



**HAL**  
open science

## **2C protein of Enterovirus: key protein of viral replication and antiviral target**

Priscila El Kazzi, Carole Yaacoub, Ziad Fajloun, Patrice Vanelle, Etienne Decroly, Bruno Coutard, Karine Barral

► **To cite this version:**

Priscila El Kazzi, Carole Yaacoub, Ziad Fajloun, Patrice Vanelle, Etienne Decroly, et al.. 2C protein of Enterovirus: key protein of viral replication and antiviral target. *Virologie*, 2023, 27 (3), pp.35-49. 10.1684/vir.2023.1002 . hal-04252121

**HAL Id: hal-04252121**

**<https://hal.science/hal-04252121>**

Submitted on 27 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **2C protein of Enterovirus: key protein of viral replication and antiviral target**

**Protéine 2C d'Entérovirus : protéine clé de la réplication virale et cible antivirale**

Priscila El Kazzi<sup>1</sup>, Carole Yaacoub<sup>2,3</sup>, Ziad Fajloun<sup>3,4</sup>, Patrice Vanelle<sup>5</sup>, Etienne Decoly<sup>1</sup>, Bruno Coutard<sup>2#</sup>, Karine Barral<sup>5#\*</sup>

<sup>1</sup> AFMB, CNRS UMR 7257, Aix-Marseille Université, Case 925, 163 Avenue de Luminy, 13288 Marseille Cedex 09, France.

<sup>2</sup> Unité des Virus Emergents (UVE), Aix-Marseille Université, IRD 190, Inserm 1207, Marseille, France

<sup>3</sup> Laboratory of Applied Biotechnology (LBA3B), Azm Center for Research in Biotechnology and its Applications, EDST, Lebanese University, Tripoli 1300, Lebanon.

<sup>4</sup> Department of Biology, Faculty of Sciences 3, Lebanese University, Michel Slayman Tripoli Campus Ras Maska, Tripoli 1352, Lebanon.

<sup>5</sup> Aix-Marseille Université, CNRS, Institut de Chimie Radicalaire (ICR) UMR7273, Equipe Pharmaco-chimie Radicalaire, Faculté de Pharmacie, 27 Boulevard Jean Moulin, CS30064, 13385 Marseille Cedex 05, France

# The authors contributed equally

\* Corresponding author: karine.barral@univ-amu.fr

## **Abstract**

Enteroviruses (EVs) include many human pathogens of increasing public health concern. These EVs are often associated with mild clinical manifestations, but they can lead to serious complications such as encephalitis, meningitis, pneumonia, myocarditis or poliomyelitis. Despite significant advances, there is no approved antiviral therapy for the treatment of enterovirus infections. Due to the high genotypic diversity of EVs, molecules targeting highly conserved viral proteins may be considered for developing a *pan-EV* treatment. In this regard, the ATPase/Helicase 2C, which is a highly conserved non-structural protein among EVs, has essential functions for viral replication and is therefore an attractive antiviral target. Recent functional and structural studies on the 2C protein led to the identification of molecules showing *ex vivo* anti-EV activity and associated with resistance mutations on the coding sequence of the 2C protein. This review presents the current state of knowledge about the 2C protein from an antiviral target perspective and the mode of action of specific inhibitors for this therapeutic target.

