Antiviral Research 89 (2011) 204–218

Contents lists available at ScienceDirect

Antiviral Research

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

Review

Picornavirus non-structural proteins as targets for new anti-virals with broad activity

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ARTICLE INFO

Article history:
Received 1 July 2009
Received in revised form 23 August 2010
Accepted 23 December 2010

Keywords:
Picornavirus
Non-structural proteins
Anti-viral compounds
Enterovirus
Polio
Structure

ABSTRACT

Picornaviridae is one of the largest viral families and is composed of 14 genera, six of which include human pathogens. The best known picornaviruses are enteroviruses (including polio, PV, and rhinoviruses), foot-and-mouth disease virus (FMDV), and hepatitis A virus (HAV). Although infections often are mild, certain strains may cause pandemic outbreaks accompanied with meningitis and/or paralysis. Vaccines are available for PV, HAV and FMDV. When the oral vaccines are given to immunocompromised individuals, they may be chronically infected, and remain secretors of vaccine-derived variants of virus for years. There is no effective prophylaxis available for these or other picornaviruses. So far, only the 3C protease from viruses in three genera has been fully characterized as an anti-viral target, whereas the mode of action of compounds targeting other non-structural proteins have remained largely unaddressed. Within the EU-supported FP6 project-VIZIER (Comparative Structural Genomics of Viral Enzymes Involved in Replication), the non-structural proteins were studied to identify conserved binding sites for broadly reactive anti-virals. The putative 2C helicase from echovirus-30 was shown to form ring-shaped hexamers typical for DNA-encoded SF3 helicases, and to possess ATPase activity. Hexamer formation of 2C from enterovirus 76 was *in vitro* shown to be dependent on the 44 N-terminal residues. Crystal structures of three enterovirus 3C proteases were solved and shown to be similar to those of other picornaviruses. A new binding site of VPg to the bottom of the thumb domain of CV-B3 3D polymerase was identified as a potential target. Broad anti-enterovirus compounds against 2C and 3A proteins were also identified, including thiazolobenzimidazoles (active against 2C) and TTP-8307 (targeting 3A). There is a need for more potent inhibitors against PV and other picornaviruses, which are potential silent reservoirs for re-emerging PV-like disease.

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doi:10.1016/j.antiviral.2010.12.007

1. Introduction

The family *Picornaviridae* is one of the largest medically and economically important families of human and animal viral pathogens (Ehrenfeld et al., 2010). The family consists of 285 different picornavirus types that form 29 species classified in eight established and five proposed genera (Stanway et al., 2005; Knowles et al., 2010). Six of these genera include viruses infecting both humans and other animals. Picornaviruses are small, icosahedral positive-sense single-stranded RNA viruses, causing a variety of diseases ranging from benign to fatal (Melnick, 1983). The best known picornavirus pathogens are enteroviruses including poliovirus (PV) and human rhinovirus (HRV), foot-and-mouth disease virus (FMDV), and hepatitis A virus (HAV). There are, however, several other picornaviruses that also cause outbreaks and serious diseases (Zhang et al., 2009; Sapkal et al., 2009; Le Guyader et al., 2008; Dussart et al., 2005).

The number of picornaviruses is steadily growing through the identification of viruses that escaped detection in the past as well as emerging viruses (Greninger et al., 2009; Zoll et al., 2009; Brown et al., 2009; Holtz et al., 2008; McErlean et al., 2008; Kapoor et al., 2008). Vaccines have so far only been produced against PV, HAV and FMDV. For other picornavirus infections, no efficient prophylaxis is currently available, and it may not be realistic to develop and produce vaccines against all major picornavirus pathogens (Xu et al., 2010). There is thus a consensus that the development of potent antiviral compounds should complement vaccines in the effort to control these infections.

The key steps in the viral life cycle, as potential targets for antiviral compounds, are virus adsorption, uncoating, RNA translation, polyprotein cleavage, RNA replication, and particle assembly. The majority of currently available antiviral compounds against picornaviruses are directed against either the virions to block early stages of infection or the 3C protease to block genome expression (De Palma et al., 2008a,b,c). There is a need for additional broadly reactive compounds against conserved target molecules and processes that may not be prone to mutation and that are delineated as most conserved in the different species or genera. To minimize the risk of possible side effects, the anti-viral compounds must recognize targets that are unique to viruses, or be capable to discriminate between viral targets and cellular homologs.

Several nonstructural proteins of picornaviruses including the proteases, the putative helicase, and the RNA-dependent RNA polymerase fall in this category. The structure of some of these proteins have been characterized for a few picornaviruses, although our knowledge is still very limited. The EU-supported FP6 project-VIZIER (Comparative Structural Genomics of Viral Enzymes Involved in Replication, project: 2004-511960) was initiated to identify potential new drug targets against RNA viruses through structural characterization of the replicative machinery of representative viruses of most RNA virus families containing human pathogens (Coutard et al., 2007; Coutard and Canard, 2010). The non-structural proteins were studied to identify conserved binding sites for broadly reactive anti-virals. In this review, we describe the importance of picornaviruses as pathogens to be considered for treatment along with some results obtained on the characterization of picornavirus proteins and their inhibitors in the VIZIER project.

1.1. Disease manifestations in humans

Six genera of the *Picornaviridae* family include pathogens infecting humans. They are *Enterovirus*, *Parechovirus*, *Hepatovirus*, *Cardiovirus*, *Kobuvirus*, and the proposed genus *Cosavirus*. The *Enterovirus* genus is the largest among the *Picornavirus* genera with 219 virus types in 10 species circulated worldwide. Seven of these species are known as enteroviruses (EVs) infecting humans or other animals; three are *human rhinovirus* species, HRV-A, B and C. Human infections caused by viruses of the *Enterovirus* genus are often mild or asymptomatic with upper respiratory tract symptoms and/or exanthemas, although they may also be associated with more severe illnesses (Table 1). Infection with the same type may be accompanied with different clinical manifestations. Chronic EV infections may also occur in patients with agammaglobulinemia,

Table 1
Clinical manifestations of infrctions by different enterovirus types.

Symptom	Enterovirus types
Asymptomatic	All enteroviruses
Exanthema	
Macular	Several coxsackievirus A (CV-A), B (CV-B) and echoviruses (E)
Vesicular	CV-A16, enterovirus 71 (EV-71), E-3, 4, 13, CV-B2, 5
Herpangina	Several CV-As
Upper respiratory infections	Human rhinovirus A (HRV-A), HRV-B, HRV-C, E-6, 11, 13, EV-68, several CV-As and CV-Bs
Epidemic myalgia	CV-B3, 5, CV-A9
Pleurodynia	Several CV-Bs, CV-A16, E-11
Pancreatitis	CV-B3, 4
Pericarditis, myocardiopathy	CV-B3, 4
Gastroenteritis	E-6, 7, 13, EV-74, 95, HPeV7, HCoSV-E1
Aseptic meningitis	Several echoviruses, CV-A, CV-B, PV 1-3, EV-71, 75
Meningoencephalitis	EV-71, several echoviruses, CV-B and PV 1-3
Pareses	
Permanent	PV1-3, CV-A7
Temporary	CV-B1–6, several echoviruses, EV-71, 93, 94, 98
Fever	Most enteroviruses
Uveitis	E-11, 19, CV-B4
Hemorrhagic conjunctivitis	EV-70, CV-A24v

chronic dilated cardiomyopathy, myocarditis, and chronic fatigue syndrome (Chapman and Kim, 2008; Huber, 2008). PV, one of the human EVs, may cause poliomyelitis, which is often called just polio or infantile paralysis, and is characterized by acute flaccid paralysis (AFP). Several decades after acute poliomyelitis infection, survivors may develop post-polio syndrome (Ramaraj, 2007). These patients have increased expression of mRNA for proinflammatory cytokines in the CNS, suggesting an ongoing inflammatory process (Gonzalez et al., 2002).

EVs have continuously posed a threat to children, and annual virus outbreaks are frequently reported. Large outbreaks of aseptic meningitis affecting different continents have occurred, caused by echovirus 30 (E-30) in mainland China and Taiwan during 2003, and by echovirus 13 in Lithuania in 2001 (Narkeviciute and Vaiciuniene, 2004; Zhao et al., 2005). These two virus types simultaneously caused outbreaks in Denmark, Belgium, France, and Turkey. Spread of hand, foot and mouth disease associated with serious complications as encephalitis or myocarditis, and being fatal was caused by large epidemics of enterovirus 71 (EV-71) in Sarawak, East Malaysia, and the Malaysian Peninsula in 1997, in Taiwan in 1998, and in mainland China, Malaysia, and Singapore in 2000 and 2008 (Fan et al., 2009). During 2008 and 2009 there was a large outbreak of EV-71 in Taiwan and in mainland China, with more than 50 fatalities (mainly children) and 700 life-threatening cases (Lee et al., 2010; Wong et al., 2010). Little is known on the factors promoting an endemic enterovirus strain to become an outbreak strain or causing chronic infection.

