens and are the two major protective antigens.

**G protein.** The attachment G protein can be divided into an intracellular domain, a transmembrane domain, and a large ectodomain. The latter contains a highly conserved central motif between aminoacids 164 and 176 that constitutes the putative receptorbinding site. The changes are concentrated in two hypervariable regions that flank the highly conserved central region of the G protein ectodomain: the first variable region is located in the amino(N)-terminal part of the protein preceding the conserved region and the second variable region is located in the carboxy(C)-terminal end. The domain located near the C-terminal region has been reported to provide a reliable proxy for the entire G gene variability and has subsequently been used in phylogenetic analysis for molecular epidemiological studies and to assign isolates to genotypes.[32]


**Fig. 3.**The 298-amino acid attachment glycoprotein of the A2 strain of HRSV is subdivided into the five domains: I, a cytoplasmic domain; II, a transmembrane domain; III and V, heavily glycosylated and variable subdomains of the ectodomain; and IV, the nonglycosylated central subdomain of the ectodomain **(**Adapted from Gorman J. J., Jennifer L. Kimm-Breschkin Mc, Norton R. S., Barnham K. J.; Antiviral Activity and Structural Characteristics of the Nonglycosylated Central Subdomain of Human Respiratory Syncytial Virus Attachment (G) Glycoprotein; 2001).[33]

**F protein.** The F protein directs viral penetration by membrane fusion and also mediates fusion of infected cells with their neighbors to form syncytia. F is synthesized as a precursor, F0, which is activated by cleavage by furin-like intracellular host protease. This cleavage occurs at two sites (amino acids 109/110 and 136/137). This yields in amino-to-carboxy-terminal order, F2 (109 amino acids), p27 (27 amino acids), and F1 (438 amino acids). F2 and F1 remain linked by a disulfide bond and represent the active form.

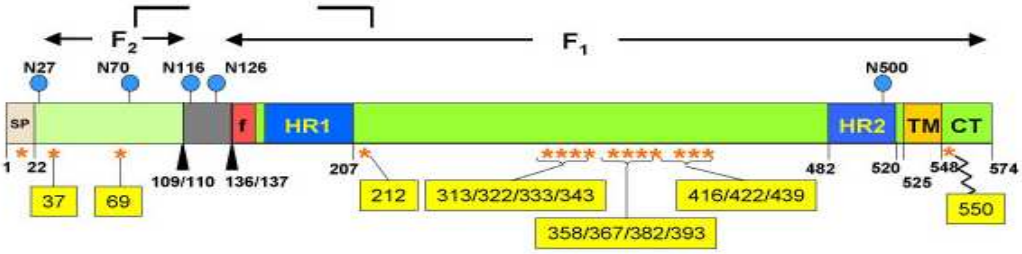



**Fig. 4.** RSV F protein structure

During its transport through the secretory pathway, the F protein is glycosylated by several enzyme activities in the endoplasmic reticulum (ER) and Golgi complex. Depending on the RSV isolate, the F protein amino acid sequence can contain up to 5 potential N-linked glycosylation sites. However, experimental evidence suggests that in the mature F protein, the F1 subunit is glycosylated at a single site, N500, while the F2 subunit is glycosylated at two different sites, namely N27 and N70. Recent reports suggest that N500 plays a role in F protein-mediated fusion possibly by interacting with the heptad repeat regions. In contrast, the role played by N27 and N70 in the functionality of the F protein remains to be established.

Evidence that maturation of the N-linked glycans of the respiratory syncytial virus (RSV) glycoproteins is required for virus-mediated cell fusion.[34] The mature F protein is expressed as a homotrimer on the cell surface. The ectodomain of the F1 transmembrane subunit contains two heptad repeat regions, HR1 and HR2, and an N-terminal fusion peptide.

Processing of the F0 precursor has been proposed to generate a “metastable” F1/F2 complex. In one proposed model, receptor binding triggers conformational changes in the metastable F protein, resulting in insertion of the N-terminal fusion peptide into the target cell membrane. The HR1 and HR2 heptad repeat regions then interact to form a six-helix bundle structure. According to this model, formation of the six-helix bundle structure facilitates membrane fusion by bringing the viral and cellular membranes into close apposition.[35]

**SH protein.** SH is present as an unmodified 7.5 kDa species. Molecular modelling studies also favored the pentamer, and suggested SH adopts a circular structure with a central pore. Several studies have indicated SH affects membrane permeability.[36] The role that the SH protein plays during virus infection is unclear. It appears to be dispensable for virus replication in tissue culture, and recombinant viruses in which the SH protein was deleted grew better in HEp-2 cells. These studies suggested that the SH protein exerted a negative effect on virus induced membrane fusion in tissue culture, providing evidence that it may play a regulatory role during membrane fusion.

However, the absence of a direct interaction between the SH and F proteins suggests that the SH protein is unlikely to assist the F protein in mediating the membrane fusion process (Low K.W. et al.; 2008).

**RSV gene expression and RNA replication.** The polymerase enters the genome at or near its 3-end, and the genes are transcribed into individual mRNAs by sequential start-stop-restart synthesis that is guided by short transcription signals flanking the genes. RNA replication involves synthesis of the full-length positive-sense antigenome that in turn is copied into progeny genomes.

With RSV, processive transcription depends on the M2-1 protein, which is essential for viral viability. In its absence, transcription terminates nonspecifically within several hundred nucleotides and results in reduced expression of NS1 and NS2 alone.

The other product of the M2 gene, the M2-2 protein, is not essential but appears to downregulate transcription in favour of RNA replication as infection progresses.[37]

## 2.0 Challenges for antiviral research vs pathogenic RNA viruses

Since RNA virus zoonoses cannot be eradicated, countermeasures will always be needed to prevent / treat the related human diseases. Some of them will increase in incidence over coming decades, as expanding population, failure of vector control, effects of global warming on vector distribution and spread, together with other factors are likely to increase the rate of contact between humans and sources of infection. Other diseases will become less prevalent, as changes in human behaviour reduce exposure to the etiologic agents and vaccines are introduced or more widely applied.

Because it is better to prevent a disease than to treat it, vaccines are an essential weapon in the fight against highly pathogenic RNA viruses. However, although being the simplest strategy, vaccination would require continuous and capillary immunization across the regions where the etiological agents persist. But this represents an unlikely prospect.

Currently, no specific antiviral therapy exist for diseases due to highly pathogenic RNA viruses. Compared to HIV/AIDS, HCV and influenza, RNA viral encephalites and hemorrhagic fevers have received much less attention: in part because these diseases occur predominantly in underdeveloped countries that lack the infrastructures for clinical trials, in part due to the relevant economic resources needed to afford expensive medications.

Only a single licensed drug, Ribavirin, is of clinical use against some of the emerging / neglected infections. However, its efficacy against a few types of viral hemorrhagic fever has been reported only in observational studies. Ebola and Marburg hemorrhagic fever present the greatest challenge to the development of new antiviral therapies for highly pathogenic RNA viral infections. The basic problem can be summarized by noting the striking contrast between the high-tech containment labs in which these diseases are studied and the impoverished locations in central Africa where they occur.

The effort to develop effective therapies against highly pathogenic emerging and/or neglected RNA viral infections is one of the most challenging in public health. Its success requires intensive research at multiple levels, from basic studies of the etiologic agents and their replication mechanisms, to medicinal chemistry, efficacy testing in animal models, performance in clinical trials.

### 2.1 Nucleos(t)ide analogues and pro-drugs

Antiviral nucleoside analogues can be divided into two main classes:

**A-** Specific inhibitors: target viral enzymes and show high selectivity indices.

i) Activated by cellular kinases:

a. Target RT (HIV and occasionally HBV). Show a narrow spectrum that depends on the high affinity of their TP metabolites for target enzymes.
b. Target RdRp ($RNA^+$ viruses). Show a wide spectrum that depends on the high affinity of their TP metabolites for target enzymes. Different target viruses share highly conserved sequences in their polymerization site.

ii) Activated by viral kinases:

a. Target DdDp (Herpesviruses). Their narrow spectrum depends on the high affinity of their TP metabolites for target enzymes following activation by kinases.

**B**- Non-specific inhibitors: target cellular enzymes and show low selectivity indices and broad-spectrum.

iii) Activated by cellular kinases:

a. Ribavirin, targets IMP dehydrogenase
b. 6-aza-UdR, targets OMP decarboxylase

Generally speaking, the current antiviral drug armamentarium for prevention and treatment of many important viral infections comprises more than 40 compounds that have been officially approved for clinical use. Generally speaking, the current antiviral drug armamentarium for prevention and treatment of many important viral infections comprises more than 40 compounds that have been officially approved for clinical use.[38] Three decades after the discovery of HIV, 30 antiretroviral drugs are currently used in the clinic.

Polymerase inhibitors are the largest class of approved antiviral drugs, and nucleosides are the largest chemical class therein. Many of them proved highly successful for the treatment of HIV (zidovudine, zalcitabine, didanosine, stavudine, abacavir, lamivudine, and emtricitabine), HBV (lamivudine, telbivudine and entecavir), and Herpes virus infections (acyclovir, valacyclovir, penciclovir, famciclovir, idoxuridine, trifluridine, ganciclovir, valganciclovir, cidofovir, fomivirsen), but only few [2'-O-methylcytidine, 2'-C-methylcytidine (and its 3'-valine ester, valopicitabine), 2'-C-methyladenosine, 2'-C-methylguanosine, 2'-deoxy-2'-fluoro-2'-C-methylcytidine, 7-deaza-2'-C-methyladenosine, 7-deaza-7-fluoro-2'-C-methyladenosine, 4'-azidocyitidine] have been proved effective against HCV in vitro,[39-41] and none has been successful in the clinic, yet. Nucleoside analogues that target viral polymerases share a common mechanism of action. They undergo intracellular activation by kinases to triphosphate forms, after which they compete with endogenous nucleoside triphosphates (NTPs) for use as substrates by viral polymerases and incorporation into nascent nucleic acid chains. Since most of nucleoside analogues lack the necessary 3'-OH group for further chain elongation (or, when present, it is unavailable at

the right intermolecular distance with the 5'-$PO_3^{--}$ of the incoming nucleotide, because of sugar structure distortions), chain termination ensues.

Although discovered 40 years ago, **ribavirin** remains the only approved small molecule drug that is active against both DNA and RNA viruses.[42] Ribavirin itself is a prodrug that is metabolized to the active 5'-monophosphate, -diphosphate and triphosphate in vivo. Ribavirin 5′-monophosphate (RMP) inhibits cellular inosine monophosphate dehydrogenase (IMPDH), thereby depleting intracellular pools of GTP for viral replication. Ribavirin 5′-triphosphate (RTP) is the major metabolite accumulated in vivo. It is an effective inhibitor of viral RNA guanylyl transferase and (guanine-7N-)-methyl transferase, contributing to the defective 5′-cap structure of viral transcripts and insufficient translation. All the 5′-phosphate forms of ribavirin have been implicated as direct inhibitors of viral RNA-dependent RNA polymerase (RdRp). Because human cells lack RdRp, the latter appear to be one of the most promising targets for antivirals against RNA viruses. Ribavirin is a broad-spectrum antiviral agent and has been used clinically to treat respiratory syncytial, hepatitis C, Lassa, Hantaan and La Crosse viruses. Ribavirin has inhibitory activity against WNV infection in cell culture.[43] However, during a WNV outbreak in Israel in 2000, a high mortality rate (41%) was observed in a group of 37 patients who received ribavirin.[44] 5-Aza-7deazaguanosine (ZX-2401) is a broad spectrum inhibitor of the RNA viruses that is less toxic and more active than Ribavirin and it inhibits YFV, DENV and WNV.[45]

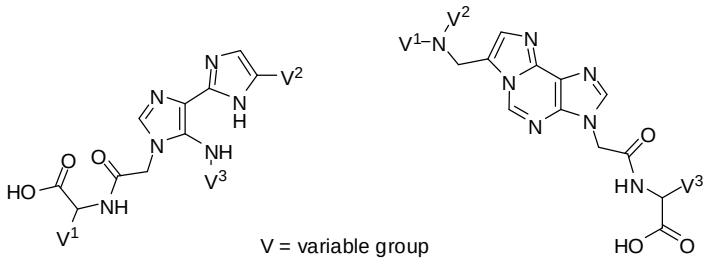
## 2.2 Non-nucleoside inhibitors

Several non-nucleoside inhibitors (NNIs) are known to target reverse transcriptases (RTs), RdRps and proteases. The Food and Drug Administration (FDA) has approved 4 non-nucleoside drugs as reverse transcriptase inhibitors (NNRTI's) of HIV-1.