**Keywords:** Enterovirus, protein 2C, inhibitors, antivirals.

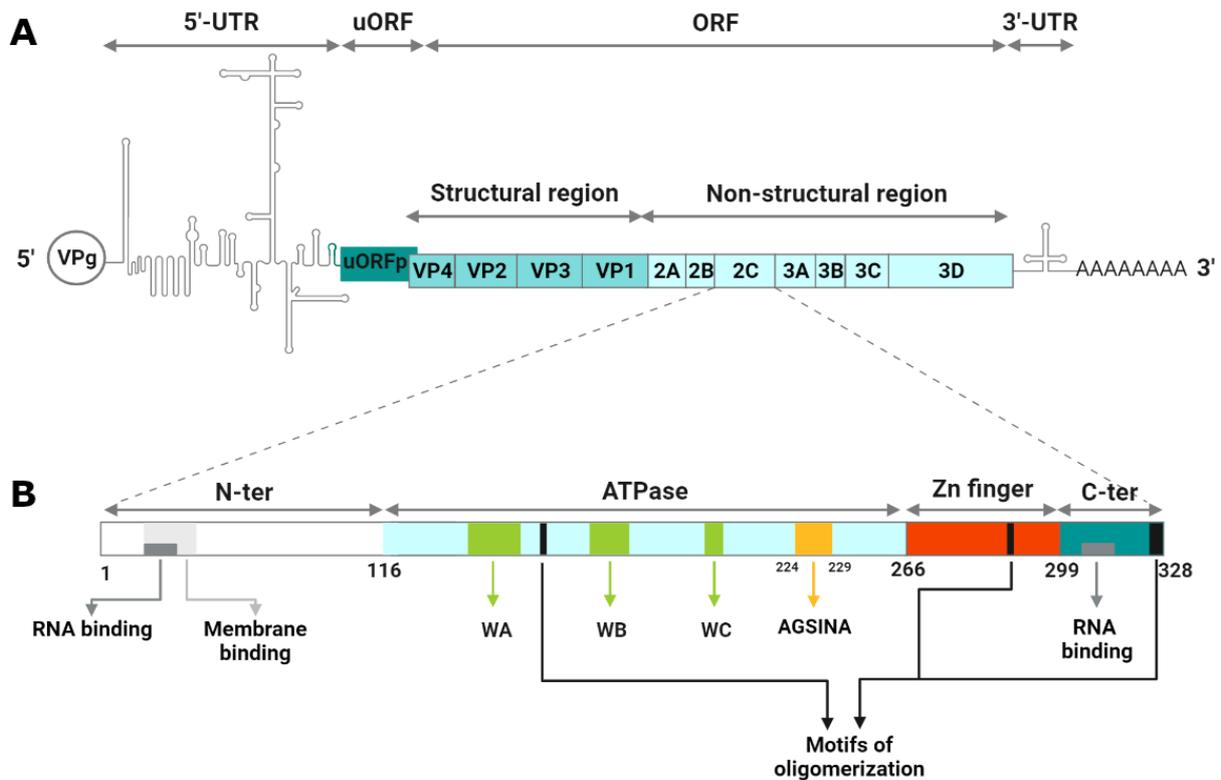
## Introduction

Several dozen enteroviruses infect human beings and cause nearly three billion infections per year. Viruses belonging to the genus *Enterovirus* (family *Picornaviridae*) include a group of viruses classified in 15 species: enteroviruses (EV) A to L and rhinoviruses (RV) A to C. Human-infectious EVs are grouped into seven species, EV-A, EV-B, EV-C, EV-D, RV-A, RV-B and RV-C. These species include, among others, polioviruses (PV; EV-C) pathogens responsible for poliomyelitis, coxsackieviruses (CVA and CVB; EV-A to EV-C) and echoviruses (E; EV-B) [1]. Non-polio EV (NPVE) infections are very common and often associated with benign clinical manifestations (mild respiratory and gastrointestinal illnesses, rash). However, they can lead to serious complications such as encephalitis, meningitis, pneumonia, myocarditis, pancreatitis or poliomyelitis, especially in children under five years of age and people with weakened immune system [2-3]. Recently, several EVs have emerged as pathogens of increasing public health concern. For example, EV-A71 in Southeast Asia [4] and EV-D68 in North America [5] are causing epidemics associated with severe neurological complications. Since 2016, in France, an increased circulation of these two viruses has been associated with severe neurological manifestations. On the other hand, rhinoviruses are one of the most common causes of respiratory disease worldwide. Most of the time, rhinovirus infections go unnoticed, causing a mild flu-like illness. They can, however, trigger some serious respiratory illnesses such as bronchiolitis in children and life-threatening pneumonia in the elderly and immunocompromised adults [6-9]. Concerning coxsackievirus B (CV-B), they can be associated with aseptic meningitis, as well as neonatal septicemia [10].