Human rhinoviruses forming three species in the *Enterovirus* genus (Palmenberg et al., 2009) and circulating worldwide are the most common causes of upper respiratory illness. Most children have had at least one rhinovirus infection by the age of 2 years, and in adults, HRV infections account for about 50% of common colds. More than 90% of the infections are symptomatic. HRV have also been linked to severe respiratory disease in hospitalized children and serious lower respiratory infections in the elderly. They have also been shown to be one of the most common causes of asthma exacerbations in both children and adults (Arden and Mackay, 2010; De Almeida et al., 2010; Dougherty and Fahy, 2009; Gern, 2010).

The human parechoviruses (HPeV) infect mainly young children and most infections are subclinical. However, gastrointestinal and respiratory tract infections, and other more severe consequences also have been ascribed to HPeV, including AFP, encephalitis, aseptic meningitis, myocarditis, neonatal sepsis, and Reye syndrome (Wolthers et al., 2008). The associated spectrum of diseases is not fully understood and probably has been underestimated, since many serotypes of HPeVs have been identified only recently. Some of the new HPeVs may be emerging, others - long circulating - escaped the identification until very recently by molecular methods, since they did not infect standard diagnostic cell lines.

Hepatitis A virus (HAV) is the only species in the genus *Hepatovirus*. Viruses of this species infect humans and primates. The disease is mostly self-limiting and only 10% of infected children are symptomatic, whereas 70% of infected adults have clinical hepatitis. The severity of the disease increases with age, with up to 3% mortality in patients over 50 years. Previously, hepatitis A virus infection was common among children in many countries, but with increasing hygiene, fewer individuals get infected during childhood (Brundage and Fitzpatrick, 2006). However, outbreaks are now often seen in day care centres, where the source often is an unvaccinated child returning from an endemic country (Hauri et al., 2006; Gervelmeyer et al., 2006). There are also large outbreaks among adults often infected through contaminated food or water (Sattar et al., 2000; Lee et al., 2008; Pontrelli et al., 2007) and in risk groups such as intravenous drug users, and men having sex with men (O'Donovan et al., 2001; Stene-Johansen et al., 2007).

The genus *Kobuvirus* is comprised of two species, *Aichi virus*, *Bovine kobuvirus* and an unassigned species Porcine kobuvirus. Aichi virus was isolated in 1989 as the likely cause of oyster-associated gastroenteritis in Japan (Yamashita et al., 1991). It has now been shown to be a common cause of gastroenteritis among adults in Asia and has recently also been described from Europe and South America (Le Guyader et al., 2008; Goyer et al., 2008; Oh et al., 2006).

The newly described human cosaviruses (HCoSV) were isolated from both healthy children and children with AFP in Pakistan (Kapoor et al., 2008). The virus has also been isolated in one patient from Scotland and from a child with acute diarrhea in Melbourne, Australia (Holtz et al., 2008), although the virus has not been identified as a causal agent of particular diseases. Several genetically divergent HCoSVs have now been isolated and classified into five genomic groups designated HCoSV-A to HCoSV-E (Kapoor et al., 2008; Holtz et al., 2008).

There are two species of *Cardiovirus*, *Encephalomyocarditis virus* (EMCV) and *Theilovirus* (ThV). EMCV circulates worldwide with rodents being its natural reservoir. Infection in other mammalian species is often fatal and associated with sporadic cases and outbreaks of myocarditis and encephalitis. Human EMCV infection and associated disease have been documented, but clinical manifestation is probably infrequent. ThV are classified into five types, two of which infects humans: Vilyuisk human encephalomyelitis virus and Saffold virus (Liang et al., 2008). They have been shown to cause respiratory diseases, gastroenteritis, myocarditis, and acute and chronic encephalitis. Recently, several new types of Saffold virus were isolated, and there are now eight types designated SAFV1–8. They are all isolated from young children with upper respiratory symptoms or gastroenteritis (Blinkova et al., 2009; Zoll et al., 2009). There may be additional, not yet identified Cardioviruses pathogenic for humans, since several of these viruses are difficult to cultivate on diagnostic cell lines.

1.2. Emerging picornaviruses

Most emerging viruses are zoonotic, with animals forming the natural reservoir of new viruses. Spontaneous mutations and recombinations may also play an important role in the emergence of new human pathogens. Viruses in most genera of the *Picornaviridae* family have been shown to infect several mammalian species (Table 2), and zoonotic transmissions have been described (Brown et al., 1973). It is also likely that many other animal species harbor yet undiscovered picornaviruses. Some of these unknown animal picornaviruses may evolve to infect and cause disease in humans.

Many enterovirus types belonging to the same species have been shown to recombine (Lindberg et al., 2003; Norder et al., 2002). Such recombinants may give rise to new emerging human or zoonotic viruses. Swine vesicular disease virus (SVDV) is an example of a recombinant between two parental human enteroviruses, CV-B5 (providing the structural region) and E-9 (non-structural region), respectively (Brown et al., 1973). Apparently, this recombinant gained properties necessary to cross the species barrier. Several new human enteroviruses have been identified during the last years. Whether they represent newly emerging or since-long circulating and undetected viruses remains unknown, although some of these are closely related to already established viruses. Besides the commonly recognized zoonotic origin, pathogenic viruses may also emerge from benign siblings infecting the same species. This concept was developed to explain the origin of the PV ancestor (Gromeier et al., 1999; Jiang et al., 2007). Accordingly, PV may have originated from an ancestral Coxsackie A virus (CAV) (Jiang et al., 2007), whose numerous contemporary descendants broadly circulate in the human population and readily recombine with PV strains in the field (Kew et al., 2002; Arita et al., 2005;

Table 2
Natural hosts for members of different species within the picornavirus family.

Genus	Natural host	Zoonotic spread
Enterovirus	Human	Swine
	Macaque	
	Sooty mangabey	
	Yellow baboon	
	Cattle	
	Swine	
Hepatovirus	Human	
Parechovirus	Human	
	Rodent	
Kobuvirus	Human	
	Cattle	
	Swine	
Cardiovirus	Rodent	Primates
	Squirrel	Elephants
	Human	Swine
Cosavirus[a]	Human	
Aphtovirus	Cattle	
	Swine	
	Goat	
	Sheep	
	Buffalo	
	Wild ruminants	
Erbovirus	Horse	
Teschovirus	Swine	
Sapelovirus[a]	Several monkeys	
	Duck	
	Swine	
Senecavirus[a]	Rodent	
Tremovirus[a]	Chicken	
Avihepatovirus[a]	Duck	
Seal picornavirus[a]	Seal	

[a] Proposed genera.

Rakoto-Andrianarivelo et al., 2007). Enteroviruses also account for a high proportion of cases of aseptic meningitis with viral origin worldwide and may cause large outbreaks affecting people on different continents. Little is known about the factors promoting an endemic enterovirus strain to become an outbreak strain with changed pathogenicity and virulence.

Members of the genus *Cardiovirus* have also been shown to spread between different mammalian species. The natural hosts are rodents, and disease transmission apparently results from close contact between rodents or their excreta and susceptible mammalians. This often results in a fatal disease with sudden death and most virus outbreaks have been associated with captive animals at primate research centers, zoos or pig-breeding facilities.

2. Taxonomy

The *Picornaviridae* family encompasses 285 virus types divided into 14 genera, each with between one and ten established or provisional species (Table 3) (Knowles et al., 2010). Eighty (28%) of these types have been discovered since 2001, 43 of them belong to the genus *Enterovirus* and 13 to *Parechovirus*. It is not known whether all these newly described viruses have been circulating since long, or whether some are emerging viruses established through cross-species transfer. Since simian enteroviruses showing high similarity to human enteroviruses have been identified, they might have given rise to human pathogens through recombinations.

The genetic classification of the different enteroviruses into types is based on divergences of the structural region of the viral genome (P1). The non-structural region in these viruses is poorly suitable for classifying strains due to frequent recombinations between related viruses. Phylogenetic analysis of the P1 region revealed two major clusters of picornaviruses. The one with the deepest bifurcation divided into parechoviruses with the newly discovered seal Picornavirus and duck hepatitis virus on one branch and the hepatoviruses and tremovirus on the other branch (Fig. 1). The second cluster includes strains from the other genera. The topology of this tree is in agreement with previous phylogenetic comparisons of the 3D region and the polyprotein (Johansson et al., 2002; Hughes, 2004), although parechoviruses formed the first split followed by hepatoviruses in these trees. This deep subdivision of the picornavirus genera into these major genetic groups shown in Fig. 1 may be considered when developing broad-spectrum inhibitors against structurally conserved viral targets.