The NS5B RdRps of HCV and other *Flaviviridae* are also seen as attractive targets for the development of NNIs. Screening of countless compounds has revealed a variety of inhibitors from different structural classes, representative of various heterocyclic systems, which bind to distinct allosteric sites on the HCV polymerase. Several small molecules have been reported to display inhibitory activity against the HCV helicase[46] and a class of benzotriazole-benzimidaole-based inhibitors have been disclosed by Bristol-Mers Squibb Pharmaceutical as potent inhibitors of RSV entry.[47]

5'-Amino-2,4'-biimidazoles constitute a scantily studied group of compounds that may afford an interesting new scaffold for lead search. They obey well the 2-0 rule of kinase-likeness,[48] they can be synthesized on-support from adenine and they contain six points of diversification. Structure of 5'-amino-2,4'-biimidazole is a rigid rod, but rotation around 2,4'-bond connecting the imidazoles is

possible, although hindered. Depending on substituents and pH, there is a possibility for intramolecular hydrogen bonding between the 5'-amino proton and the pyridine-like nitrogen of the second imidazole ring. This hydrogen bond results in formation of a pseudo six-membered ring between the imidazoles thus making the ring system planar. Different twisted conformations between the imidazole rings can be evoked by the choice of substituents. The multifaceted core structure makes it possible to design a spatially diverse set of compounds using a single scaffold. For these reasons 5´-amino-2,4´-biimidazoles form a reasonable basis for finding leads to rare RNA viruses though the antiviral activity of this kind of compounds has not been so far demonstrated. Another class of potential new leads is offered by 7-substituted 3H-imidazo[2,1-i]purines.




Recently a number of different aromatic heterocyclic systems have been proposed as anti-RNA virus agents, as illustrated in Figure 1.

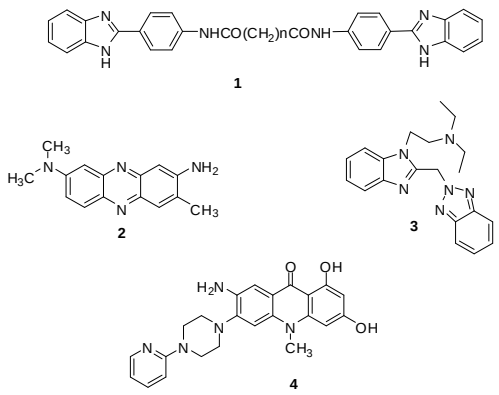



Figure 1: Templates for anti-RNA-virus agents.

Derivatives 1 have been proposed as RNA-dependent RNA helicase inhibitors.[49] Derivative 2 has been proposed as internal ribosome entry site (IRES) inhibitors.[50] Derivative 3 has been proposed as fusion inhibitors.[51] Derivative 4 have been proposed as RNA-dependent RNA polymerase (RdRp) inhibitors.[52]

| TABLE 1. RNA VIRUSES CAUSING EMERGING (EI) AND/OR NEGLECTED (NTD) INFECTIONS | | | | | | | |
|---|---|---|---|---|---|---|---|
| Disease | Type | # cases / deaths (%CFR) per year | Transmission | Prevention / Therapy | Agent | Family / Genus | BSL |
| Dengue fever (DF); dengue hemorrhagic fever (DHF) | EI, NTD | DF: 50-100 million<br>DHF: up to 500.000 (CFR 1-5%)<br>2.5 billion people at risk | Arthropod-borne (mosquitoes) | None | Dengue virus (DENV) | Flaviviridae/ Flavivirus $RNA^{+}$ | 3 |
| Yellow fever | EI, NTD | 200.000 / 30.000<br>610 million African people at risk | Arthropod-borne (mosquitoes) | Vaccine / No therapy | Yellow Fever virus(YFV) | Flaviviridae / Flavivirus $RNA^{+}$ | 3 |
| West Nile encephalitis | EI | 18.000 / 800<br>No valid statistics for # people infected worldwide | Arthropod-borne (mosquitoes) | Supportive treatment only | West Nile virus (WNV) | Flaviviridae / Flavivirus $RNA^{+}$ | 3 |
| Hantavirus hemorrhagic fever | EI | HFRS: 200.000 (CFR 1- 2%)<br>HPS: 200 (CFR 40%) | Contact with infected rodents (or their excretions) | None | Hantavirus | Bunyaviridae / Hantavirus $RNA^{-}$ | 3 |
| Rift Valley hemorrhagic fever | EI | 2006-2007 outbreaks: 684/155 (CFR: 23%) in Kenya<br>Older outbreaks usually CFR 2% | Arthropod-borne (mosquitoes) | Supportive treatment only | Rift Valley Fever virus (RVFV) | Bunyaviridae / Phlebovirus $RNA^{-}$ | 3 |
| Ebola hemorrhagic fever | EI | 1976 – 2008 outbreaks: 2246 / 1507 (CFR 67%) | Source unknown. Contact with infected biological fluids or organs. | None / Supportive treatment only | Ebola virus | Filoviridae / Ebola-like $RNA^{-}$ | 4 |
| Hendra encephalitis, pneumonia | EI | 1994-2009, 9 cases | Mode of transmission unknown (respiratory route is suspected. Human infection most commonly associated with direct contact with infected horses. | None | Hendra virus | Paramyxoviridae / Henipavirus $RNA^{-}$ | 4 |
| Nipah encephalitis | EI | 1998 - 2008 outbreaks: 477 / 252 (CFR 52%) | Mode of transmission unknown, although a respiratory route is suspected. Human infection most commonly associated with direct contact with infected pigs. | None / Supportive treatment only | Nipah virus | Paramyxoviridae / Henipavirus $RNA^{-}$ | 4 |
| Lassa hemorrhagic fever | EI | 300.000 / 5000 (CFR 15-20%) | Contact with infected rodents (or their excretions) | None / Ribavirin | Lassa virus (LASV) | Arenaviridae / Old World Arenavirus $RNA^{A}$ | 4 |
| Venezuelan hemorrhagic fever | EI | 200 | Contact with infected rodents (or their excretions) | None / Supportive treatment only | Guanarito virus (GTOV) | Arenaviridae / New World Arenavirus $RNA^{A}$ | 4 |
| Argentine hemorrhagic fever | EI | 30.000 cases since 1951 | Contact with infected rodents (or their excretions) | None / Ig only | Junin virus (JUNV) | Arenaviridae / New World Arenavirus $RNA^{A}$ | 4 |
| Bolivian hemorrhagic fever | EI | | Contact with infected rodents (or their excretions) | None / Supportive treatment only | Machupo virus (MACV) | Arenaviridae / New World Arenavirus $RNA^{A}$ | 4 |

# 3.0 Materials and Methods

*Test compounds*

Compounds were dissolved in DMSO at 100 mM, and then diluted in culture medium.

*Cells and Viruses*

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of RNA and DNA viruses were the following: $CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4); Madin Darby Bovine Kidney (MDBK) [ATCC CCL 22 (NBL-1) Bos Taurus]; Baby Hamster Kidney (BHK-21) [ATCC CCL 10 (C-13) Mesocricetus auratus] and Monkey kidney (Vero 76) [ATCC CRL 1587 Cercopithecus Aethiops]. Viruses were purchased from American Type Culture Collection (ATCC), with the exception of Yellow Fever Virus (YFV). Viruses representative of positive-sense, single-stranded RNAs ($ssRNA^+$) were: i) Flaviviridae: yellow fever virus (YFV) [strain 17-D vaccine (Stamaril Pasteur J07B01)] and bovine viral diarrhoea virus (BVDV) [strain NADL (ATCC VR-534)]; ii) Picornaviridae: human enterovirus B [coxsackie type B5 (CVB-5), strain Ohio-1 (ATCC VR-29)], and human enterovirus C [poliovirus type-1 (Sb-1), Sabin strain Chat (ATCC VR-1562)]. Viruses representative of negative-sense, single-stranded RNAs (ssRNA-) were: iii) Paramyxoviridae: human respiratory syncytial virus (RSV) [strain A2 (ATCC VR-1540)]; iv) Rhabdoviridae: vesicular stomatitis virus (VSV) [lab strain Indiana (ATCC VR 1540)]. The virus representative of double-stranded RNAs (dsRNA) Reoviridae was reovirus type-1 (Reo-1) [simian virus 12, strain 3651 (ATCC VR-214)]. DNA virus representatives were: v) Poxviridae: vaccinia virus (VV) [vaccine strain Elstree-Lister (ATCC VR-1549)]; vi) Herpesviridae: human herpes 1 (HSV-1) [strain KOS (ATCC VR-1493)].

*Cytotoxicity Assays*

Cytotoxicity assays were run in parallel with antiviral assays. Exponentially growing MT-4 cells were seeded at an initial density of $1x10^5$ cells/ml in 96-well plates in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G and 100 μg/mL streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% $CO_2$ atmosphere, in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 hrs at 37°C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. MDBK and BHK cells were seeded in 24-well plates at an initial density of $6x10^5$ and $1x10^6$ cells/mL, respectively, in Minimum Essential Medium with Earle's salts (MEM-E), L-glutamine,

1mM sodium pyruvate and 25mg/L kanamycin, supplemented with 10% horse serum (MDBK) or 10% foetal bovine serum (FBS) (BHK). Cell cultures were then incubated at 37 °C in a humidified, 5% $CO_2$ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48-96 hrs at 37°C by the MTT method. Vero-76 cells were seeded in 24-well plates at an initial density of $4x10^5$ cells/mL, in Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine and 25mg/L kanamycin, supplemented with 10% FBS. Cell cultures were then incubated at 37 °C in a humidified, 5% $CO_2$ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48-96 hrs at 37 °C by the crystal violet staining method.

*Antiviral assays*

Compound's activity against YFV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected with a m.o.i. of 0.01. Compound's activity against BVDV was based on inhibition of virus-induced cytopathogenicity in MDBK cells acutely infected with a m.o.i. of 0.01. Briefly, BHK and MDBK cells were seeded in 96-well plates at a density of $5x10^4$ and $3x10^4$ cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified $CO_2$ (5%) atmosphere. Cell monolayers were then infected with 50 μL of a proper virus dilution in maintenance medium [MEM-Earl with L-glutamine, 1mM sodium pyruvate and 0.025g/L kanamycin, supplemented with 0.5% inactivated FBS] to give an m.o.i of 0.01. After 1 hr, 50 μL of maintenance medium, without or with serial dilutions of test compounds, were added. After a 3-/4-day incubation at 37 °C, cell viability was determined by the MTT method.

Compound's activity against CVB-5, Sb-1, VV, HSV-1 and RSV was determined by plaque reduction assays in infected cell monolayers. To this end, Vero 76-cells were seeded in 24-well plates at a density of $2x10^5$ cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium [Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine and 4500 mg/L D-glucose and 0.025g/L kanamycin, supplemented with 10% FBS] at 37 °C in a humidified $CO_2$ (5%) atmosphere. Then, monolayers were infected for 2 hrs with 250 μL of proper virus dilutions to give 50-100 PFU/well. Following removal of unadsorbed virus, 500 μL of maintenance medium [D-MEM with L-glutamine and 4500 mg/L D-glucose, supplemented with 1% inactivated FBS] containing 0.75% methyl-cellulose, without or with serial dilutions of test compounds, were added. Cultures were incubated at 37°C for 2 (Sb-1 and VSV), 3 (CVB-5, VV and HSV-1) or 5 days (RSV) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques were then counted.

*Linear regression analysis*

The extent of cell growth/viability and viral multiplication, at each drug concentration tested, were expressed as percentage of untreated controls. Concentrations resulting in 50% inhibition ($CC_{50}$ or $EC_{50}$) were determined by linear regression analysis.