There is a great genetic diversity in enteroviruses, which can be explained by frequent recombination events and by the mutagenic properties of the viral polymerase [11-13]. Although vaccination has almost eradicated polio worldwide and several EV-A71 vaccines are on the market in China [4], there is, to date, no effective antiviral treatment for human EVs, highlighting the need to develop new broad-spectrum therapeutic strategies. The risk associated with EVs is illustrated by the recent resurgence of Poliovirus infection in Europe and Israel, despite high vaccination coverage. Two therapeutic approaches have been considered, one targeting the host factors responsible for viral spread and the other relying on the development of direct-acting antivirals (DAAs) targeting viral proteins [14].

EVs are small (30 nm in diameter), non-enveloped, icosahedral capsid viruses with a single-stranded RNA genome of positive polarity of about 7.2 to 8.5 kb. Their genome, lacking a cap, has an IRES (Internal Ribosome Entry Site) structure in its 5' region and is polyadenylated at its 3' end (Figure 1). The genomic RNA is composed of two "ORFs" (Open Reading Frame), named ORF<sub>u</sub> and ORF. ORF<sub>u</sub> encodes a small 8 kDa protein (uORF<sub>p</sub>) involved in pathogenesis [15] and ORF encodes a long polyprotein of 250 kDa that is cleaved by the mature viral proteases 2A<sub>pro</sub>, 3C<sub>pro</sub> and 3CD<sub>pro</sub> into ten proteins (Figure 1), including three capsid proteins (VP2/VP4, VP1 and VP3) and seven non-structural replication proteins (2A-2C and 3A-3D). Currently, DAAs described in the literature are either directed against structural proteins to block viral infection at an early stage [16-17] or directed against classical antiviral targets, such as 3C protease and 3D polymerase, to inhibit viral replication/transcription mechanisms [16- 17]. Several inhibitors of these viral proteins have

been identified and some have been tested in clinical trials, mainly to treat rhinovirus infections, but the trials have failed due to their low efficacy or toxicity [18-20].



**Figure 1.** Schematic representation of the EV genome and 2C protein architecture. **(A)** Genomic and protein organization of EVs. The EV genome is composed of two open reading frames (uORF and ORF) flanked by two untranslated regions, 5'UTR and 3'UTR. The uORF overlaps the 5'UTR and ORF, and encodes a small 8 kDa protein (uORFp). The main ORF of EV encodes four structural proteins (VP1-VP4) and seven non-structural proteins (2A- 2C, 3A-3D). **(B)** Diagram of the organization of the 2C protein. The N-terminal (N-ter) portion of the 2C protein has an amphipathic helix interacting with intracellular membranes (light gray). The ATPase domain (light blue) contains the conserved Walker A (WA), Walker B (WB) and Walker C (WC) motifs (green) characteristic of the SF3/AAA+ superfamily helicases. The location of the AGSINA sequence is also highlighted in orange on this domain. Downstream of the ATPase domain is the zinc finger domain (red), a Cys-rich region involved in zinc coordination that adopts a characteristic zinc finger fold. The amphipathic C-terminal helix (C-ter) has an oligomerization motif (black) that mediates 2C-2C interaction and self-oligomerization of 2C proteins by interacting with two other motifs located at the interface of the ATPase domain and the Zn finger of the adjacent 2C. The 2C protein has two putative RNA-binding sites (dark gray), the first is on the N-ter amphipathic helix and the second on the C-ter.

The 2C protein is highly conserved among Enteroviruses and plays a crucial role in almost all stages of the virus life cycle (Figure 1). Recent structural and functional studies led to a better understanding of its pleiotropic roles during viral infection and, *de facto*, to the identification of specific inhibitors. The 2C protein is, thus, considered an attractive target for the development of broad-spectrum DAAs to treat EV infections [21].