3. Genomic organization

All picornaviruses have a similar genomic organization that is conserved in some but varies in other regions (Racaniello, 2007; Ehrenfeld et al., 2010). The genome consists of a positive-stranded RNA molecule of approximately 6700–8800 nucleotides (Table 3) containing one single large open reading frame (ORF) preceded by a long 5′-untranslated region and followed by a much smaller 3′-untranslated region and a genetically encoded poly-(A) tail. A small viral protein, VPg, is covalently linked to the 5′ end of the viral genome. The ORF is translated into a single large polyprotein, of approximately 2200 amino-acid residues, which is subsequently

Table 3
Number of virus types within each species and genus in the family *Picornaviridae*.

Genus	Species	Number of virus types	Genomic size (number of nucleotides)
Enterovirus	*Human enterovirus A* (HEV-A)	21	7400–7500
	HEV-B	59	
	HEV-C	19	
	HEV-D	3	
	Simian enterovirus A (SEV-A)	3	
	Bovine enterovirus	2	
	Porcine enterovirus A (PEV-A)	1	
	PEV-B	2	
	Human rhinovirus A (HRV-A)	74	
	HRV-B	25	
	HRV-C	10	
		Subtotal: 219	
Parechovirus	*Human parechovirus*	14	7300
	Ljunganvirus	1	7600
		Subtotal: 15	
Hepatovirus	*Hepatitis A virus*	1	7500
Cardiovirus	*Encephalomyocarditis virus*	1	7700–8100[a]
	Theilovirus	12	
		Subtotal: 13	
Cosavirus[b]	*Human Cosavirus* (HcoSV A-E)	5	
Aphtovirus	*Foot-and-mouth disease virus*	7	7700–8200[a]
	Equine rhinitis A virus	1	
	Bovine rhinovirus	3	
		Subtotal: 11	
Tremovirus	*Avian encephalomyelitis-like viruses*	1	7100
Erbovirus	*Equine rhinitis B virus*	3	8800[a]
Kobuvirus	*Aichi virus*	1	8300–8400[a]
	Bovine kobuvirus	1	
	Porcine kobuvirus	1	
Teschovirus	*Porcine teschovirus*	11	>7200[a,c]
Avihepatovirus	*Duck hepatitis virus*	3	7700
Seneca virus	*Seneca valley virus*	1	7300[a]
Unassigned[b]	*Seal picornavirus* (SePV)	1	6700
		Total: 285	

[a] Encodes a leader protein at the 5′ end of the coding region.
[b] Proposed genera.
[c] Complete genomes are lacking.

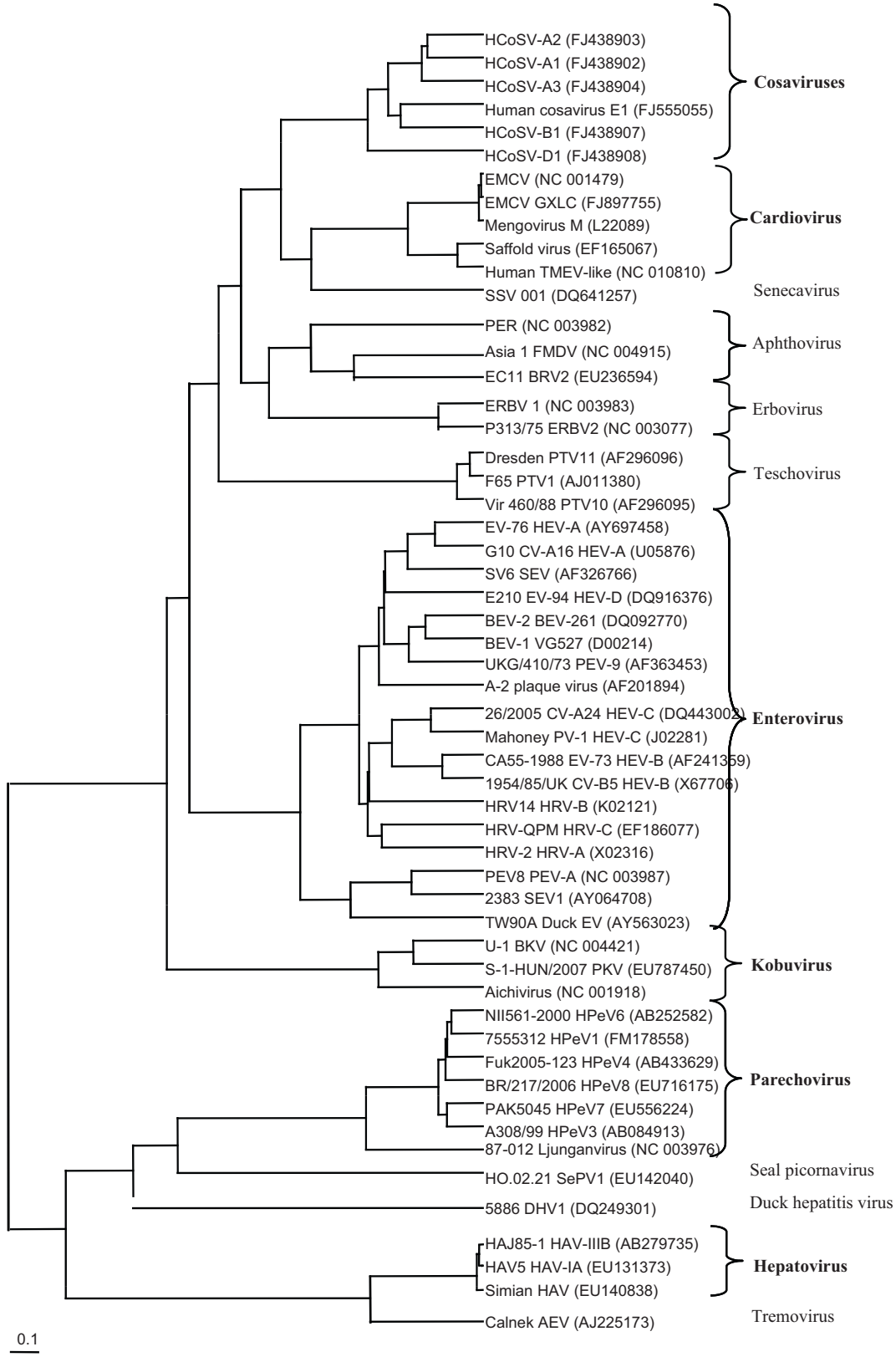

Fig. 1. Phylogenetic tree based on the deduced amino-acid sequence of the structural P1 region of representatives of the 13 different genera within *Picornaviridae*. Within the genera, types infecting humans among other species are shown in bold.

cleaved by viral protease(s) into mature proteins and their intermediates, which may be quite stable. For the purpose of common nomenclature, the ORF is divided into three consecutive parts, P1, P2 and P3; in a subset of picornaviruses a region L precedes P1 (Fig. 2). Processing of the P1 region yields the capsid-forming (structural) proteins 1A–1C, also known under their traditional names as VP4, VP2, VP3, and VP1, respectively. VP4 may not be formed in every picornavirus. Processing of the P2 and P3 regions yields

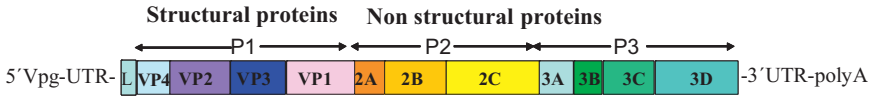

Fig. 2. Principal organization of picornavirus genomes. The actual genome organization may deviate in some picornaviruses.

the nonstructural replication proteins 2A–2C and 3A–3D, respectively, as well as cleavage intermediates. In some picornaviruses, two or three unrelated 2A proteins may be formed while in others, two or three paralogous copies of 3B (also known as VPg) are (predicted to be) produced. The L region encodes for a leader protein in viruses of five genera (Table 3). The L and 2A proteins may not be homologous, thus performing lineage-specific functions in different picornaviruses (Table 4) (Agol and Gmyl, 2010; Gorbalenya and Lauber, 2010).

4. Structural studies on non-structural target proteins

The initial effort in the antipicornaviral drug development was directed toward designing compounds that target viral attachment (and/or uncoating). As result, soluble intercellular adhesion molecule 1, pirodavir, and pleconaril were developed. The latter compound belongs to capsid binding agents, commonly referred to as "WIN" compounds (referring to Sterling-Winthrop, were they were originally developed) (7, 8). Even if some of these compounds seem efficient, more compounds are needed both against the structural and non-structural proteins to be used in combination or alone to avoid development of resistance.