*HCV replicon assays*

A human hepatoma cell line (Huh-7) bearing the HCV genotype 1b replicon (GS4.1 cells), kindly provided by C. Seeger (Fox Chase University, Philadelphia, PA, USA) through Idenix Pharmaceuticals, was grown in D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.5 mg/mL G418 (Invitrogen). For dose-response testing, GS4.1 cells were seeded in 96-well plates at a density of $7.5x10^3$ cells/well in 50 µL medium containing two-fold serial dilutions of test compounds (highest concentration, 75 µM). Huh-7 cells lacking the HCV replicon served as negative controls. Plates were then incubated for 72 hrs at 37 °C in a humidified, 5% $CO_2$ incubator. Inhibition of HCV replication was measured by quantification of the viral NS4A protein using an enzyme-linked immunosorbent assay (ELISA) as follows: plates were fixed for 1 min with 1:1 acetone–methanol, washed twice with PBS containing 0.1% Tween 20, left for 1 h at room temperature with TNE buffer containing 10% FBS, and then incubated for 2 hrs at 37 °C with the anti-NS4A mouse monoclonal antibody A-236 (ViroGen, Watertown, MA, USA) diluted in the same buffer. After three washes with PBS containing 0.1% Tween 20, plates were incubated for 1 h at 37 °C with anti-mouse immunoglobulin G–peroxidase conjugate in TNE buffer containing 10% FBS. After further washing as above, the reaction was developed with o-phenylenediamine (Zymed, San Francisco, CA, USA). The reaction was stopped after 30 min with 2N $H_2SO_4$ and absorbance was determined at 492 nm using a Sunrise Tecan (Durham, NC, USA) spectrophotometer. $EC_{50}$ values were determined from % inhibition vs compound concentration data, using a sigmoidal non-linear regression analysis based on four parameters, with Tecan Magellan software. For cytotoxicity evaluation, Huh-7 and GS4.1 cells were treated with compounds as described above, and cellular viability was monitored using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega). $CC_{50}$ values were determined from the % cytotoxicity vs compound concentration data with Tecan Magellan software, as described above.

*Molecular analysis of drug-resistant mutants*. Plaque purified virus was subjected to RNA extraction, RT-PCR and genome sequencing to identify the mutation patterns responsible for drug resistance. Viral RNAs from wt and drg-resistant mutants were obtained using the QIAamp viral

RNA minikit (QIAGEN), starting from 140 μL of cell-free viral suspensions, each containing about $10^6$ PFU/mL, in order to determine the nucleotide sequence of the mutated genes. Reverse transcriptions were carried out using the Superscript II enzyme (INVITROGEN) and cDNAs were amplified by PCR using Platinum Pfx polymerase (INVITROGEN), following the manufacturer's protocol.

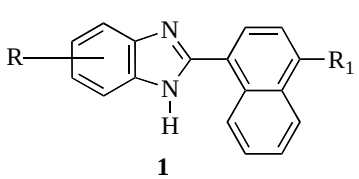
PCR fragments were purified using the QIAquick PCR Purification kit (QIAGEN) and analyzed using the cycle-sequencing method (CIBIACI service, University of Firenze). Both DNA strands were sequenced with specific primers. The comparative analysis of the chromatograms allowed us to deduce the mutation pattern responsible for drug resistance.

# 4.0 5-Acetyl-2-Arylbenzimidazoles as potent and selective inhibitors of BVDV and HCV

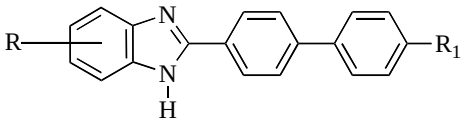
## Premises

Our interest on the chemistry and biological properties of benzimidazoles has led us to synthesize numerous derivatives endowed with analgesic-antinflammatory[53,54] and choleretic[55,56] activities, as well as with a chloropromazine-like activity in the conditioned avoidance response[57,58]and, more recently, with antiproliferative and antiviral activities.[59-62]

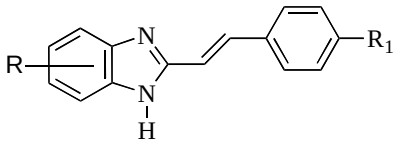
In this context, we have described the series of 2-[4-substituted naphtyl]- (1),[59] 2-[4-substituted diphenyl]- (2),[60] 2-[4-substituted styryl]-benzimidazoles (3)[61] and 1-substituted-2-[(benzotriazol-1/2-yl)methyl benzimidazoles (4) and (5),[62] endowed with selective activity, at micromolar level, against Respiratory Syncytial virus (RSV) and some members of the *Flaviviridae* family (*Fig. 1*).



**1**



**2**



**3**

R=H; 5-Me; 5,6-diMe, 5-Cl; 5,6-diCl; 5-$CF_3$; 5-$COCH_3$;
$R_1$= H; $NO_2$; $NH_2$; $NHCOCH_3$;

YFV ($EC_{50}$ μM)

**1a**, 5,6-diMe; $R_1$=H 6
**1b,** 5-Cl; $R_1$= $NH_2$ 27
**1c,** 5,6-diCl; $R_1$=$NH_2$ 16

**2d,** R=$COCH_3$; $R_1$=$NO_2$

YFV ($EC_{50}$ =0.5μM); CVB-2 ($EC_{50}$ =1 μM)

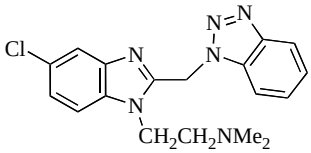
**3e**, R=5-$COCH_3$; $R_1$=$NO_2$

BVDV( EC50=1.7 μM)

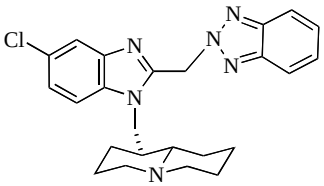
**3f;** R=H; $R_1$=$NH_2$;
**3g;** R=Cl; $R_1$=$NH_2$

CVB-2, ( $EC_{50}$ values of 7 - 8 μM respectively)

**3h**, R=$CF_3$; $R_1$=$NHCOCH_3$
RSV ($EC_{50}$= 1 μM)



**4**

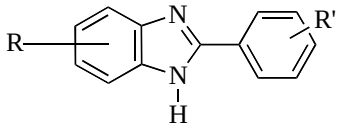


**5**

Anti RSV $EC_{50}$=0.03 μM

*Fig. 1*

As part of a pluriennal program in this area,[59-62] very recently we described new 2-phenyl-benzimidazoles [compounds **6** in *Fig. 2*], which have been evaluated in cell-based assays for cytotoxicity and activity against a panel of RNA and DNA viruses.[76] Thirty-nine compounds exhibited antiviral activity at concentrations comprised between 0.1 and 10 µM, and four of them were outstanding for their potency against VV (**6a**, R=5,6-diCl; $R_1$=4-$NO_2$) or BVDV (**6b**, R=5-$NO_2$; $R_1$=H; **4c**, R=5-$NO_2$; $R_1$=$COCH_3$; **6d**, R=5,6-diCl; $R_1$=$COCH_3$), with $EC_{50}$ = 0.1, 1.5, 0.8 and 1.0 µM, respectively. In enzyme assays, **6b** and **6d** inhibited at low µM concentrations the RNA-dependent RNA polymerase (RdRp) of BVDV and HCV, respectively. This suggested that 2-phenylbenzimidazoles could be attractive leads for further development of inhibitors of poxviruses, pestiviruses and even HCV. Therefore, we decided to carry on our program to evaluate whether this type of compounds, purposely modified, could be improved in both potency and spectrum of antiviral activity.




**6**

R=H; 5-$CF_3$; 5-$COCH_3$; 5,6-diCl;
R'=2-$NO_2$; 2,4-di$NO_2$; 4-OH; 4-$OCH_3$; 3-$OCH_3$; 2,4-di$OCH_3$; 3,5-di$OCH_3$; 2,3,4-tri$OCH_3$; 4-F;2,6-diF; 4-$NO_2$; 2-$NO_2$; 4-$OCH_3$;2-$NH_2$; 4-$NH_2$ ; 4-$OCH_3$

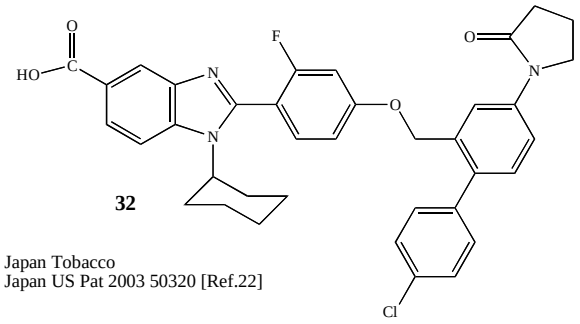
*Fig. 2*

Focusing on the derivatisation of the 5-acetyl group on the benzimidazole, by using typical reagents of carbonyl group as hydroxylamine, semicarbazide and thiosemicarbazide, we designed and synthesized derivatives **7-31** (Table 1) in order to evaluate the influence of this modification on the antiviral activity.

In support of our hypothesis was the example of the many benzimidazole derivatives reported in the literature, where the presence of a carboxylic or tetrazolyl group at position 5 of the benzimidazole ring [as in compounds **32-34** (*Fig. 3*)] allowed inhibition of the HCV RdRp at very low $IC_{50}$ values.[77-79]

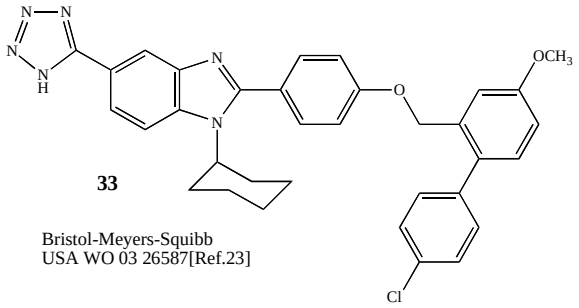
In our opinion, the presence of an electron withdrawing substituent, such as the acetyl group in compounds **7-12** and their derivatives **13-31**, could represent a good strategy to improve the pharmacophoric properties of this class of compounds. Thus, we decided to proceed in two stages. First, we afforded the simple modification of the acetyl group, as in compounds **7-31**. Once verified that this type of substitution could lead to selective BVDV inhibitors, we prepared benzimidazoles **35-61** (Table 2) in form of both simple acetyl derivatives and corresponding thiosemicarbazones

and semicarbazones, characterized by an increased lipophilicity due to the cyclohexyl substitution at position 1, as for the above mentioned compounds **32-34**.



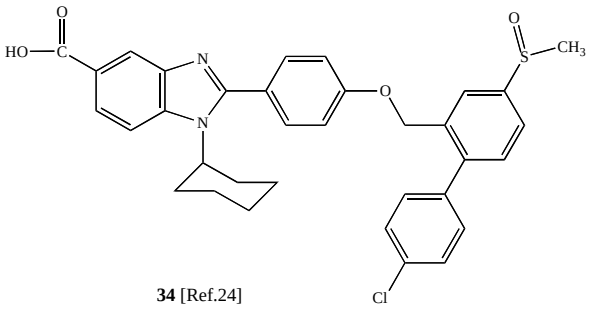
**32**

Japan Tobacco
Japan US Pat 2003 50320 [Ref.22]



**33**

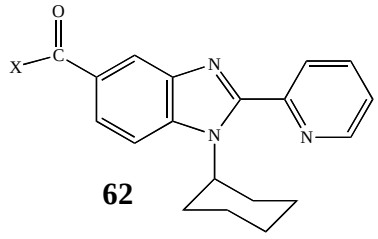
Bristol-Meyers-Squibb
USA WO 03 26587[Ref.23]



**34** [Ref.24]

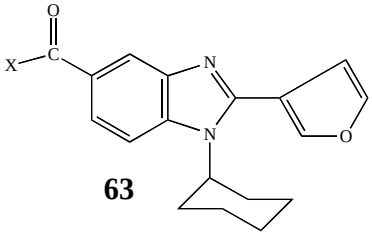
*Fig. 3*

In addition, we tested the effect of the isosteric replacement, at position 2, of unsubstituted and substituted phenyl rings with different aromatic rings, such as furan and pyridine, which are present in the potent HCV RdRp inhibitors **62-63** (*Fig. 4*) described by other Authors.[80,81]



**62**

X= OH HCV NS5B polymerase $IC_{50}$= 4.3 μM
[Ref.25]



**63**

X= OH HCV NS5B polymerase $IC_{50}$= 1.6 μM
[Ref.26]

*Fig. 4*

## Biology

5-acetyl-2-arylbenzimidazole derivatives were tested in cell-based assays for cytotoxicity and antiviral activity against representative members of a number of virus families (Tables 1 and 2). In addition to the *Flaviviridae* BVDV and YFV, among $ssRNA^+$ viruses we tested two picornaviruses, (human enterovirus B (coxsackie virus type B5, CVB-5,) and human enterovirus C (polio virus type-1, Sb-1). Among double-stranded RNA (dsRNA) viruses, we tested reovirus type-1 (Reo-1). Among $ssRNA^-$ viruses we tested a paramyxovirus (human respiratory syncytial virus, RSV) and a rhabdovirus (vesicular stomatitis virus, VSV). Two representatives of DNA viruses were also included: human herpesvirus 1 (herpes symplex type-1, HSV-1) and vaccinia virus (VV). 2'-C-methyl-guanosine, 2'-C-methyl-cytidine, 6-aza-uridine, mycophenolic acid and acycloguanosine were used as reference inhibitors. Cytotoxicity was evaluated in parallel with the antiviral activity.