In this review, we present the current knowledge of the structure and functions about the 2C protein of EVs, and we describe advances in the development of specific 2C inhibitors and their mode of action.

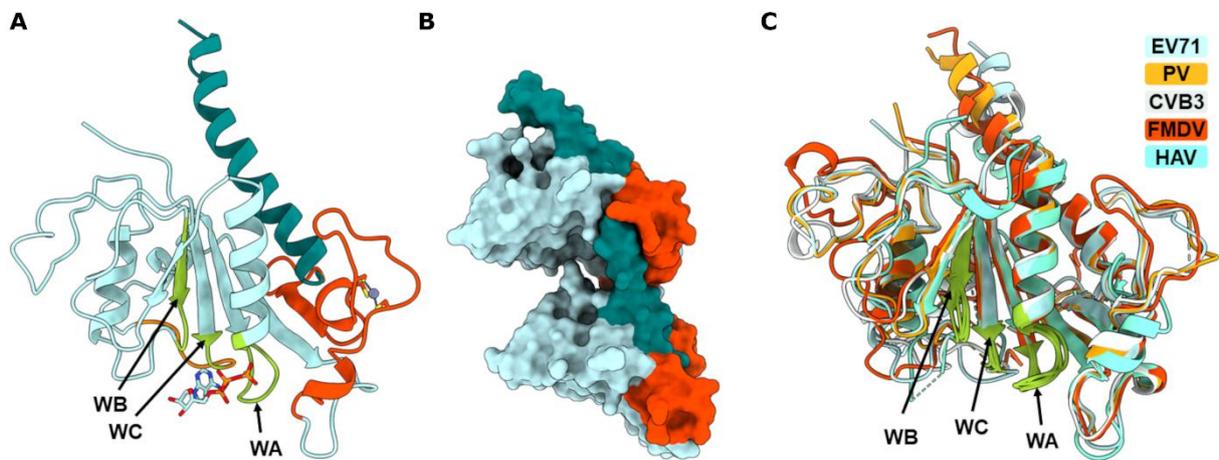
### **Structure and functions of the 2C protein in Enterovirus life cycle**

In the 1990s and 2000s, many biological functions of the 2C protein in the viral cycle were highlighted. Studies have shown an ATPase/Helicase activity of this protein [22-23], its involvement in the RNA replication process, the viral decapsidation [24-28], the rearrangement of the host cell membrane [29-31], the recruitment of host factors [32], the encapsidation and the viral morphogenesis [33] as well as the disruption of the antiviral cellular response [34-35]. An unconventional single-stranded ribonuclease (sbRNase) activity has also been attributed to the 2C [36]. However, the biological role of this activity remains poorly understood. Furthermore, the cellular E3 ligase TRIM7, which plays an antiviral role against human EVs, has been described as an actor of the ubiquitination of the C-terminal Glutamine of the 2C protein, leading to its degradation by addressing the cellular proteasome [37-38].

By sequence homology, EV 2C proteins were classified as superfamily 3 (SF3) helicases. In 2015, Xia *et al.* demonstrated the catalytic helicase activity associated with the 2C protein, confirming the prediction of sequence analyses and the central role of the 2C protein in viral RNA replication [39]. In addition to RNA-helicase-ATP-dependent activity, 2C protein possesses an ATP-independent chaperone activity that destabilizes helices bidirectionally, facilitates strand hybridization and the formation of complex RNA structures, independently of ATP hydrolysis. The catalytic helicase activity is carried by the ATPase domain of the 2C protein of EV-A71, while the C-terminus is essential for its ability to chaperone RNA.