The non-structural proteins of picornaviruses were characterized from few members within three of the 14 genera with regard to the structure and suitability as targets for anti-viral compounds (Table 5). The VIZIER consortium has expanded the characterization considerably starting from sequencing the non-structural region of wild-type strains from seven genera within Picornaviridae. Guided by bioinformatics (Gorbalenya et al., 2010), the focus was put on putative helicases (protein 2C), proteases (3C) and polymerases (3D) due to their family-wide conservation and critical functions. They are thus attractive classes of viral proteins towards which chemotherapeutic agents could be produced. As shown in Table 5, there was no structural information before VIZIER on protein 2C while the structures were solved for few $3C^{pro}$ (Matthews et al., 1994; Mosimann et al., 1997; Allaire et al., 1994; Bergmann et al., 1997; Birtley et al., 2005; Sweeney et al., 2007) and $3D^{pol}$ with or without its protein primer VPg (protein 3B) (Ferrer-Orta et al., 2006a,b, 2007; Thompson et al., 2007). One structure was recently described of the precursor $3CD^{pro}$ (Marcotte et al., 2007).

4.1. Protein 2C, the putative helicase

4.1.1. Ring-shaped structure of echovirus 30 protein 2C

Protein 2C is one of the most conserved non-structural viral proteins within the *Picornaviridae* family (Gorbalenya and Lauber, 2010). It was predicted to have a helicase activity due to the presence of conserved motifs A, and B found in NTP-binding proteins

Table 4
Major demonstrated and predicted functions of picornavirus proteins.

Genomic region	Protein designation	Genus	Protein function	Reference
L	Leader	*Aphthovirus*, *Erbovirus*	Papain-like cysteine protease implicated in virus–host interaction	Guarné et al. (2000)
L	Leader	*Cardiovirus*	Involved in internal ribosome entry site-mediated translation of viral RNA	Dvorak et al. (2001)
L	Leader	*Kobuvirus*	No protease activity; involved in both viral RNA replication and encapsidation	Sasaki et al. (2003)
P1	VP2, VP3, VP1	All	Major capsid proteins	Fry and Stuart (2010)
	VP4	Majority	Small capsid protein implicated in virion uncoating that is present in viruses of most genera	Chow et al. (1987)
P2	2A	*Enterovirus*	Chymotrypsin-like cysteine protease releasing capsid precursor from the nascent polypeptide; implicated in the control of RNA synthesis	Toyoda et al. (1986), Martinez-Salas and Ryan (2010)
	2A	*Cardiovirus Aphthovirus Parechovirus Senecavirus Erbovirus Teschovirus*	Small protein whose synthesis is accompanied by termination and re-initiation of translation to separate 2A and 2B proteins	Martinez-Salas and Ryan (2010) and Ryan and Drew (1994)
	2A	*Hepatovirus*	Structural protein	Agol and Gmyl (2010) and Martinez-Salas and Ryan (2010)
	2A	*Parechovirus Tremovirus*	Putative acyltransferase implicated in virus–host interaction	Hughes and Stanway (2000)
	2B	All	Membrane–anchoring protein for the virus replication complex	Van Kuppeveld et al. (1997)
	2C	All	Multifunctional protein with ATPase and predicted helicase activity implicated in capsid assembly, virion uncoating, and RNA synthesis	Takeda et al. (1986) and Palmenberg et al. (2010)
P3	3A	*Enterovirus* and likely all	Membrane–anchoring protein for the virus replication complex; inhibits ER to Golgi membrane and secretory traffic	van Kuppeveld et al. (2010)
	3B VPg	All	Protein primer for the initiation of RNA-synthesis	Paul et al. (1998)
	3C	All	Chymotrypsin-like cysteine protease mediating most cleavages in polyprotein	Porter (1993) and Martinez-Salas and Ryan (2010)
	3D	All	RNA-dependent RNA polymerase	Porter (1993)
	3CD	*Enterovirus*	Stable intermediate of 3C and 3D that is responsible for processing capsid P1 precursor and regulation of RNA synthesis through binding to two RNA cis signals	Marcotte et al. (2007)

Table 5
Predicted domains in the non-structural region investigated during the VIZIER project, in relation to solved structures that were available before the beginning of VIZIER.

Genus	Number of types sequenced in VIZIER	Structures solved of nonstructural proteins before VIZIER							
		$2A^{pro}$	2B	2C	3A	3B (VPg)	$3C^{pro}$	$3D^{pol}$	$3CD^{pro}$
Enterovirus	14	CV-B4 HRV2	0	0	PV	0	PV HRV-2 HRV-14	PV HRV-1 HRV-14 HRV-16	PV
Parecho-virus	2	0	0	0	0	0	0	0	0
Hepatovirus	1	0	0	0	0	0	HAV	0	0
Cardiovirus	1	0	0	0	0	0	0	0	0
Aphthovirus	1	0	0	0	0	FMDV	FMDV	FMDV	0
Erbovirus	1	0	0	0	0	0	0	0	0
Kobuvirus	1	0	0	0	0	0	0	0	0

(Walker et al., 1982), as well as motif C, which is a typical feature of members of the helicase superfamily 3 (Gorbalenya and Koonin, 1993; Gorbalenya et al., 1990). The ATPase activity has been demonstrated for several picornavirus 2C proteins (Rodriguez and Carrasco, 1993; Klein et al., 1999; Pfister and Wimmer, 1999; Samuilova et al., 2006). However, no helicase activity has ever been described, nor are structural data available for any 2C protein (Table 5). The technical difficulties in solving the 2C structure may be linked to the membrane affinity of this protein that is mediated by an amphipathic helix located at the N-terminus (Paul et al., 1994; Teterina et al., 2006). In agreement with this property, the 2C protein is also thought to function as a membrane-anchoring protein (Echeverria et al., 1998; Teterina et al., 1997) as well as an agent in structural rearrangements of intracellular membranes (Aldabe and Carrasco, 1995; Bienz et al., 1987, 1990; Teterina et al., 1997), in addition to its putative helicase function.

Echovirus 30 (E-30) belongs to HEV-B, which is one of the largest species of human pathogens within the *Enterovirus* genus (Table 3). A soluble derivative of the E-30 2C protein was studied by negative-staining transmission electron microscopy (TEM), small-angle X-ray scattering (SAXS), dynamic light-scattering (DLS) and size exclusion chromatography (SEC) (Papageorgiou et al., 2010). The purified protein exhibited ATPase activity while the TEM measurements showed that it adopted a hexameric shape reminiscent to that of proven DNA-based SF3 helicases. The fraction of hexamer particles which was observed by TEM increased by a factor of 10 in the presence of 3 mM ADP. No monomer or hexamer particles were observed by SAXS, DLS, or SEC, due to their very low yield. The measurements indicate aggregation of the main fraction of the purified protein, leaving a small fraction of monomers and an even smaller fraction of hexamers that are below the detection limit of SAXS, DLS or SEC.

Typical TEM micrographs of the hexamer particle are shown in Fig. 3, together with a 3D reconstruction of the hexamer particle calculated out of 1500 collected particles. The hexameric structure observed by TEM is in line with results obtained for other SF3 helicases with known crystal structure that form hexameric oligomers (Hickman and Dyda, 2005; Neuwald et al., 1999). This hexameric arrangement has also been demonstrated for the polyomavirus SV40 LTag (Gomez-Lorenzo et al., 2003; Li et al., 2003). However, the crystallized adeno associated virus type 2 (AAV2) Rep40 as well as the human papilloma virus type 18 (HPV18) RepE1 (James et al., 2003) were found to be monomeric, although, in the latter structure, the interaction with the activation domain of E2 prevents hexamerisation of E1 (Abbate et al., 2004). The TEM-based result is in agreement with genetic evidence for 2C functioning as an oligomer (Tolskaya et al., 1994) and supports the helicase function of 2C of E-30, and, by implication, other picornaviruses.

The N-terminal domain, encompassing an amphipathic helix (Paul et al., 1994; Teterina et al., 2006), is essential for hexamer formation of picornaviral 2C. A similar role for the N-terminal region was shown for the SF3 DNA helicase from simian virus 40 (Li et al., 2003). The oligomerization interfaces of 2C helicases could therefore be an excellent target for antiviral compounds. This would represent an entirely novel strategy to inhibit viral replication, as illustrated below by the thiazolobenzimidazole compounds (see Section 5.1).