As far as the activity against *Flaviviridae* is concerned, nine compounds (**26**-**28**, **30**, **31**, **53**, **56**, **57** and **60**) exhibited selective activity against BVDV in the low micromolar range ($EC_{50}$ = 0.8-8.0 μM). In particular, derivatives **28** and **31** showed the most potent anti-BVDV activity ($EC_{50}$ = 0.83 ± 0.09 and 0.80 ± 0.06 μM, respectively; see dose-response curve in *Fig. 5A*) and, like compounds **26**, **57** and **60**, were non cytotoxic at concentrations up to 100 μM.

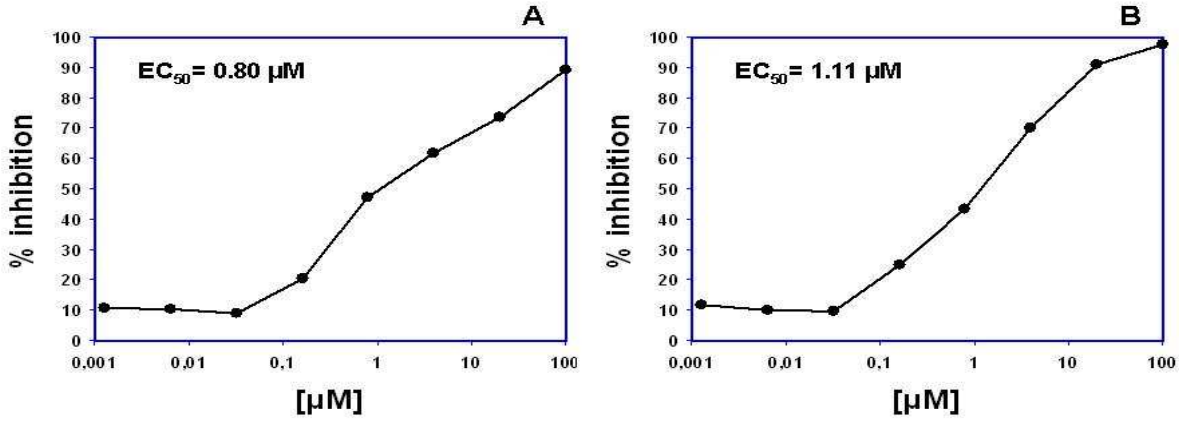



*Fig. 5. Dose-response curves of compd **31** from cell-based assays against BVDV (A) and HCV (B).*

Other compounds (**11**, **20**, **36**-**38**, **42** and **61**) exhibited activity against BVDV at higher μM concentrations ($EC_{50}$ = 16-23 μM). Notewhorthy, compound **38** exhibited a selective, although not very potent, activity also against YFV ($CC_{50}$ >100 μM; $EC_{50}$ = 13.4 ± 1.1 μM), whereas compound **35** showed activity against YFV ($CC_{50}$ >100 μM; $EC_{50}$ = 13.2 ± 1.5 μM), but not against BVDV.

When tested against representatives of other virus families (Table 1 and 2), 5-acetyl-2-arylbenzimidazoles resulted mainly inactive. Exceptions were: compound **20** and **35** which, besides BVDV, also inhibited CVB-5 ($CC_{50}$ >100 μM; $EC_{50}$ = 43 μM) and RSV ($CC_{50}$ >100 μM; $EC_{50}$ = 25 μM), respectively; and compound **43**, active only against CVB-5 ($CC_{50}$ >100 μM; $EC_{50}$ = 47 μM).

SAR studies allow to conclude that derivatisation of the 5-acetyl group led to a significant improvement in the anti-BVDV activity (compounds **7**-**11**); transformation into ketoximes allowed to achieve higher anti-BVDV potency (**20**); the conversion into semicarbazones also allowed to achieve an increase in potency (**26**); finally, the replacement of a semicarbazide with a thiosemicarbazide to obtain the thiosemicarbazones allowed to achieve fairly low $EC_{50}$ values (**28** and **31**). On the contrary, no significant extension of the antiviral spectrum was obtained, the only exception being compounds **20**, **35** and **37**.

Introduction of a cyclohexyl substituent at position 1 (derivatives **35**-**61**) failed to prove useful in improving the anti-BVDV potency. However, compounds **35**-**38**, **42**, **48**, **53**, **57**, **59**-**61** resulted active against this virus, the most potent being, again, the thiosemicarbazones (**53**, **57**, **60**, **61**). Moreover, susbstitution with cyclohexyl (derivatives **35**-**41**) allowed the appearance of antiviral activities against different viruses: YFV and RSV, in the case of compounds **35** and **37**, and YFV in the case of compound **38**.

Structure-activity relationship studies indicate that the acetyl group as such, or its corresponding hydroxyethyl derivatives, are unable to promote antiviral activity in the series of N-1-unsubstituted benzimidazoles. Only when the phenyl bears a 2,4-dimethoxy group at position 2 (as in the above cited compound **11**), the improvement in anti-BVDV activity resulted higher than that obtained previously.[21] As a cycloexyl substitution at position 1 of the benzimidazole takes place, some compounds (**35**-**38**, **42**, **48**, **53**, **57**, **59**-**61**) exhibit anti-BVDV activity, the most active being those bearing the most lipophilic susbstituents in the phenyl ring (2,4-$OCH_3$ = Cl > $OCH_3$). Bioisosteric replacement of the phenyl with a furanyl or pyridinyl ring was partially successful in the case of **59** ($EC_{50}$ = 26 μM) and fairly good in the case of compound **60** ($EC_{50}$ = 8 μM).

Due to its interesting activity against BVDV, compound **31** was also tested against HCV in a subgenomic replication assay that allows viral replication in a human hepatoma cell line (GS4.1).

In this assay, **31** selectively inhibits the HCV replication with an $EC_{50}$ of 1.11 ± 0.15 µM (Table 3 and dose-response curve in *Fig. 5B*).

**Table 3.** Comparative activity of compd **31** against BVDV and HCV in cell-based assays.

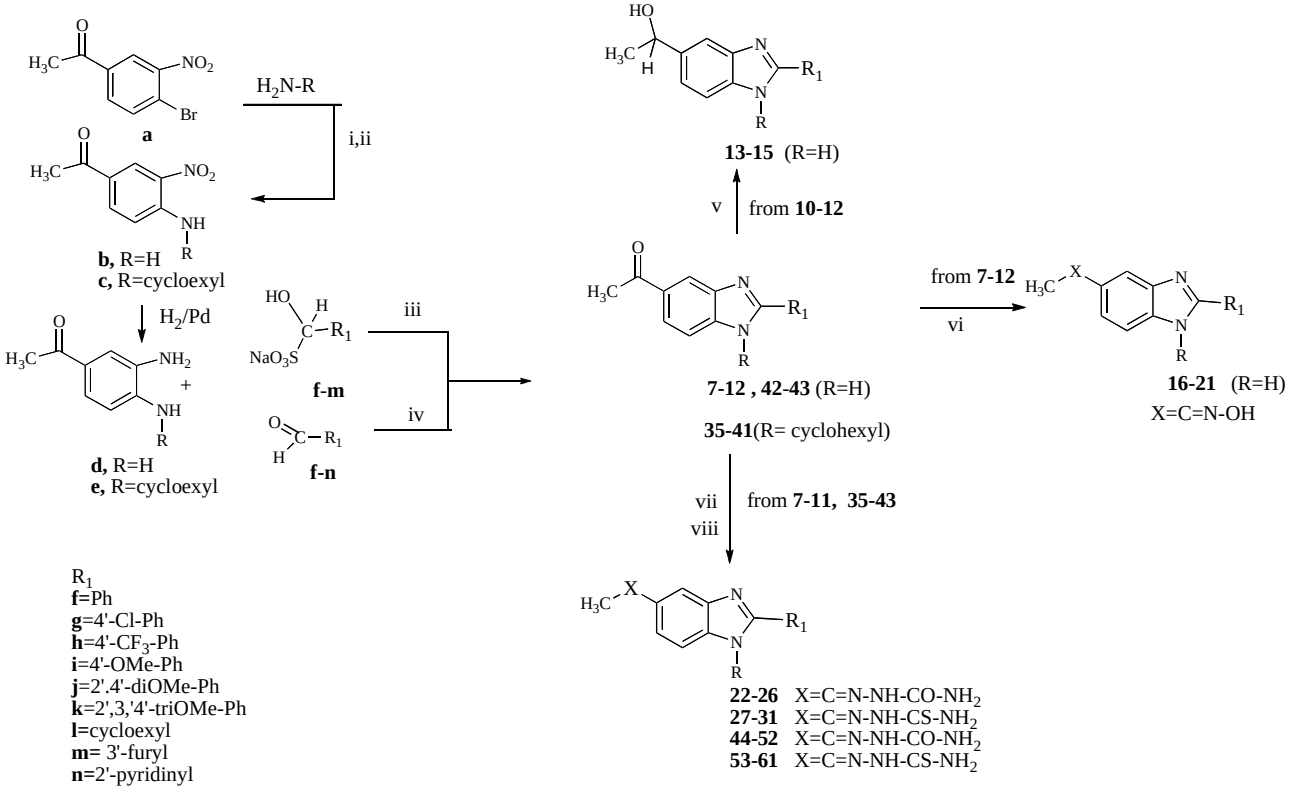
| | [a]Cell-based | | | |
|---|---|---|---|---|
| **Compd** | **BVDV** | | **HCV-1b** | |
| | [b]$CC_{50}$ | [c]$EC_{50}$ | [d]$CC_{50}$ | [e]$EC_{50}$ |
| **31** | >100 | 0.80 ± 0.06 | 11.3 ± 1.5 | 1.11 ± 0.15 |

[a]Data represent mean values for three independent determinations.
[b]Compound concentration (µM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method.
[c]Compound concentration (µM) required to achieve 50% protection of MDBK cells from BVDV-induced cytopathogenicity, as determined by the MTT method.
[d]Compound concentration (µM) required to reduce the viability of GS4.1 cells by 50%, as described in the Experimental section.
[e]Compound concentration (µM) required to achieve 50% protection of GS4.1 cells from cytopathogenicity, as described in the Experimental section.

## Chemistry

The preparation of compounds **7-31** and **35-61** was achieved according to the sequence of reactions of Scheme 1. From the commercially available bromoacetofenone (a) (Jansen), we prepared the necessary intermediates (b,c) following a known procedure for (b)[58], whereas (c) was obtained for the first time in DMSO at 70°C for 3h. The diamine (d) was previously described by W. Borsche and J. Barthenheier,[82] while (e) is a new compound. Ring closure to benzimidazoles was accomplished in two different ways. As the N-unsubstitued diamine (d) was used, condensation with the bisulfite salts of the aldheydes f-m yielded the desired compounds **7-12**, **42**, **43**. In the case of the N-cyclohexyl substituted diamine (e), we had to carry on the reactions with the aldehydes f-n in DMF/water (30:1), at room temperature for 1h, in the presence of oxone to obtain compounds **35-41**. In both cases yields were very satisfactory (ranging from 58-98%). The intermediates **7**, **8**, **10-12,** have been described previously by some of us (G.P., M.L), while compounds **9**, **42**, **43** are described here for the first time. Compounds **13-15** were obtained on reduction by means of $NaBH_4$ in aqueous ethanol of the parent **10-12**. The ketoximes **16-21** were obtained from the ketones **7-12** by reaction with hydroxylamine. The acetylderivatives **7-12** and **35-43** were alternatively converted into the corresponding semicarbazones **22-26** and **44-52**, respectively, and thiosemicarbazones **27-31** and **53-61**, respectively, using in turn semicarbazide hydrochloride and thiosemicarbazide in refluxing ethanol and in the presence of sodium acetate or glacial acetic acid, respectively.