Like all superfamily 3 (SF3) helicases, the 2C protein requires multimeric structural organization in order to acquire its helicase activity. The multimerization of recombinant 2C protein from PV1 and E30 has been previously observed [40-41]. Given the relationship between oligomerization and helicase and/or ATPase activity, the multimerization interface could be an interesting antiviral target, as recently demonstrated for the 2C protein of EV-A71 [42] and foot-and-mouth disease virus (virus of the genus *Aphtovirus* of the *Picornaviridae* family [43]. Structural characterization of the 2C protein of two EVs, EV-A71 (PDB 5GQ1 and 5GRB) and PV-1 (PDB 5Z3Q), has led to a better understanding of the organization of the different functional domains [44-45]. The crystallographic structure of the soluble part of the 2C protein (116-329 aa) of EV-A71 was solved to 2.5 Å by Guan *et al.* in 2017. For this purpose, the N-terminal region (115 amino acids) corresponding to a region predicted as partially helical and amphipathic was deleted. This is the first high-resolution structure of 2C protein of the *Picornaviridae* family. Structurally, this 2C protein consists of an ATPase domain, an atypical cysteine-rich zinc-binding domain, and a C-terminal helical domain of amphipathic nature (Figure 2A). The ATPase domain of protein 2C has the characteristic signature of the AAA+ superfamily of ATPases and is formed by the conserved

Walker A and B motifs as well as the Walker C motif typical of SF3 helicases (Figure 1B and 2A).



**Figure 2.** Overall structure of the 2C protein. (A) Ribbon model of the crystal structure of EV-A71 protein 2C- $\Delta$ 115 complexed with ATP- $\gamma$ -S (PDB 5GRB). The ATPase, zinc finger, and C-terminal domains are highlighted in light blue, red, and duck blue, respectively. Walker A (WA), Walker B (WB), and Walker C (WC) motifs of the ATPase domain catalytic site are highlighted in green. (B) Overview of the assembly process of two protomers of EV-A71 protein 2C (PDB 5GRB). Polymerization of the 2C protein occurs through a specific interaction between the C-terminal portion of one protomer (blue duck) and the zinc finger domain of the adjacent protomer (red). (C) Superposition of the 2C crystal structure of EV-A71 (PDB 5GRB, chain B) with the 2C of PV (PDB 5Z3Q, chain A), CV-B3 (PDB 6T3W, chain A), FMDV (PDB 7E6V, chain A) and HAV (PDB 7XT3, chain A). The WA, WB, and WC motifs of the ATPase domain of various 2C proteins are shown in light green.

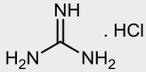
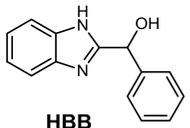
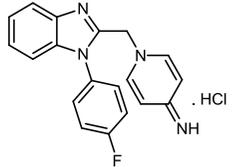
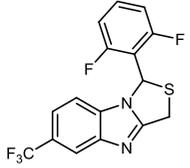
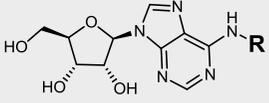
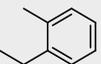
Structure resolution of the ATPase domain of the 2C protein from various *Picornaviridae* has revealed that this domain is highly conserved beyond the EV genus, which is not the case for the specific zinc finger domain found specifically in EV (Figure 2C). Unlike other ATPases, the C-terminus of 2C proteins forms a helix that participates in homo-oligomerization by a specific interaction with the neighboring protomer at a concave pocket formed between the ATPase site and the zinc finger domain (Figure 2B). This homo-oligomerization is required for ATPase activity of the 2C protein and replication of EV-A71, and allows the formation of an ATP binding site at the interface between two protomers [46]. This assembly leads to the formation of a hexamer, which was modeled in structural studies of EV-A71 and PV 2C proteins, and then experimentally confirmed by electron microscopy experiments performed with a chimeric recombinant 2C protein from CV-B3. Recently, Hurdiss *et al.* identified the presence of a site adjacent to the ATP binding site in the 2C protein of CV-B3 [46]. Mutations in this site are generally deleterious for viral replication. In addition, synthetic molecules capable of interacting with the oligomerization domains inhibit both the ATPase activity of the 2C protein and viral replication. These studies confirm that inhibition of 2C protein