4.2. Proteins $3C^{pro}$ and $3D^{pol}$ – the main protease and the RNA-dependent RNA polymerase

4.2.1. Elucidation of several $3C^{pro}$ structures

Proteases play essential roles in the enteroviral life cycle. The virus polyprotein of approximately 2200 amino-acid residues is co- and post-translationally processed by the viral proteases $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$, the latter being the precursor of $3C^{pro}$ and the RNA-dependent RNA polymerase, $3D^{pol}$. *In vivo*, most steps of poliovirus polyprotein processing are carried out by the $3CD^{pro}$ precursor proteinase rather than by the $3C^{pro}$ (Ypma-Wong et al., 1988; Kräusslich and Wimmer, 1988; Marcotte et al., 2007). However, the first cleavage reaction in all enteroviral polyproteins is catalyzed by the $2A^{pro}$. The three-dimensional structures of rhinovirus $2A^{pro}$ as well as of its homologue from coxsackievirus B4 have been determined revealing a chymotrypsin-like fold with a cysteine at the catalytic site (Table 5; Petersen et al., 1999; Baxter et al., 2006) in agreement with prior bioinformatics and genetic studies (reviewed in Dougherty and Semler, 1993; Gorbalenya and Snijder, 1996).

Since the early 1990s, crystal structures have been determined for the 3C proteases ($3C^{pro}$s) of HRV-14, HRV-2, PV-1, HAV, and FMDV (Table 5). Similarly to the findings for the enterovirus 2A proteases and in line with bioinformatics and genetic data (Gorbalenya and Snijder, 1996), these studies revealed two-domain structures with a chymotrypsin-like fold and a cysteine residue instead of a serine in the catalytic site (Matthews et al., 1994; Mosimann et al., 1997; Allaire et al., 1994; Bergmann et al., 1997; Birtley et al., 2005; Sweeney et al., 2007). Conserved histidine and glutamic or aspartic acid residues complete a catalytic triad (Cys. . .His. . .Glu/Asp). Recently, the crystal structure of the $3CD^{pro}$ of PV1 was determined (Marcotte et al., 2007). Proteolytic cleavage of substrate (poly)proteins occurs preferentially at Gln-Gly dipeptides with alanine in P4 and a proline residue in the P2′ position (see Schechter and Berger, 1967, for nomenclature). In addition to viral substrate proteins, several host cellular proteins were described to be cleaved by the $3C^{pro}$ upon enterovirus infections: TFIIIC, TFIID, TAF110, Oct-1, CREB, MAP-4, PABP (Dougherty et al., 2010).

Within the VIZIER project, we have determined the crystal structures of the protease $3C^{pro}$ of CV-B3 and enterovirus 93 (EV-93), within HEV-B, and enterovirus 68 within HEV-D (Tan et al., in press). The overall protein fold seen in these structures is similar to that of other picornavirus $3C^{pro}$s. As shown in Fig. 4, following an N-terminal helix of 14 amino-acid residues, the $3C^{pro}$ in EV-93 folds into two β barrels (residues 15–79 and 99–173) formed

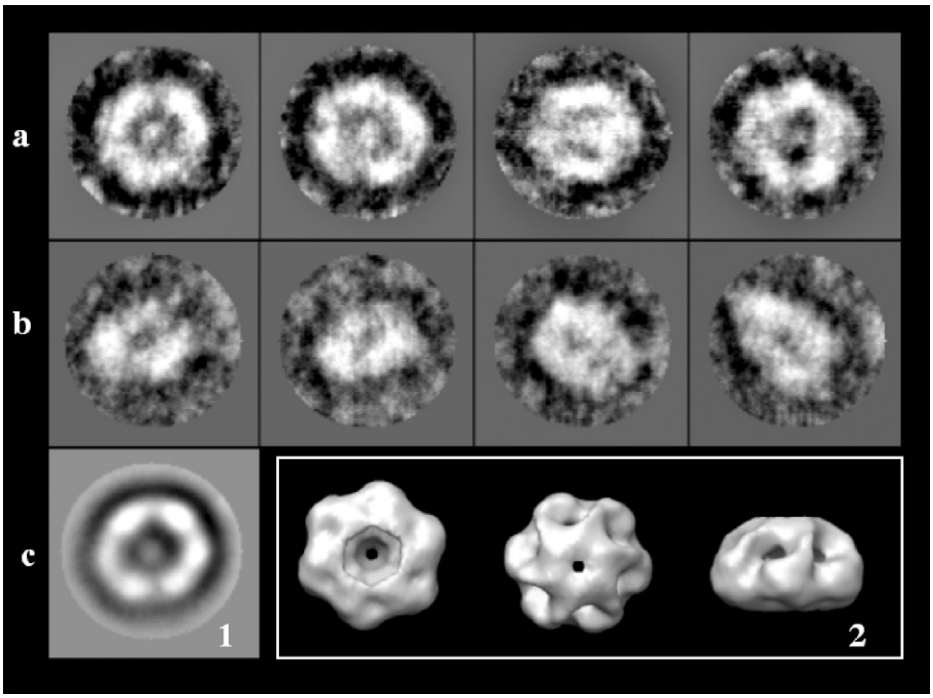

Fig. 3. Negative staining TEM results (HITACHI HNAR 300 keV) showing (a) typical top-view micrographs of the hexameric particle, (b) typical lateral view micrographs, (c1) class average particle with no symmetry constraints calculated out of 800 "top-view" collected particles, clearly showing the 6-fold symmetry adopted by the protein, (c2) 3D reconstruction out of 1500 particles done by EMAN 1.7.

by six antiparallel strands, each with a 3-1-1 topology. The two barrels pack together, with a relative orientation of ~90°, to form an extended groove for substrate binding. Overall, the structure of $3C^{pro}$ in EV-93 adopts a chymotrypsin fold similar to those of other closely and distantly related 3C proteases. For example, the rmsd between EV93-$3C^{pro}$ and the structure of CV-B3 $3C^{pro}$ is below 0.5 Å. The rmsd with other picornavirus $3C^{pro}$s are 0.77 Å for rhinovirus (Matthews et al., 1999) and 0.71 Å for poliovirus (Mosimann et al., 1997), underlining the high conservation of the fold among the enterovirus $3C^{pro}$s.

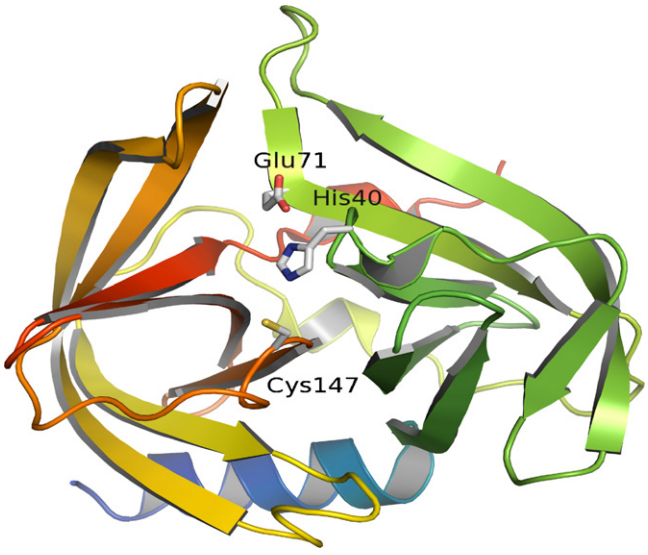

Fig. 4. Crystal structure of the 3Cpro of EV-93. Ribbon colored from blue to red from the N to the C terminus. The protease folds into two β barrels, (the first one in green tones and the second in orange tone), forming the chymotrypsin-like fold. Catalytic triad residues are highlighted in stick representation. The nucleophilic Cys^{147} (from the second barrel) and the general acid/base pair His^{40} and Glu^{71} (from the first barrel) form the catalytic triad.

The proteolytic active site is located in the cleft between the two β barrels and consists of a catalytic triad and an electrophilic oxyanion hole, which stabilizes the tetrahedral intermediate of the substrate covalently linked to the protease. The nucleophilic Cys^{147} (from the second barrel) and the general acid/base pair His^{40} and Glu^{71} (from the first barrel) form the catalytic triad, whereas the oxyanion hole consists of the amide groups of Gly^{145}, Gln^{146} and Cys^{147}.

4.2.2. Structure of coxsackievirus B3 $3D^{pol}$ in complex with its protein-primer VPg

The RNA-dependent RNA polymerase (RdRp) of RNA viruses plays a vital role in viral replication. It has no close homologs in the host cell. The clinical use of inhibitors against the HIV reverse transcriptase, the hepatitis B virus DNA polymerase and the herpes virus DNA polymerase has validated viral polymerases as therapeutic targets (De Clercq, 2005). Concerning viral RdRps, inhibitors of the RdRp of hepatitis C virus (HCV) are currently undergoing clinical trials (De Francesco and Carfi, 2007). Thus, RdRps can be considered prime targets in the search of anti-picornavirus drugs.