The elucidation of all novel compounds was supported by elemental analyses and $^{1}$H-NMR spectra, that are fully consistent with the described structures. In addition, for certain compounds (**14**, **17**, **22**, **24**, **25**, **29**, **39**, **48**, **50**, **53**, **55**, **61**) $^{13}$C-NMR spectra were recorded to further ascertain the localisation of the carbon atoms external to the hetero-rings. Examination of these $^{13}$C-NMR spectra (vide infra) allowed us to confirm that a few variations are concerned with the presence of substituents in the phenyl moiety at position 2 of the benzimidazole ring, whereas the chemical shifts of C-8, C-9, and of C=S and C=O in side chains, were almost coincident.




Scheme1- i, in ethanol in sealed tube at 100°C; ii, in DMSO at 70°C for 3 h; iii, in refluxing ethanol for 4-5h; iv, in DMF at r.t for 1h in the presence of oxone; v, $NaBH_4$ in ethanol/water; vi, $NH_2OH$ in ethanol ; vii, semicarbazidehydrochloride in ethanol and in the presence of sodium acetate; viii, thiosemicarbazide in ethanol , water and acetic acid.

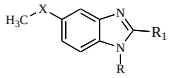


**Table 1**. Cytotoxicity and antiviral activity of **2-phenylbenzimidazoles**, and reference compds, against representatives of ssRNA$^+$ (BVDV, YFV, CBV-5, Sb-1), ssRNA$^-$ (RSV, VSV), dsRNA (Reo-1) and dsDNA (VV, HSV-1) viruses°.

| Compd | X | R | R' | MDBK $CC_{50}$[a] | BVDV $EC_{50}$[b] | BHK $CC_{50}$[c] | YFV $EC_{50}$[d] | Reo-1 $EC_{50}$[e] | Vero-76 $CC_{50}$[f] | CVB-2 $EC_{50}$[g] | Sb-1 $EC_{50}$[h] | RSV $EC_{50}$[i] | VSV $EC_{50}$[j] | VV $EC_{50}$[k] | HSV-1 $EC_{50}$[l] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **7** | C=O | H | Ph | >100 | 100 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **8** | C=O | H | Ph(4'-Cl) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **9** | C=O | H | Ph(4'- $CF_3$) | >100 | 15 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **10** | C=O | H | Ph(4'-$OCH_3$) | 58 | >58 | 40 | >40 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **11** | C=O | H | Ph(2',4'-$OCH_3$) | 73 | 21 (3) | 50 | >50 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **12** | C=O | H | Ph(2',3',4'-$OCH_3$) | >100 | >100 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **13** | CH-OH | H | Ph(4'-$OCH_3$) | >100 | >100 | >100 | >100 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **14** | CH-OH | H | Ph(2',4'-$OCH_3$) | >100 | >100 | >100 | >100 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **15** | CH-OH | H | Ph(2',3',4'-$OCH_3$) | 60 | >60 | 60 | >60 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **16** | CN-OH | H | Ph | >100 | >100 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **17** | CN-OH | H | Ph(4'-Cl) | 37 | 18 | 33 | >33 | ND | ND | ND | ND | ND | ND | ND | ND |
| **18** | CN-OH | H | Ph(4'- $CF_3$) | 55 | >55 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **19** | CN-OH | H | Ph(4'-$OCH_3$) | 44 | >44 | 44 | >44 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **20** | CN-OH | H | Ph(2',4'-$OCH_3$) | >100 | 17 (5) | >100 | >100 | >100 | >100 | 43 (2) | >100 | >100 | >100 | >100 | >100 |
| **21** | CN-OH | H | Ph(2',3',4'-$OCH_3$) | >100 | >100 | >100 | >100 | ND | >100 | >100 | >100 | ND | ND | >100 | ND |
| **22** | C=N-NH-CO-$NH_2$ | H | Ph | >100 | 50 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **23** | C=N-NH-CO-$NH_2$ | H | Ph(4'-Cl) | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **24** | C=N-NH-CO-$NH_2$ | H | Ph(4'- $CF_3$) | >100 | 45 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **25** | C=N-NH-CO-$NH_2$ | H | Ph(4'-$OCH_3$) | 25 | 11 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **26** | C=N-NH-CO-$NH_2$ | H | Ph(2',4'-$OCH_3$) | >100 | **2.5** (40) | $\geq$100 | >100 | >100 | 80 | >80 | >80 | ND | >80 | 80 | ND |
| **27** | C=N-NH-CS-$NH_2$ | H | Ph | 32 | **3.8** (8) | 32 | >32 | ND | >100 | >100 | >100 | ND | >100 | >100 | ND |
| **28** | C=N-NH-CS-$NH_2$ | H | Ph(4'-Cl) | >100 | **0.8** (125) | >100 | >100 | ND | >100 | >100 | >100 | ND | >100 | >100 | ND |
| **29** | C=N-NH-CS-$NH_2$ | H | Ph(4'- $CF_3$) | 5 | >5 | 5 | >5 | ND | 22 | >22 | >22 | ND | ND | ND | ND |
| **30** | C=N-NH-CS-$NH_2$ | H | Ph(4'-$OCH_3$) | 13 | **1.8** (7) | 13 | >13 | >13 | >100 | >100 | >100 | ND | >100 | >100 | ND |
| **31** | C=N-NH-CS-$NH_2$ | H | Ph(2',4'-$OCH_3$) | >100 | **0.8** (125) | 28 | >28 | >28 | >100 | >100 | >100 | >100 | >100 | ND | >100 |
| | *Reference Compds | | | | | | | | | | | | | | |
| | 2'-C-methyl-guanosine | | | >100 | **2.0**±0.1 (>50) | >100 | **1.8**±0.1 (>56) | **0.7**±0.2 (143) | >100 | **55**±2 | **50**±0.5 | >100 | >100 | >100 | >100 |
| | 2'-C-methyl-cytidine | | | >100 | **3.2**±1.0 (>31) | >100 | >100 | **17**±1.0 (>6) | >100 | **18**±1.5 (>6) | **8.7**±1.4 (>11) | >100 | >100 | >100 | >100 |
| | 6-Aza-uridine | | | >100 | >100 | >100 | >100 | >100 | 12.5 | >12.5 | >12.5 | **1.4**±0.2 (9) | >12.5 | >12.5 | >12.5 |
| | Mycophenolic acid | | | 2.8 | >2.8 | >100 | >100 | **20** | 13 | >13 | >13 | >13 | >13 | **1.7**±0.1 (8) | >13 |
| | Acycloguanosine | | | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | **3.0**±0.1 (>33) |

° Data represent mean values ± SD for three independent determinations. For values where SD is not shown, variation among duplicate samples was less than 15%.
[a] Compound concentration (µM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method.
[b] Compound concentration (µM) required to achieve 50% protection of MDBK cells from BVDV-induced cytopathogenicity, as determined by the MTT method.
[c] Compound concentration (µM) required to reduce the viability of mock-infected BHK cells by 50%, as determined by the MTT method.
[d] Compound concentration (µM) required to achieve 50% protection of BHK cells from YFV-induced cytopathogenicity, as determined by the MTT method.

[e] Compound concentration (µM) required to achieve 50% protection of BHK cells from Reo-1-induced cytopathogenicity, as determined by the MTT method.
[f] Compound concentration (µM) required to reduce the viability of mock-infected Vero-76 cells by 50%. as determined by the MTT method.
[g] Compound concentration (µM) required to reduce the plaque number of CVB-2 by 50% in Vero-76 monolayers.
[h] Compound concentration (µM) required to reduce the plaque number of Sb-1 by 50% in Vero-76 monolayers.
[i] Compound concentration (µM) required to reduce the plaque number of RSV by 50% in Vero-76 monolayers.
[j] Compound concentration (µM) required to reduce the plaque number of VSV by 50% in Vero-76 monolayers.
[k] Compound concentration (µM) required to reduce the plaque number of VV by 50% in Vero-76 monolayers.
[l] Compound concentration (µM) required to reduce the plaque number of HSV-1 by 50% in Vero-76 monolayers.
( ) Selectivity Index: $CC_{50}/EC_{50}$
ND= not determined

**Table 2**. Cytotoxicity and antiviral activity of **2-phenylbenzimidazoles**, and reference compds, against representatives of $ssRNA^+$ (BVDV, YFV, CBV-5, Sb-1), $ssRNA^-$ (RSV, VSV), dsRNA (Reo-1) and dsDNA (VV, HSV-1) viruses°.

| Compd | X | R | R' | MDBK $CC_{50}$[a] | BVDV $EC_{50}$[b] | BHK $CC_{50}$[c] | YFV $EC_{50}$[d] | Reo-1 $EC_{50}$[e] | Vero-76 $CC_{50}$[f] | CVB-2 $EC_{50}$[g] | Sb-1 $EC_{50}$[h] | RSV $EC_{50}$[i] | VSV $EC_{50}$[j] | VV $EC_{50}$[k] | HSV-1 $EC_{50}$[l] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **35** | C=O | c-hexyl | Ph | >100 | 60 | >100 | 13 | >100 | 80 | >80 | >80 | 25 | ND | >80 | >80 |
| **36** | C=O | c-hexyl | Ph(4'-Cl) | >100 | 23 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | ND | >100 | >100 |
| **37** | C=O | c-hexyl | Ph(4'-$CF_3$) | 52 | 23 | 33 | >33 | >33 | 35 | >35 | >35 | 20 | ND | >35 | >35 |
| **38** | C=O | c-hexyl | Ph(4'-$OCH_3$) | >100 | 21 | >100 | 13 | >100 | 30 | >30 | >30 | >30 | >30 | >30 | >30 |
| **39** | C=O | c-hexyl | Ph(2',4'-$OCH_3$) | 21 | >21 | 8.5 | >8.5 | >8.5 | 70 | >70 | >70 | >70 | >70 | >70 | >70 |
| **40** | C=O | c-hexyl | 3'-Furyl | >100 | >100 | 77 | >77 | ND | 80 | >80 | ND | ND | ND | >80 | >80 |
| **41** | C=O | c-hexyl | 2'-Pyridinyl | >100 | >100 | ≥100 | >100 | ND | 90 | >90 | ND | >90 | ND | >90 | >90 |
| **42** | C=O | H | 3'-Furyl | ≥100 | 22 | 98 | >98 | ND | >100 | >100 | ND | >100 | ND | >100 | >100 |
| **43** | C=O | H | c-hexyl | >100 | >100 | >100 | >100 | >100 | >100 | 47 | >100 | >100 | ND | >100 | >100 |
| **44** | C=N-NH-CO-$NH_2$ | c-hexyl | Ph | >100 | >100 | >100 | >100 | >100 | ND | ND | ND | ND | ND | ND | ND |
| **45** | C=N-NH-CO-$NH_2$ | c-hexyl | Ph(4'-Cl) | >100 | >100 | >100 | >100 | >100 | ND | ND | ND | ND | ND | ND | ND |
| **46** | C=N-NH-CO-$NH_2$ | c-hexyl | Ph(4'-$CF_3$) | >100 | >100 | >100 | >100 | >100 | ND | ND | ND | ND | ND | ND | ND |
| **47** | C=N-NH-CO-$NH_2$ | c-hexyl | Ph(4'-$OCH_3$) | 54 | >54 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **48** | C=N-NH-CO-$NH_2$ | c-hexyl | Ph(2',4'-$OCH_3$) | 36 | 22 | 19 | >19 | ND | 20 | >20 | >20 | ND | ND | >20 | >20 |
| **49** | C=N-NH-CO-$NH_2$ | c-hexyl | 3'-Furyl | >100 | 100 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **50** | C=N-NH-CO-$NH_2$ | c-hexyl | 2'-Pyridinyl | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **51** | C=N-NH-CO-$NH_2$ | H | 3'-Furyl | >100 | 60 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **52** | C=N-NH-CO-$NH_2$ | H | c-hexyl | >100 | 44 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **53** | C=N-NH-CS-$NH_2$ | c-hexyl | Ph | 24 | **4.5** | 38 | 9 | >38 | 20 | >20 | >20 | >20 | >20 | >20 | >20 |
| **54** | C=N-NH-CS-$NH_2$ | c-hexyl | Ph(4'-Cl) | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **55** | C=N-NH-CS-$NH_2$ | c-hexyl | Ph(4'-$CF_3$) | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **56** | C=N-NH-CS-$NH_2$ | c-hexyl | Ph(4'-$OCH_3$) | 32 | 7 | ND | ND | ND | 40 | >40 | >40 | >40 | ND | >40 | >40 |
| **57** | C=N-NH-CS-$NH_2$ | c-hexyl | Ph(2',4'-$OCH_3$) | >100 | **4 (24)** | 17 | >17 | >17 | 20 | >20 | >20 | >20 | >20 | >20 | >20 |
| **58** | C=N-NH-CS-$NH_2$ | c-hexyl | 3'-Furyl | >100 | >100 | >100 | >100 | ND | 25 | >25 | >25 | ND | >25 | >25 | >25 |
| **59** | C=N-NH-CS-$NH_2$ | c-hexyl | 2'-Pyridinyl | 41 | 26 | 24 | >24 | ND | 20 | >20 | >20 | ND | >20 | >20 | >20 |
| **60** | C=N-NH-CS-$NH_2$ | H | 3'-Furyl | >100 | **8 (12)** | >100 | >100 | >100 | 100 | >100 | 100 | >100 | >100 | >100 | >100 |
| **61** | C=N-NH-CS-$NH_2$ | H | c-hexyl | >100 | 16 | 24 | >24 | ND | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| | Reference Compds | | | | | | | | | | | | | | |
| | 2'-C-methyl-guanosine | | | >100 | **2.0**±0.1 (>50) | >100 | **1.8**±0.1 (>56) | **0.7**±0.2 (143) | >100 | **55**±2 | **50**±0.5 | >100 | >100 | >100 | >100 |
| | 2'-C-methyl-cytidine | | | >100 | **3.2**±1.0 (>31) | >100 | >100 | **17**±1.0 (>6) | >100 | **18**±1.5 (>6) | **8.7**±1.4 (>11) | >100 | >100 | >100 | >100 |
| | 6-Aza-uridine | | | >100 | >100 | >100 | >100 | >100 | 12.5 | >12.5 | >12.5 | **1.4**±0.2 (9) | >12.5 | >12.5 | >12.5 |
| | Mycophenolic acid | | | 2.8 | >2.8 | >100 | >100 | **20** | 13 | >13 | >13 | >13 | >13 | **1.7**±0.1 (8) | >13 |
| | Acycloguanosine | | | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | **3.0**±0.1 (>33) |