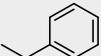
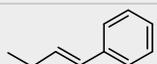
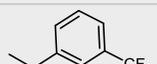
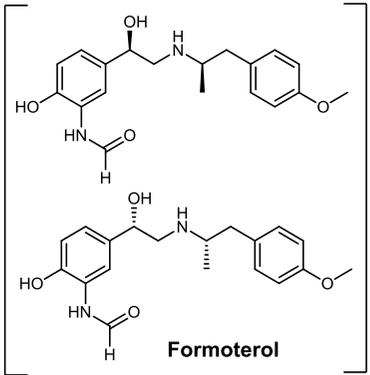
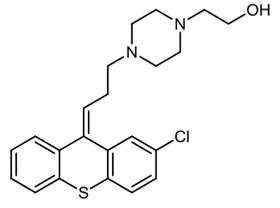
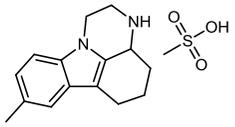
functions, by disrupting 2C-2C self-oligomerization or by inhibiting its catalytic activities, is a relevant strategy for inhibiting EV replication.

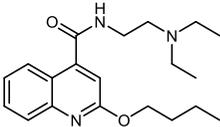
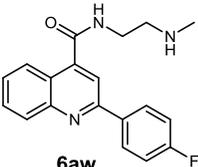
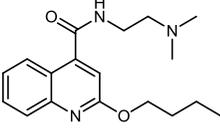
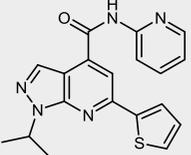
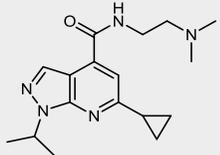
### **Antivirals targeting the 2C protein of Enteroviruses**

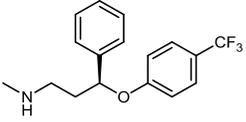
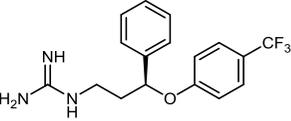
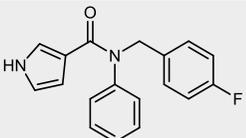
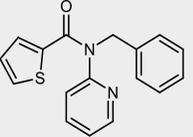
So far, about twenty molecules demonstrating *ex vivo* anti-EV activity and associated with resistance mutations on the coding sequence of the 2C protein have been identified (Table 1). These include Guanidine-HCl, HBB, MRL-1237, TBZE-029, Metrifudil, N6-benzyladenosine and its analogues, Formoterol, Zuclopenthixol, Pirlindole mesylate, Dibucaine and its analogues, pyrazolopyridine derivatives, Fluoxetine and its analogues, N-benzylamide derivatives, and the 2CL peptide. The inhibition properties of these molecules are described in the following subchapters. For some of them, their mode of action is not completely elucidated. Guanidine-HCl and Fluoxetine, identified by a drug repositioning approach, are the most studied 2C inhibitors.

**Table 1.** Chemical structure, anti-enteroviral properties and EVs targeted by the known 2C inhibitors.

Molecules	Enteroviruses tested	Residues of resistance mutations	EC <sub>50</sub> (μM)	SI	References
 <p><b>Guanidine-HCl</b></p>	PV-1 EV-A71 RV-87 EV-D68 CV-B3 E-9	I142, A143, N179, M187, S225 I227, A233 I127, T173, M187, M193, S224 nd I127, T173, M187, M193, S224 A224, I227, A229 E64, A133	nd 305 91 91 (Fermon) nd nd	12 40 40 nd nd	48, 50, 51 53, 54 54 54, 55 46, 57 52
 <p><b>HBB</b></p>	E-9 PV-1 CV-B3	I227, A229 nd A224, I227 and A229	nd 26 (Sabin 2) 70	15 nd	59, 52 62 46, 57
 <p><b>MRL-1237</b></p>	PV-1 CV-B3	I120, F164 A224, I227 and A229	11 (Mahoney) 2.9 (Sabin 2) nd	51 190 nd	62 46
 <p><b>TBZE-029</b></p>	CV-B3	A224, I227 and A229	0.18	nd	57
 <p><b>R =</b>  <b>Metrifudil</b></p>	EV-A71	E325	1.3	> 38	64