Before VIZIER, the 3D structures of RdRps of PV, FMDV and several HRVs were elucidated either as unliganded proteins (Table 5; reviewed in Ferrer-Orta et al., 2005) or in a complex with primers, template and/or NTP substrates (Ferrer-Orta et al., 2006a,b, 2007; Thompson et al., 2007). The complex structure of FMDV RdRp with its protein primer VPg (also called 3B) showed VPg-pU (VPg with one UMP bound covalently to Tyr3) bound near in the active center (Ferrer-Orta et al., 2006a,b). VPg-pU corresponds to the product of the first step of minus- and plus-strand RNA synthesis, which is followed by a second uridylation and then processive primer elongation. In the framework of VIZIER, we determined the crystal structure of CV-B3 RdRp at 2.5 Å resolution in complex with VPg, bound to a different site, and a pyrophosphate (PPi), corresponding to the reaction co-product of RNA polymerization (Gruez et al., 2008; Jabafi et al., 2007).

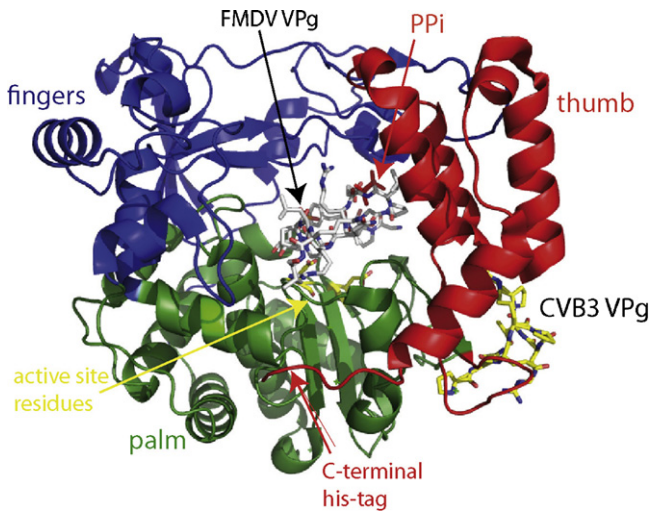

Fig. 5. Structure of Coxsackievirus B3 $3D^{pol}$ in complex with VPg and PP_i Ribbon representation of the "front" view of the protein with the accessible active site in the center. Palm, fingers and thumb subdomains are shown in green, blue and red, respectively. Catalytic residues Asp233 and Asp329 are shown as sticks with C-atoms in yellow. The PPi ligand (shown in sticks and colored in red) is situated in the NTP tunnel formed by the connection between fingers and thumb subdomain at the "back" of the protein. CVB3 VPg binds to the bottom of the thumb subdomain. It is represented in sticks and colored according to the atom type (C-atoms in yellow, N in blue, O in red). FMVD VPg was taken from the complex structure with FMDV RdRp (Ferrer-Orta et al., 2006a,b), after the two RdRp structures have been superposed. It is represented in sticks and colored according to the atom type (C-atoms in white, N in blue, O in red). The recombinant protein bears a C-terminal his_6-tag, which was fully resolved in the structure. The figure has been generated with Pymol.

As shown in Fig. 5, CV-B3 RdRp adopts a canonical RdRp fold (Ferrer-Orta et al., 2005) resembling a cupped right hand with fingers, palm and thumb subdomains. The fingers and thumb subdomains are involved in template, primer and NTP substrate binding. *Picornaviridae* RdRps present a small thumb subdomain typical for primer-dependent RdRps, which leaves the active site open and accessible. Fingers and thumb subdomains are in contact with each other, thus the RdRps' active site is encircled. The structurally most conserved RdRp subdomain is the catalytic palm subdomain where, as in most other polymerases (Castro et al., 2009), two catalytic aspartic acid residues coordinate two divalent metal ions essential for catalysis. In Fig. 5, the FMDV VPg (Ferrer-Orta et al., 2006a,b) is shown bound to the corresponding putative primer-binding site near the active site of CVB3 RdRp after superposition of the two RdRps. VPg residues 1-GPYAGPLERQPRPLKV-15 were visible in the FMDV 3D-VPg complex structure (Ferrer-Orta et al., 2006a,b) and residue Y3 in the active site had been uridylated. In contrast, in the CVB3 3D-VPg complex structure, VPg binds to a second binding site at the bottom of the thumb subdomain. VPg residues 7-PNQKPRVPT-15 were visible whereby Pro7 was 20 Å apart from Val15 of FMDV VPg (see Fig. 5).

The existence of this second site had been evidenced before on PV RdRp by a series of genetic and biochemical experiments (Hope et al., 1997; Lyle et al., 2002; Tellez et al., 2006) and our structure (Gruez et al., 2008) is thus the first structural evidence for it. Given the distance between the resolved part of VPg and the active site, the binding mode of VPg to CVB3 $3D^{pol}$ at this site excludes its uridylation by the carrier $3D^{pol}$. We suggest that VPg is uridylated by another $3D^{pol}$ molecule. Elongation may take place after translocation of the uridylated VPg to the primer-binding site. The latter can either be the same $3D^{pol}$ or another $3D^{pol}$. Alternatively, VPg at the second binding site may play a mere scaffolding role. It may stabilize a second VPg bound to the primer-binding site of the same carrier $3D^{pol}$ molecule. Or, it may be part of a non-productive $3D^{pol}$-VPg complex (with VPg at the bottom of the thumb subdomain), which stabilizes a productive complex (with VPg at the primer-binding position). The PPi is situated in the NTP tunnel within the active site cavity (see Fig. 5). It could represent a new pre-interrogation site for NTP substrates or a site for the PPi coproduct when leaving the active site.

The newly described VPg and the PPi binding sites on *Picornaviridae* RdRps (Gruez et al., 2008) as well as other substrate binding sites (Ferrer-Orta et al., 2006a,b, 2007; Thompson et al., 2007) represent putative binding sites of inhibitors. These binding sites may be exploited by virtual screening and/or the ligands might serve as starting points for drug design. The search for *Picornaviridae* RdRp inhibitors is just beginning. There are some reports on non-nucleoside inhibitors of PV RdRp: gliotoxin (Rodriguez and Carrasco, 1992) and amiloride derivatives (Harrison et al., 2008) with unknown binding sites. We have found that UTP analogs, especially 2′fluoro-2′deoxy-UTP, inhibited RNA synthesis by CVB3 RdRp on polyA/dT (B. Selisko and B. Canard, unpublished data) and other nucleoside inhibitors have been reported (Graci et al., 2008; Harki et al., 2006, 2007). Clearly, the search for *Picornaviridae* antivirals targeting the RdRp will gain momentum in the near future.

5. Antiviral compounds against picornaviruses

As previously mentioned, picornaviruses form a large family of pathogens that affect both humans and animals. Notwithstanding their enormous clinical impact, no antiviral drugs have been approved for the treatment of infections with picornaviruses (De Palma et al., 2008a,b,c). There is consensus that drugs that can be used to treat several forms of picornavirus infections are urgently needed. A few examples that support the role of antivirals in the battle against picornavirus-induced diseases are briefly discussed.

A major group of pathogens within the enterovirus genus are the rhinoviruses, which comprise more than 100 different types (Table 3). These viruses are the primary etiologic agent of the common cold, which accounts for more than 40 million days of absence from work or school, enhances improper use of antibiotics and hence poses a considerable socio-economical burden (Rotbart, 2000; Turner, 1998). Moreover, increasing evidence is presented that rhinovirus infections trigger a majority of exacerbations of both asthma and chronic obstructive pulmonary disease (COPD) (Hershenson and Johnston, 2006; Mallia et al., 2007). These conditions are predicted by the World Health Organization (WHO) to be the third leading cause of death worldwide by the year 2030 (http://www.who.int/respiratory/copd/en/). Given the multitude of types, it is unlikely that vaccination against rhinoviruses will ever be feasible, in particular since all serotypes are equally associated with disease manifestations. Therefore, antiviral therapy remains the only therapeutic/prophylactic option.

Antivirals directed against human rhinoviruses could be used to treat the common cold, but could also be employed therapeutically or prophylactically to prevent asthma and COPD exacerbations in high-risk patients. Pleconaril and rupintrivir, respectively a capsid binding compound and a protease inhibitor, have been in clinical development for the treatment of the common cold. Unfortunately, pleconaril was rejected by the FDA due to side effects and rupintrivir was halted because of unsatisfactory activity in natural rhinovirus infection studies (Patick, 2006; Senior, 2002). Currently, pleconaril is in clinical development again for the treatment of rhinovirus infections in high-risk patients with chronic lung diseases (De Palma et al., 2008a,b,c). Besides pleconaril, another capsid binding agent (BTA-798) is in clinical development at Biota Holdings for the same application.