**Legend as in Table 1**

# 5.0 1-substituted 2-[(benzotriazol-1/2 yl)methyl]benzimidazoles as potent and selective inhibitors of RSV

## Premises

Recently, Yu et al. reported that 1-[(dialkylamino)alkyl]-2-[(benzotriazol-1/2-yl)methyl] benzimidazoles are endowed with potent anti-RSV activity.[83,84] Interestingly, replacement of the benzotriazole element with a benzimidazol-2-one or a 6/7-azabenzimidazol-2-one, and the introduction of substituents on both the benzimidazole and benzimidazolone N-atoms, led to even more potent anti-RSV agents.[85,86] (Fig. 1).

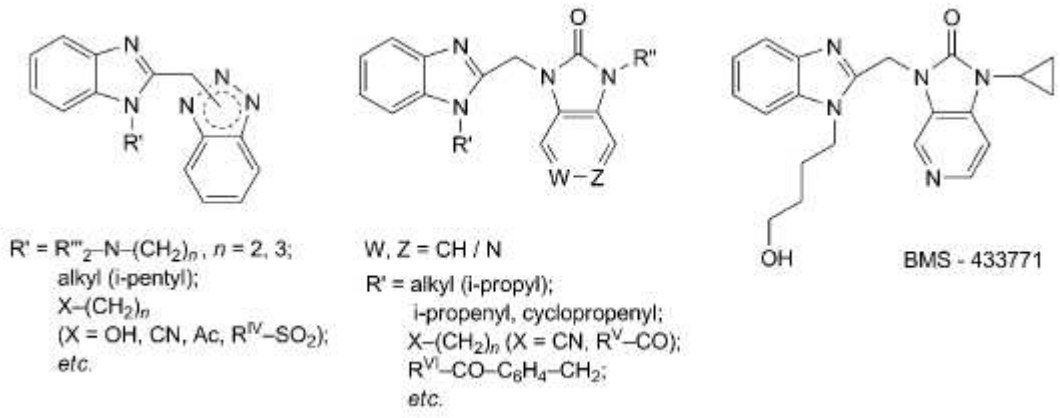



*Fig. 1. Known benzimidazole derivatives with anti-RSV activity.*

Indeed, the (dimethylamino)ethyl chain, which characterized first-generation compounds, conferred excellent antiviral properties; however, it was also associated with metabolic lability.[87] The introduction on said N-atoms of 4-hydroxybutyl and cyclopropyl substituents, respectively, produced a compound (BMS-433771) endowed with anti-RSV activity in animals following oral administration.

The 1-[(dialkylamino)alkyl]-2-[(benzotriazol-1/2-yl)methyl]benzimidazoles, evaluated for antiviral activity by Yu et al., had been prepared by Prof. Boido and his group as analgesics, as well as for other pharmacological purposes.[88,89] Therefore, following the report from Bristol researchers, the antiviral activity of related 1-substituted 2-[(benzotriazol-1/2-yl)methyl]benzimidazoles, prepared in the past but not examined by Yu et al., has been evaluated in our lab. In particular, some 5-substituted derivatives, as well as compounds bearing at position 1 the peculiar and cumbersome (quinolizidinyl)alkyl residues, have been the object of our previous study.

The 1-substituents consisted of either simple (dialkylamino)alkyl chains or, as was the case for 18 derivatives, lupinyl, epilupinyl, and homolupinyl residues characterized by the presence of the bulky, strongly basic and lipophilic quinolizidine (octahydro-2H-quinolizine) nucleus (*Fig. 2*).

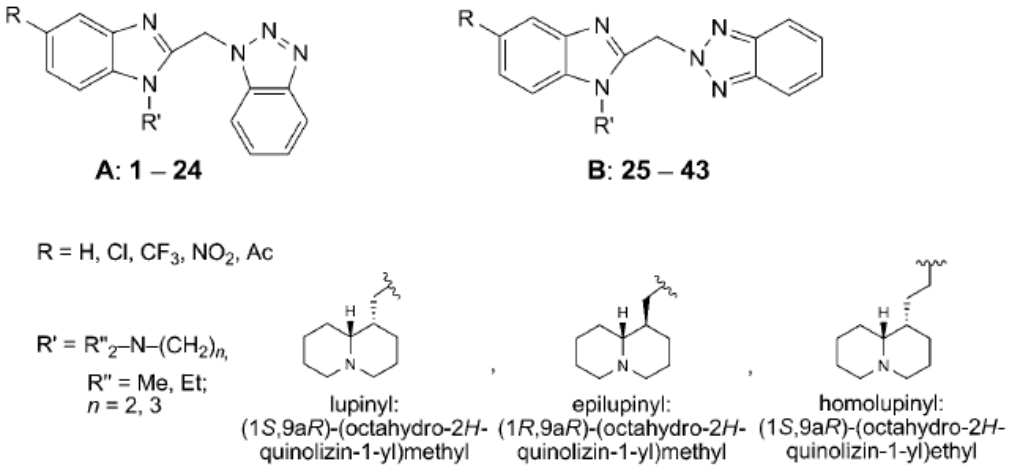



*Fig. 2. 1-substituted 2-[(benzotriazol-1/2 yl)methyl]benzimidazoles.*[90]

A total of fortythree 1-substituted 2-[(benzotriazol-1/2-yl)methyl]benzimidazoles have been tested for cytotoxicity and antiviral activity against a panel of RNA and DNA viruses and, among them, thirtynine compounds exhibited potent activity against RSV, in many cases with $EC_{50}$ values below 50 nm.[90] Briefly, SAR studies suggested that:

- the presence of substituents at position 5 of the benzimidazole ring leads to an increase in cytotoxicity;
- the most potent antiviral activity correlates with Cl in R and either $EtNMe_2$ or $PrNMe_2$ in R';
- replacement of Cl in R with H, $NO_2$, $CF_3$ or $COCH_3$ leads to lower potency;
- replacement in R' of $EtNMe_2$ with $EtNEt_2$ or lupinyl leads to a progressive increase of potency;
- homolupinyl derivates are the most potent, regardless of whether R is H or Cl.

## Biology

Due to the fact that the presence of substituents at position 5 of the benzimidazole ring led to an increase in cytotoxicity, especially in (quinolizidinyl)alkyl derivatives, two new analogues (ASCC

459 and ASCC 460) bearing a methyl group at this position (R') were synthesized and evaluated in cell-based assays for cytotoxicity and anti-RSV activity (Table 1). Like their counterparts (P-149-1 and FP-FS-8) carrying a Cl at the R' position, ASCC 459 and ASCC 460 resulted potent RSV inhibitors ($EC_{50}$ = 0.030 μM). Noteworthy, the presence of a methyl group at position 5 of the benzimidazole ring led to a two-fold decrease of the cytotoxicity for MT-4 cells of the (quinolizidinyl)alkyl derivative (compare ASCC 460 with FP-FS-8).

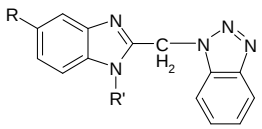



**Table 1**. Cytotoxicity for MT-4 and Vero-76 cell lines and anti-RSV activity of new benzimidazole derivatives.

| Compd achronim | R | R' | MT-4 [a]$CC_{50}$ | Vero76 [b]$CC_{50}$ | RSV [c]$EC_{50}$ |
|---|---|---|---|---|---|
| ASCC 459 | $CH_3$ | $CH_2CH_2N(CH_3)_2$ | >100 | >100 | **0.030 ± 0.002** |
| ASCC 460 | $CH_3$ | $CH_2CH_2$-Lupinyl | 41 | >100 | **0.030 ± 0.003** |
| P-149-1 | Cl | $CH_2CH_2N(CH_3)_2$ | ≥100 | >100 | **0.030 ± 0.001** |
| FP-FS-8 | Cl | $CH_2CH_2$-Lupinyl | 16 | 45 | **0.020 ± 0.003** |
| Reference compd | | | | | |
| 6-aza-uridine | | | 0.3 | ≥12.5 | **1.4 ± 0.2** |

[a]) Compound concentration [μM] required to reduce the viability of MT-4 cells by 50%, as determined by the MTT method.
[b]) Compound concentration [μM] required to reduce the viability of Vero-76 cells by 50%, as determined by the MTT method.
[c]) Compound concentration ± SD [μM] required to reduce the plaque number of RSV by 50% in Vero-76 monolayers.

In order to define the spectrum of the their antiviral activity, ASCC 459 and ASCC 460 were tested against representatives of $ssRNA^+$ (BVDV, YFV, CBV-5, Polio-1), $ssRNA^-$ (VSV), dsRNA (Reo-1) and dsDNA (VV, HSV-1) viruses.

As shown in Table 2, ASCC 459 and ASCC 460 did not exhibit cytotoxicity for the other cell lines tested, nor activity against viruses different from RSV. As previously reported,[90] FP-FS-8 exhibited a moderate anti-BVDV and -CVB2 activity, whereas ASCC 460 failed to inhibit the latter two viruses.