<p>R =  <b>N6-Benzyladenosine</b></p>	EV-A71	H118, I324	0.10	33000	64
<p>R =  <b>7</b></p>	EV-A71	nd	4.3	> 101	65
<p>R =  <b>6</b></p>	EV-A71	nd	0.66	645	66
<p>R =  <b>10</b></p>	EV-A71	nd	0.068	> 3456	67
<p> <b>Formoterol</b></p>	CV-B3 EV-A71 PV-1 EV-D68 RV-A2 RV-B14	nd	2.36 0.83 0.88 0.51 0.30 0.34	nd	68
<p> <b>Zuclopenthixol</b></p>	CV-B3	A224, I227 and A229	nd		68
<p> <b>Pirlindole mesylate</b></p>	CV-B3 EV-D68	A224, I227 and A229	9.91 9.24	nd	68

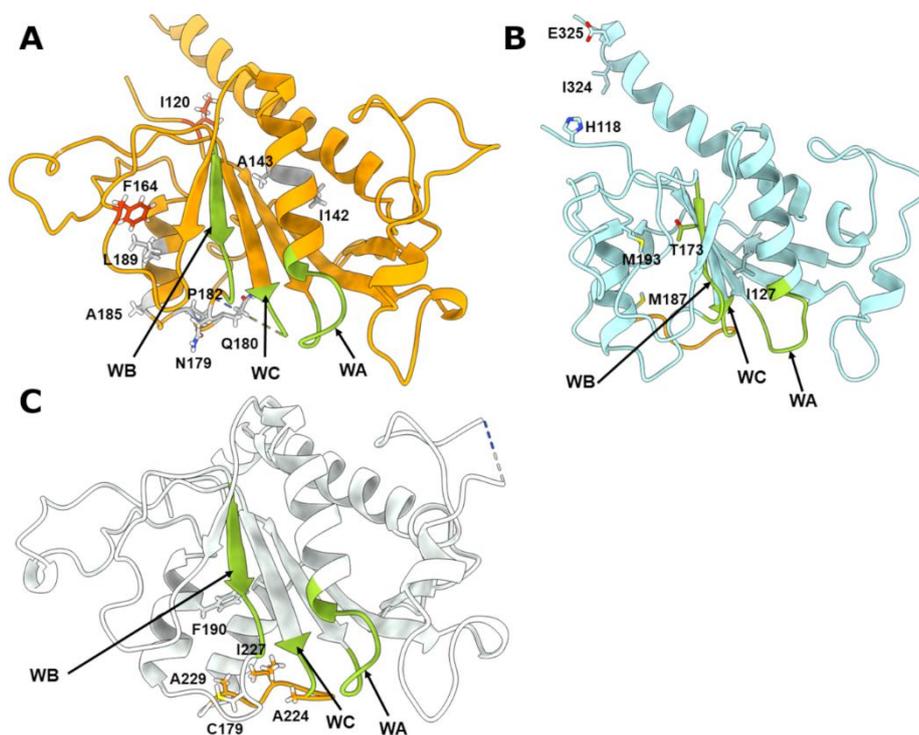
 <p><b>Dibucaine</b></p>	CV-B3 EV-A71 EV-D68	A224, I227 and A229	1.60 7.59 3.03	nd	68
 <p><b>12a</b></p>	EV-D68	nd	0.04	1057	70
 <p><b>6aw</b></p>	EV-D68 EV-A71 CV-B3	nd	0.2 3.1 0.2	162 11 200	71
 <p><b>6i</b></p>	EV-A71	nd	1.2	85	72
 <p><b>JX040</b></p>	EV-A71 CV-B3	nd	0.5 0.8	>400 >250	74
 <p><b>7d</b></p>	EV-D68 EV-A71 CV-B3	D183, D323	0.1 0.1 0.2	2000 2000 625	75