Poliovirus, the prototype of the *Enterovirus* genus and a serious threat in western countries during the past decades, caused many cases of AFP, often with life-long sequelae (Table 1). The use of polio vaccines led to the eradication of polio in the western hemisphere,

although polio remains endemic in several countries in Africa and Asia. Recently, at the request of the WHO, a panel of experts at the National Research Council (NRC) of the USA concluded that additional tools should be developed to tackle polio (Couzin, 2006). More specifically, the development of antivirals against poliovirus was claimed as a necessary condition for the successful worldwide eradication of polio (Couzin, 2006). To date, no such antivirals have so far been approved (Collett et al., 2008).

In the pediatric setting, enteroviruses often infect neonates. Most infections subside mildly or asymptomatically but in some cases, patients develop severe or even life-threatening diseases, such as meningitis, encephalitis, pancreatitis, myocarditis or acute paralysis (Table 1; Sawyer, 2002). Treatment options for children that are hospitalized for these conditions remain symptomatic, as no specific therapy for infections with enteroviruses has been developed so far. As mentioned earlier, EV-71 has (re-)emerged in recent years and more and more often causes epidemics, particularly in Asia (Qiu, 2008). These epidemics usually affect young adults and might result in severe complications, such as meningoencephalitis, AFP or pulmonary edema (which may be fatal). Health care authorities worldwide are insisting upon the rapid development of specific therapy to contain EV-71 epidemics.

The discussed cases clearly illustrate the substantial impact of enteroviruses on human health, in particular in the pediatric setting. Therefore, it is of utmost importance that research focusing on antiviral therapies be urged. First and foremost, it is important to study which enteroviral proteins are the targets of choice for inhibition of viral replication. Research so far has focused mainly on the viral capsid and the proteases of these viruses, whereas replication inhibitors targeting other nonstructural enteroviral proteins have remained largely unaddressed. Within the VIZIER Framework, it was the aim to study non-structural viral proteins as targets for replication inhibition. Several new inhibitors and their viral target were identified.

5.1. Identification of a novel inhibitor of the non-structural enterovirus protein 2C

A first class of inhibitors that we identified were the thiazolobenzimidazoles, which were found to inhibit the replication of several enteroviruses in a dose-dependent manner. More than 20 analogs on an initially identified lead compound were synthesized, and the study of structure-activity relationships revealed that the antiviral activity of the compound could be substantially improved by chemical modification with specific substituents (De Palma et al., 2007). One compound (TBZE-029) with an IC_{50} value of 7 μM (SI > 83) was withdrawn for mode of action studies. Time-of-drug-addition studies indicated that this class of molecules acts at a stage in the viral replication cycle between the early (attachment, entry, uncoating) and late (release) events (De Palma et al., 2008a,b,c).

Next, we demonstrated that specifically viral RNA synthesis but not polyprotein synthesis and/or processing were inhibited by the thiazolobenzimidazoles. Pheno- and genotyping of drug-resistant CV-B3 clones revealed that drug resistance maps to the non-structural protein 2C (De Palma et al., 2008a,b,c). This well conserved protein is essential for viral replication and contains as mentioned earlier three motifs (A, B and C) typical for the ATPase/helicase SF3 (Gorbalenya et al., 1990). Motifs A and B are well documented to be associated with the ATPase activity of the protein, whereas the third motif is presumed to mediate a putative helicase activity. The drug resistance mutations selected for by the thiazolobenzimidazoles were A224V, I227V and A229V. Interestingly, these amino-acid mutations appeared to be clustered just downstream from the ATPase/helicase motif C, which is presumed to be essential for the putative helicase function of the protein. By means of reverse genetics, the role of the identified mutations in the resistant phenotype was confirmed. In particular, the mutations at positions 227 and 229 appeared to be essential to confer drug resistance. Moreover, the generated recombinant viruses proved cross-resistant with previously identified enterovirus 2C-targeting compounds (GuaHCl, HBB and MRL-1237), resistance to which may also be mapped to the same 2C locus (Baltera and Tershak, 1989; Tolskaya et al., 1994). In contrast, they lacked cross-resistance with compounds that were shown to target viral proteins different from 2C (enviroxime and rupintrivir). Studies with the drug-resistant recombinant viruses indicated that the growth kinetics of the mutated viruses were hampered and that they generated smaller plaques than wildtype virus, indicating an essential role in efficient viral replication for the mutated amino acids of motif C.

For poliovirus, it was previously reported that protein 2C exhibits ATPase activity (as predicted by the presence of motifs A and B) (Gorbalenya et al., 1990; Pfister and Wimmer, 1999). Since this activity was never demonstrated for coxsackievirus, we cloned, expressed and purified GST-tagged 2C protein from CV-B3. As expected, time- and concentration dependent ATPase activity could be demonstrated for this protein. An active site mutant containing a mutation in motif A lacked such enzymatic characteristics, confirming specific activity of the protein. Despite the fact the 2C was identified as the viral target of the antiviral thiazolobenzimidazole compounds, the ATPase activity of 2C was not inhibited to any extent in the presence of the compound. Moreover, none of the other previously reported anti-enterovirus compounds targeting 2C could inhibit this enzymatic activity. These observations led us to conclude that either (i), 2C inhibitors that target 2C do inhibit the enzymatic ATPase activity, but that this inhibition cannot be mimicked using the purified 2C protein (since the conditions are different from those of the complete replication complex in a cellular context) or that (ii), 2C inhibitors interfere with a 2C function that is different from its ATPase activity. More detailed studies on the mode of action of the thiazolobenzimidazoles are ongoing.

5.2. Identification of a novel inhibitor of the non-structural enterovirus protein 3A

Another compound that was identified to selectively inhibit the replication of several entero- and rhinoviruses was TTP-8307 (De Palma et al., 2009). This compound exhibited an IC_{50} value of 1.2 μM against CVB3 (SI > 83) and inhibited viral RNA synthesis in a dose-dependent manner. Akin to the thiazolobenzimidazoles, TTP-8307 had no effect on the synthesis and processing of the viral polyprotein (De Palma et al., 2009). A genotypic feature that was shared by all TTP-8307-resistant variants was the presence of at least one mutation in non-structural protein 3A (De Palma et al., 2009). As far as known, this protein serves as a key scaffolding partner in the viral replication complex, and interacts with several other viral proteins, possibly to assemble the replication complex in a conformationally correct manner. Four mutations (I8T, V45A, I54F and H57Y) that were identified in this protein were reintroduced in an infectious full-length clone of CVB3, which successfully led to a reconstruction of the resistant phenotype (De Palma et al., 2009). Moreover, cross-resistance was observed with enviroxime, the sole 3A-targeting enterovirus inhibitor reported to date (Heinz and Vance, 1995, 1996). It is hypothesized that TTP-8307 disrupts certain interactions of 3A with other cellular and/or viral proteins. Prior to the discovery of TTP-8307, only one compound (enviroxime, reported more than 20 years ago) was known to inhibit enterovirus replication by targeting protein 3A. The concept of viral inhibition by targeting 3A is thus confirmed and extended by our findings. TTP-8307 can now be added to the toolbox to study the role of proteins involved in replication complex formation.

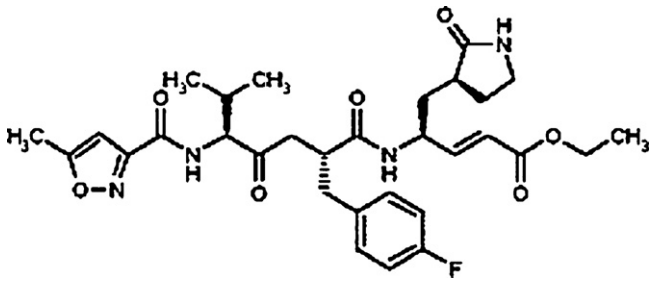

Fig. 6. Rupintrivir (AG7088) (Patick et al., 1999; Binford et al., 2007).

5.3. Enteroviral 3C proteases as targets for antiviral chemotherapy

In comparison to other viral targets, the enteroviral proteases offer the advantage of convenient assays being available. Also, at least the 3C proteases (3C^{pro}s) are usually relatively easily produced in a soluble form by recombinant DNA technology. So far, most activities in the field of 3C^{pro} inhibitor design focused on the enzyme from human rhinoviruses (Matthews et al., 1999; Johnson et al., 2002).

Rupintrivir (AG7088) (Fig. 6), an inhibitor of HRV 3C^{pro} (Matthews et al., 1999; Binford et al., 2005) was shown to block the CV-B3 enzyme with a half-maximal effective concentration (EC_{50}) of 1.3 μM. It has been demonstrated that replacement of the ethyl ester in position P2′ of AG7088 by large aromatic moieties will lead to a significant enhancement of affinity to the enzyme (Lee et al., 2007). This can be ascribed to a hydrophobic interaction with residue Tyr22 of the 3C^{pro}. Repetitive cultivation of CV-B3 in the presence of rupintrivir raised three resistance mutations in the 3C^{pro}, T68A and N126Y. We have tried to understand the effects of these mutations on the basis of the three-dimensional structure (Tan et al., in preparation). They occur in loops which, at first glance, are quite remote from the inhibitor-binding site (see Fig. 7). Both mutant proteins were crystallized and subjected to X-ray diffraction analysis. The preliminary results indicate that the structure of the T68A mutant is close to that of the wild-type enzyme, whereas the N126Y mutation apparently leads to major rearrangements of the structure. How these changes affect rupintrivir binding remains to be studied in detail. Recombinant viruses harboring these mutations have been demonstrated to be resistant against rupintrivir.