## Mode of action studies

To get more insights into the step of the RSV replication cycle inhibited by ASCC 459, a time-dependent drug addition assay was performed. In this kind of experiment, the contemporary infection of all cultured cells, so as to avoid repeated infection cycles, is fundamental. Thus, Vero cells were infected for 1 h at 20° C with RSV, using a multiplicity of infection (m.o.i.) of 5 PFU/cell. The unadsorbed virus was removed by extensive washing and ASCC 459 (50 μM), Dextran Sulfate

(40 μg/mL), and Ribavirin (80 μM) were added to duplicate cultures at different times after the end of infection, i.e. (immediately, or 2, 4, 6, 8, 10, 12, 14, 16 hrs thereafter). Virus titres (PFU/mL) were determined at the end of a 3 day incubation at 37°C. As reference inhibitors we used Ribavirin, which blocks the viral RNA synthesis, and Dextran Sulphate (DS), which is an inhibitor of RSV adsorption, as well as of the subsequent fusion of the viral envelope with the cell membrane.[91]

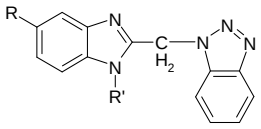



**Table 2.** Cytotoxicity and antiviral activity of 1-substituted 2-[(benzotriazol-1/2 yl) methyl] benzimidazoles derivatives against representatives of ssRNA$^+$ (BVDV, YFV, CBV-5, Sb-1), ssRNA$^-$ (VSV), dsRNA (Reo-1) and dsDNA (VV, HSV-1) viruses°.

| Compd achronim | R | R' | MDBK $CC_{50}$[a] | BVDV $EC_{50}$[b] | BHK $CC_{50}$[c] | YFV $EC_{50}$[d] | Reo-1 $EC_{50}$[e] | Vero-76 $CC_{50}$[f] | CVB2 $EC_{50}$[g] | Sb-1 $EC_{50}$[h] | VSV $EC_{50}$[i] | VV $EC_{50}$[j] | HSV-1 $EC_{50}$[k] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ASCC 459 | $CH_3$ | $CH_2CH_2N(CH_3)_2$ | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| ASCC 460 | $CH_3$ | $CH_2CH_2$-Lupinyl | >100 | >100 | 55 | >55 | >55 | >100 | >100 | >100 | >100 | >100 | >100 |
| P-149-1 | Cl | $CH_2CH_2N(CH_3)_2$ | ND | ND | ND | ND | ND | >100 | ND | ND | ND | ND | ND |
| FP-FS-8 | Cl | $CH_2CH_2$-Lupinyl | 55 | 14 | 20 | >20 | ND | 45 | 26 | ND | ND | ND | ND |
| Reference Compds | | | | | | | | | | | | | |
| 2'-C-methyl-guanosine | | | >100 | **2.0**±0.1 (>50) | >100 | **1.8**±0.1 (>56) | **0.7**±0.2 (143) | >100 | **55**±2 | **50**±0.5 | >100 | >100 | >100 |
| 2'-C-methyl-cytidine | | | >100 | **3.2**±1.0 (>31) | >100 | >100 | **17**±1.0 (>6) | >100 | **18**±1.5 (>6) | **8.7**±1.4 (>11) | >100 | >100 | >100 |
| 6-Aza-uridine | | | >100 | >100 | >100 | >100 | >100 | 12.5 | >12.5 | >12.5 | >12.5 | >12.5 | >12.5 |
| Mycophenolic acid | | | 2.8 | >2.8 | >100 | >100 | **20** | 13 | >13 | >13 | >13 | **1.7**±0.1 (8) | >13 |
| Acycloguanosine | | | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | **3.0**±0.1 (>33) |

° Data represent mean values ± SD for three independent determinations. For values where SD is not shown, variation among duplicate samples was less than 15%.
[a] Compound concentration (μM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method.
[b] Compound concentration (μM) required to achieve 50% protection of MDBK cells from BVDV-induced cytopathogenicity, as determined by the MTT method.
[c] Compound concentration (μM) required to reduce the viability of mock-infected BHK cells by 50%, as determined by the MTT method.
[d] Compound concentration (μM) required to achieve 50% protection of BHK cells from YFV-induced cytopathogenicity, as determined by the MTT method.
[e] Compound concentration (μM) required to achieve 50% protection of BHK cells from Reo-1-induced cytopathogenicity, as determined by the MTT method.
[f] Compound concentration (μM) required to reduce the viability of mock-infected Vero-76 cells by 50%. as determined by the MTT method.
[g] Compound concentration (μM) required to reduce the plaque number of CVB-2 by 50% in Vero-76 monolayers.
[h] Compound concentration (μM) required to reduce the plaque number of Sb-1 by 50% in Vero-76 monolayers.
[i] Compound concentration (μM) required to reduce the plaque number of VSV by 50% in Vero-76 monolayers.
[j] Compound concentration (μM) required to reduce the plaque number of VV by 50% in Vero-76 monolayers.
[k] Compound concentration (μM) required to reduce the plaque number of HSV-1 by 50% in Vero-76 monolayers.
( ) Selectivity Index: $CC_{50}/EC_{50}$
ND = not determined

As shown in Fig. 3, ASCC 459 behaved like Dextran Sulphate, giving rise to a significant inhibition of the virus titre only if added within two hrs after the end of infection. Viceversa, consistently with previous reports.[92] Ribavirin completely inhibited the production of RSV infectious progeny even if added as late as 10 hours after the end of infection.

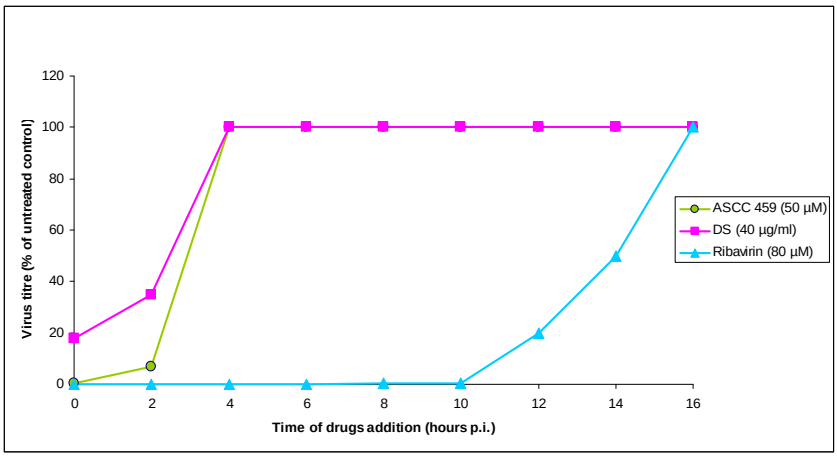



*Fig. 3. Time-dependent drug addition assay. See text for details.*

To assess whether ASCC 459 is targeted at virus entry (a two-step process consisting of adsorption of virus to cell receptors, followed by fusion of its envelope with the cell membrane), an experiment was performed (Table 3) by infecting Vero monolayers with RSV (m.o.i. > 5 PFU/cell) for 2 hrs at 37°C, in the absence or presence of ASCC 459. At the end of infection, cell monolayers were washed three times with cold D-MEM to remove both the unadsorbed virus and the drug, and were incubated for additional 96 hrs either in the absence or in the presence of the same ASCC 459 concentrations used in the 2 hr infection period. It is worth noting that length and temperature of this particular infection procedure allows both adsorption of the virus to cell receptors and subsequent fusion of its envelope with the cell membrane. Therefore, unlike what happened in the experiment shown in Fig. 3, the addition of ASCC 459 at the end of infection did not impair the final virus yield, compared to untreated controls.

Table 3. Different ASCC 459 treatment modes in an RSV acute infection assay at high m.o.i.

| | **Virus Titre (PFU/mL)** | | |
|---|---|---|---|
| **ASCC 459 [µM]** | ***Drug present only by the end of the 2 hr infection period*** | ***Drug present only during the 2 hr infection period*** | ***Drug present during and after the 2 hr infection period*** |
| 10 | $3.6 \times 10^5$ | $5.3 \times 10^5$ | $2.9 \times 10^4$ |
| 1 | $5.1 \times 10^5$ | $3.4 \times 10^5$ | $2.0 \times 10^4$ |
| 0.1 | $3.8 \times 10^5$ | $2.6 \times 10^5$ | $6.7 \times 10^4$ |
| 0.01 | $4.6 \times 10^5$ | $6.7 \times 10^5$ | $3.0 \times 10^5$ |
| No drug | | $3.9 \times 10^5$ | |

As also shown in Table 3, when ASCC 459 was removed after the 2 hr infection period, full RSV multiplication took place, no matter how high was the drug concentration used during the infection period. Viceversa, when the treatment was prolonged after the infection period, ASCC 459 was able to reduce significantly the virus titre even in the presence of drug concentrations as low as 0.1 µM. On the one hand, these results suggest that ASCC 459 does not inhibit the virus adsorption step; in fact, if it had, RSV would have been removed during washing of the inoculum, thus preventing infection of the monolayer. On the other hand, a fusion inhibitor would permit virus adsorption, thereby allowing the infection to proceed upon removal of the drug.

A characteristic of the RSV infection *in vitro* is that infected cells can fuse with adjacent uninfected cells to form giant syncytia. Also this process is mediated by the envelope F protein that mediates entry of the virus into the cell by fusion of the viral envelope with the cell membrane. Therefore, if ASCC 459 were a fusion inhibitor, it could also be expected to prevent syncytia formation in a co-culture consisting of a mix of RSV-infected and uninfected cells. Therefore, 1 x $10^4$ Vero cells infected with RSV (at a m.o.i. = 0.1 PFU/cell) were cocultured with 1x $10^5$ uninfected Vero cells in the absence or in the presence of various concentrations of ASCC 459 or Dextran Sulfate. The number of syncytia was determined with a light microscope when the untreated control co-cultures showed 90% CPE (day 3).

As shown in Fig. 4, ASCC 459 inhibited syncytia formation whit an $EC_{50}$ of 0.03 µM. Dextran Sulphate, which was used as reference compound, showed an $EC_{50}$ of 3.7 µg/mL. This result further confirms that ASCC 459 is a fusion inhibitor.

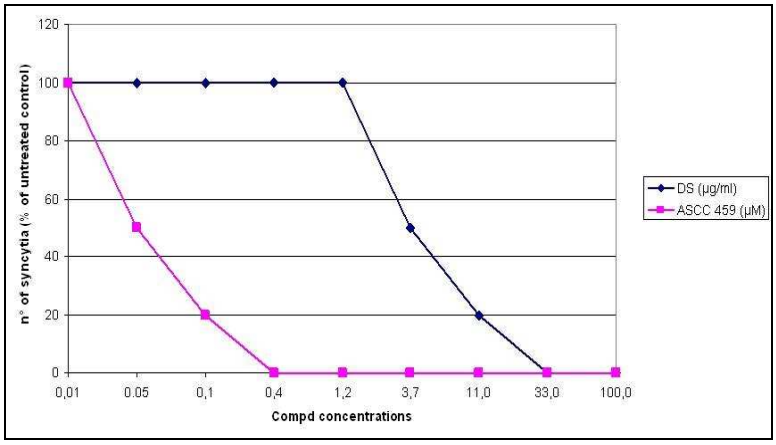



*Fig. 4. Cocultures of RSV-infected and uninfected Vero cells. See text for details.*

## *In vitro* selection and genotype mapping of P-149-1 and ASCC 459-resistant viruses

Selective inhibitors of the virus multiplication targeted at specific virus-coded proteins usually allow the *in vitro* selection of resistant variants carrying, in the genes coding for the targeted proteins, mutations allowing elusion of inhibition.

RSV codes for three envelope proteins [G (attachment), F (fusion), and SH (membrane permeability)] that are involved in virus entry. In order to get more insights into the mode of action of ASCC 459 (and the structurally related P-149-1 congener) and, possibly, to identify their target protein(s), resistant variants were selected in vitro by passaging RSV in Vero cells in the presence of increasing concentrations of ASCC 459 (or P-149-1). The starting drug concentrations were 0.03 μM. After 7 passages, mutants resistant to drug concentrations of 3.84 μM (128 fold the $EC_50$) were obtained. The control RSV wt was grown in parallel in the absence of drugs and the susceptibility of the resistant strain to compound was determined by titration in the presence or in absence of the inhibitor. Resistant viruses grown in the absence or presence of the selecting drugs showed similar titres, which were also in the same magnitude order of that of the RSV wt. Interestingly, the ASCC 459- and the P-149-1-resistant mutants showed cross-resistance to P-149-1 and ASCC459, respectively. For this reason, it was decided to give priority to the sequencing of the P-149-1 resistant mutant.

An aliquot of the latter mutant was subjected to RNA extraction, RT-PCR and genome sequencing in order to identify the mutation pattern responsible for drug resistance.

Purified fragments were added to different specific primers, in order to sequence both strands. Analysis of the received chromatograms (with Chromas software, www.technelysium.com.au), regarding the RSV wild-type strain, allowed us to obtained the entire nucleotide sequence of G, F and SH genes.