In addition to their proteolytic activity, the 3C proteases also bind to viral RNA through recognizing special structural features near the 5′ terminus of the genome. However, whereas the proteolytic substrate pocket of 3C^{pro} is quite well conserved in entero- and rhinoviruses, the details of RNA binding may not. For example, the mature poliovirus 3C^{pro} was reported to have only very low affinity to RNA, whereas its precursor 3CD^{pro} interacts with a loop of a 5′-terminal cloverleaf-like RNA element (the so-called D-loop) in the presence of the host-cell's poly(rC)-binding protein 2 (PCBP2) or the viral 3AB protein. The ribonuclear protein complex formed this way is necessary for the initiation of replication (Andino et al., 1993; Gamarnik and Andino, 1997; Harris et al., 1994; Xiang et al., 1995). On the other hand, mature 3C^{pro} of CV-B3, HRV-14, and HAV, as well as the precursor protein 3CD^{pro} of CV-B3 interact with stem-loop D (which is a subdomain of the 5′-cloverleaf RNA) in the absence of additional protein factors (Leong et al., 1993; Zell et al., 2002; Ohlenschläger et al., 2004; Ihle et al., 2005; Kusov and Gauss-Müller, 1997). It is the conformation of the D-loop rather than its sequence that determines the interaction with the viral protease (Zell et al., 2002; Ohlenschläger et al., 2004; Ihle et al., 2005). The 3C^{pro} binds viral RNA mainly through a conserved loop of the sequence Lys-Phe-Arg-Asp-Ile that connects the two β-barrel domains and is located on the side of the molecule opposite to the protease active site (Gorbalenya et al., 1989; Hämmerle et al., 1992; Matthews et al., 1994). Residues within the FMDV 3C protein have been shown to be involved in the interaction with viral RNA and with VPg uridylylation activity and virus replication (Nayak et al., 2006). Once the interaction between the 3C^{pro} and the 5′-terminal RNA of the virus will be better characterized, it could also become a target for structure-based drug discovery.

5.4. Comparative study on the activity of anti-enterovirus compounds against poliovirus

Polio eradication is within sight. In bringing the world close to this ultimate goal, the Global Polio Eradication Initiative (GPEI) has relied almost exclusively on the live, attenuated oral poliovirus vaccine (OPV). However, as eradication nears, continued OPV use becomes less tenable due to the incidence of vaccine associated paralytic poliomyelitis (VAPP) and disease caused by vaccine-derived polioviruses (VDPV) that have reverted to neurovirulence. Once wild poliovirus transmission is interrupted globally, OPV use will stop. This will leave the inactivated poliovirus vaccine (IPV) as the only weapon to defend a polio-free world. Outbreaks are expected post-OPV cessation, and there are doubts regarding the ability of IPV to control outbreaks. A study group convened by the US National Research Council concluded that the development of antivirals against poliovirus is absolutely required to successfully finalize the global eradication of polio, since immunocompromised individuals may become long-term

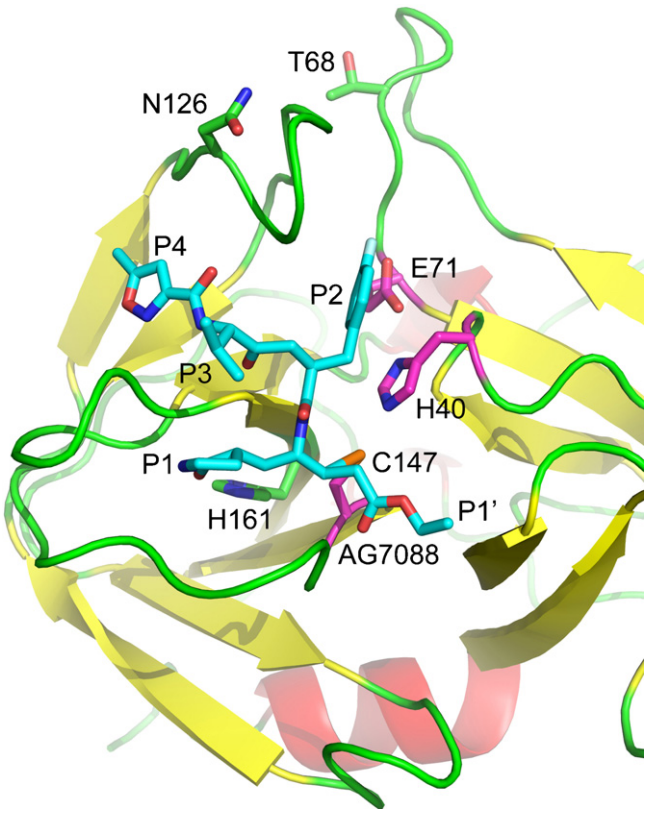

Fig. 7. Model of the complex between rupintrivir (AG7088) and the 3C protease of coxsackievirus B3. The side chains P4–P1′ of the inhibitor, filling the subsites S4–S1′ of the enzyme, are shown. Residues involved in the catalytic triad are indicated, as is His161 which interacts with the P1 lactam moiety of the inhibitor at the bottom of the S1 subsite. The nucleophilic Cys147 forms a covalent bond with the vinylogous ethyl ester. The sites where mutations confer resistance against rupintrivir (Asn126, Thr68) are located on loops relatively remote from the inhibitor-binding site; however, there replacement by Tyr and Ala, respectively, may remodel the loops shaping the S2 pocket (filled by the P2 fluorobenzylgroup in the rupintrivir complex).

excretors of drifted OPV strains. Since little data are reported on anti-poliovirus compounds, we initiated a comparative study on the anti-poliovirus activity of previously and newly discovered enterovirus inhibitors. This study revealed that several compounds, including TTP-8307 (IC_{50} = 0.51 μM against PV-1, SI > 196) which was discovered during this project, have excellent and potent activity against *in vitro* replication of all tested strains (De Palma et al., 2008a,b,c) (4). As antivirals against polio are the final missing key to the global eradication of polio, it is crucial to have molecules at hand that can be advanced to anti-poliovirus drugs. This study revealed that several of the evaluated molecules are potent and selective inhibitors of poliovirus replication. Moreover, some of these compounds [e.g. rupintrivir, developed by Pfizer, see above (IC_{50} = 22 nM, SI > 4545)] have already been used in clinical trials (against rhinovirus infections) and could therefore more rapidly be developed as a drug to be used in humans. Some of the pharmaceutical companies are currently considering further development of some of these compounds into anti-poliovirus drugs.

6. Conclusions

The design of new antiviral compounds that target specific steps in the viral replication cycle and the modification of existing antiviral compounds are the current approaches for developing antiviral drugs. However, the emergence of drug-resistant variants could occur due to high mutation rates of EVs. The use of drug combinations seems to be a way to delay or prevent the appearance of drug-resistant viruses. For example, 'cocktail therapy' extends the life of HIV-infected patients who suffer from infection with drug-resistant viruses. Many potent EV inhibitors, mentioned earlier, act on various targets in viral replication cycles. Some have been or are being tested in clinical trials. These compounds, used alone or in combination, may have the potential for the treatment of EV infection. To date, no powerful prophylaxis of non-polio EV infection is available. No antiviral agent has been approved by the FDA for treating EVs. The continued development of drugs for the treatment of picornaviral infection is essential. Moreover, many other potentially interesting targets need to be further explored.

7. Future perspectives

In the course of the VIZIER project, several inhibitors of picornavirus replication were identified (some of which are not listed in this review). A major challenge remains to determine the precise molecular interaction of these inhibitors, in particular those targeting the non-structural proteins 2C and 3A. To this end, crystallographic and molecular-biological tools will be employed and are expected to generate new insights into the function of these proteins. The investigations on antiviral compounds against picornaviruses have so far been focused on enteroviruses, since they have potential to cause larger outbreaks, and some types have been shown to lead to chronic infections. There are, however, now several new non-enterovirus human pathogens discovered during the past few years, some of these with severe disease manifestations. There may thus be a need for broader anti-picornavirus compounds. The development of such compounds may be facilitated when more structures will have been determined of the viral proteins from members of several genera within the *Picornaviridae*.

Acknowledgements

This work was supported the EU (Integrated Project VIZIER FP-6 contract No. 511960).

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