## G, F and SH nucleotide sequences of the RSV wt

**G gene [894nt]**

ATGTCCAAAAACAAGGACCAACGCACCGCTAAGACATTAGAAAGGACCTGGGACACT
CTCAATCATTTATTATTCATATCATCGTGCTTATATAAGTTAAATCTTAAATCTGTAGCA
CAAATCACATTATCCATTCTGGCAATGATAATCTCAACTTCACTTATAATTGCAGCCAT
CATATTCATAGCCTCGGCAAACCACAAAGTCACACCAACAACTGCAATCATACAAGAT
GCAACAAGCCAGATCAAGAACACAACCCCAACATACCTCACCCAGAATCCTCAGCTTG
GAATCAGTCCCTCTAATCCGTCTGAAATTACATCACAAATCACCACCATACTAGCTTCA

ACAACACCAGGAGTCAAGTCAACCCTGCAATCCACAACAGTCAAGACCAAAAACACA
ACAACAACTCAAACACAACCCAGCAAGCCCACCACAAAACAACGCCAAAACAAACCA
CCAAGCAAACCCAATAATGATTTTCACTTTGAAGTGTTCAACTTTGTACCCTGCAGCAT
ATGCAGCAACAATCCAACCTGCTGGGCTATCTGCAAAAGAATACCAAACAAAAAACCA
GGAAAGAAAACCACTACCAAGCCCACAAAAAAACCAACCCTCAAGACAACCAAAAAA
GATCCCAAACCTCAAACCACTAAATCAAAGGAAGTACCCACCACCAAGCCCACAGAA
GAGCCAACCATCAACACCACCAAAACAAACATCATAACTACACTACTCACCTCCAACA
CCACAGGAAATCCAGAACTCACAAGTCAAATGGAAACCTTCCACTCAACTTCCTCCGA
AGGCAATCCAAGCCCTTCTCAAGTCTCTACAACATCCGAGTACCCATCACAACCTTCAT
CTCCACCCAACACACCACGCCAG

**F gene [1722nt]**

ATGGAGTTGCTAATCCTCAAAGCAAATGCAATTACCACAATCCTCACTGCAGTCACATT
TTGTTTTGCTTCTGGTCAAAACATCACTGAAGAATTTTATCAATCAACATGCAGTGCAG
TTAGCAAAGGCTATCTTAGTGCTCTGAGAACTGGTTGGTATACCAGTGTTATAACTATA
GAACTAAGTAATATCAAGAAAAATAAGTGTAATGGAACAGATGCCAAGGCAAAATTG
ATAAAACAAGAATTAGATAAATATAAAAATGCTGTAACAGAATTGCAGTTGCTCATGC
AAAGCACACAAGCAACAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAATT
ATACACTCAACAATGCCAAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAAGAA
GATTTCTTGGTTTTTTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCGTTGCTGTATCTA
AGGTCCTGCACCTAGAAGGGGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAAA
CAAGGCTGTAGTCAGCTTATCAAATGGAGTTAGTGTCTTAACCAGCAAAGTGTTAGAC
CTCAAAAACTATATAGATAAACAATTGTTACCTATTGTGAACAAGCAAAGCTGCAGCA
TATCAAATATAGAAACTGTGATAGAGTTCCAACAAAAGAACAACAGACTACTAGAGAT
TACCAGGGAATTTAGTGTTAATGCAGGTGTAACTACACCTGTAAGCACTTACATGTTAA
CTAATAGTGAATTATTGTCATTAATCAATGATATGCCTATAACAAATGATCAGAAAAA
GTTAATGTCCAACAATGTTCAAATAGTTAGACAGCAAAGTTACTCTATCATGTCCATAA
TAAAAGAGGAAGTCTTAGCATATGTAGTACAATTACCACTATATGGTGTTATAGATAC
ACCCTGTTGGAAACTACACACATCCCCTCTATGTACAACCAACACAAAAGAAGGGTCC
AACATCTGTTTAACAAGAACTGACAGAGGATGGTACTGTGACAATGCAGGATCAGTAT
CTTTCTTCCCACAAGCTGAAACATGTAAAGTTCAATCAAATCGAGTATTTTGTGACACA
ATGAACAGTTTAACATTACCAAGTGAAGTAAATCTCTGCAATGTTGACATATTCAACCC
CAAATATGATTGTAAAATTATGACTTCAAAAACAGATGTAAGCAGCTCCGTTATCACA
TCTCTAGGAGCCATTGTGTCATGCTATGGCAAAACTAAATGTACAGCATCCAATAAAA
ATCGTGGAATCATAAAGACATTTTCTAACGGGTGCGATTATGTATCAAATAAAGGGGT

GGACACTGTGTCTGTAGGTAACACATTATATTATGTAAATAAGCAAGAAGGTAAAAGT
CTCTATGTAAAAGGTGAACCAATAATAAATTTCTATGACCCATTAGTATTCCCCTCTGA
TGAATTTGATGCATCAATATCTCAAGTCAACGAGAAGATTAACCAGAGCCTAGCATTT
ATTCGTAAATCCGATGAATTATTACATAATGTAAATGCTGGTAAATCCACCACAAATAT
CATGATAACTACTATAATTATAGTGATTATAGTAATATTGTTATCATTAATTGCTGTTG
GACTGCTCTTATACTGTAAGGCCAGAAGCACACCAGTCACACTAAGCAAAGATCAACT
GAGTGGTATAAATAATATTGCATTTAGTAAC

**SH gene [192nt]**

ATGGAAAATACATCCATAACAATAGAATTCTCAAGCAAATTCTGGCCTTACTTTCACTA
ATACACATGATCACAACAATAATCTCTTTGCTAATCATAATCTCCATCATGATTGCAAT
ACTAAACAAACTTTGTGAATATAACGTATTCCATAACAAAACCTTTGAGTTACCAAGA
GCTCGAGTCAACACA

When compared to the above wt counterparts, the analysis of the **G** nucleotide sequence of the P-149-1-resistant variants showed the substitution of adenine (AAA-codon) with guanine (GAA-codon) in the first nucleotide of the triplet coding for lysine in wt (and glutamic acid in the resistant mutant) at position 212.

An identical mutation was found in the triplet coding for the aa at position 315 of the **F** protein.

The SH nucleotide sequence of the resistant virus revealed no mutations whith respect to wt virus.

The results are summarized in Tab. 5 and Figs. 5 and 6.

Table 4. Mutations in the P-149-1-resistant RSV.

| **Resistant mutant** | **Protein / aminoacid position** | **Triplet aminoacid** |
|---|---|---|
| $RSV^{P\text{-}149\text{-}1}$ | G 212 | AAA→GAA Lys→Glu |
| | F 315 | AAA→GAA Lys→Glu |

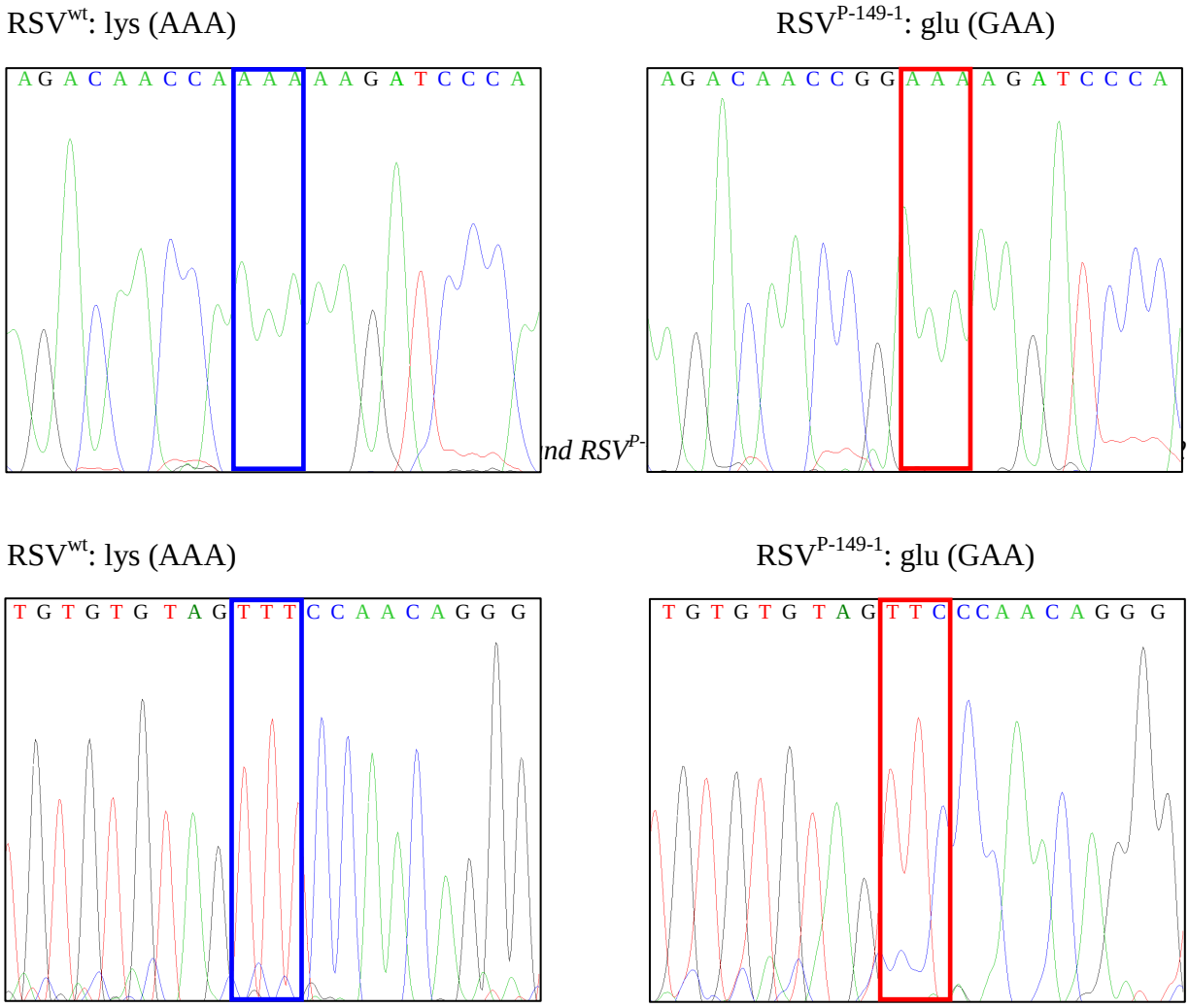



*Fig. 6. Chromatograms of F protein in RSV$^{wt}$ and RSV$^{P-149-1}$ showing the mutated triplet at position 315. In this case, the reverse DNA strands are shown.*

In conclusion, the sequence analysis of RSV$^{P-149-1}$-resistant mutant showed no mutations in the SH gene, but one mutation each in the G and F genes at positions 212 and 315, respectively.

These results suggest that mutations K212E for the G protein and K315E for the F protein correlate with the emergence of mutants resistant to P-149-1. Identical results were obtained with ASCC 459.

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Tonelli, M.; Vettoretti, G.; Tasso B.; Novelli, Federica; Boido, V.; Sparatore, F.; Busonera, B.; Ouhtit, A.; **Farci, P.**; Blois, S.; Giliberti, G.; La Colla, P. Acridine derivatives as anti-BVDV agents: A preliminary study. *Antiviral Research,* **2011**, *91,* 133–141.

Cottiglia, F.; Casu, L.; Leonti, M.; Caboni, P.; Floris, C.; Busonera, B.; **Farci, P.**; Ouhtit, A.; Sanna G. Cytotoxic Phloroglucinols from the Leaves of Myrtus communis. *Journal of natural products,* **2012**, *75,* 225-229.

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# Abstracts of P. Farci

SIV Congress, 11-14 September 2011, Orvieto (Italy):
Sanna, G.; Busonera, B.; Giliberti, G.; **Farci, P.**; Marongiu, M.E.; La Colla, P. Phenyl-benzotriazoles: new potent and selective inhibitors of Respiratory Syncytial and Hanta viruses.

Congress La Chimica della Vita: da alimenti funzionali a principi attivi, 29-30 September 2011, Catanzaro (Italy):
Cottiglia, F.; Casu, L.; Leonti, M.; Caboni, P.; Floris, C.; Busonera, B.; **Farci, P.**; Ouhtit A.; Sanna, G. Bioguided-isolation of cytotoxic phloroglucinols from the leaves of myrtus communis.