 <p><b>(S)-Fluoxetine</b></p>	CV-B3 EV-D68 RV-A2 RV-B14	C179, F190, A224, I227, A229	0.42 0.67 7.95 6.3	71 42 3.6 4.5	77
 <p><b>(S)-2b</b></p>	CV-B3 EV-D68 RV-A2 RV-B14	C179, A224, I227 and A229	0.19 0.11 7.49 0.98	315 545 8 61	83
 <p><b>12b</b></p>	EV-A71 CV-B3 PV-1 CV-A24 EV-D68 RV-A2 RV-B14	nd	0.49 0.0029 1.39 1.33 0.086 2.75 0.095	nd	84
 <p><b>R523062</b></p>	EV-D68 EV-A71	I227, Q322	4.1 18	29 7	85
YGRKKRRQRRRGSGREYNNRSAIGNTIEALFQ <b>2CL peptide</b>	EV-A71 CV-A16 CV-B3 E-11		1.35 2.16 2.29 0.38	>111 nd nd nd	42

EC<sub>50</sub>, 50% effective (virus-inhibiting) concentration; SI, selectivity index (CC<sub>50</sub> / EC<sub>50</sub>); nd, not determined.

### ***Guanidine-HCl***

Guanidine-HCl is a low molecular weight molecule (Table 1), approved by the FDA for the treatment of Lambert-Eaton myasthenic syndrome (LEMS) [47] but is no longer commonly used because of its toxicity (nephrotoxicity, hepatotoxicity, myelotoxicity and cardiotoxicity). Guanidine-HCl inhibits the replication of several EVs in cell culture, including PV [48-51], E-9 [52], EV-A71 [53-54], RV-87 [54] and several strains of EV-D68 ( $EC_{50} \sim 100 \mu\text{M}$ ) [54]. Despite its low *ex vivo* antiviral potency, Guanidine-HCl has shown *in vivo* antiviral efficacy in respiratory and neurological infection models of EV-D68 [55]. In 1997, Barton and Flanagan [56] demonstrated that Guanidine-HCl inhibits one or more function(s) carried by the 2C protein that is involved in the initiation of negative-stranded, but not positive-stranded, RNA synthesis and elongation. In addition, several studies have reported that Guanidine-HCl can inhibit ATPase activities [23] and helicase activities of EV-A71 protein 2C [53] and CV-B3 [57]. Its mechanism of antiviral action has been particularly studied in PV. These studies revealed that resistance to Guanidine-HCl is located in the 2C region (Figure 3A). This resistance is mainly attributed to two mutations, a "class N" mutation (Asn mutated at position 179) or a "class M" mutation (Met mutated at position 187), located in a loop adjacent to the Walker B motif. In addition to these two main mutations, a few additional mutations in the PV 2C protein at positions 142, 143, 225, 227 and 233 confer resistance to Guanidine-HCl (Figure 3A).



**Figure 3.** Mapping of resistance and/or dependency mutations on different motifs of protein 2C. (A) Resistance and/or dependency motifs to Guanidine-HCl as well as to MRL-1237 are shown on the structure of 2C protein of PV (PDB 5Z3Q) in gray and red, respectively. (B) Residues of resistance to Guanidine-HCl (I127, T173 WB motif; M187 and M193), Metrifudil (E325), and N6-benzyladenosine (H118 and I324 key C-terminal part for hexamerization) are shown on the structure of EV-A71 protein 2C (PDB 5GRB). (C) Resistance motifs to Guanidine-HCl, HBB, MRL-1237, TBZE-029, Dibucaine, Pirlindole, Zuclopenthixol, and Fluoxetine are shown on the structure of the 2C protein of CV-B3 (PDB 6T3W). Residues A224, I227, and A229 of the AGSINA loop (orange) are resistance residues common to the different inhibitors listed.

Residues C179 and F190 are for Fluoxetine only. (A-C) Residues of WA, WB and WC motifs of the ATPase catalytic site domain are highlighted in green.

The action of Guanidine-HCl has been studied in other EVs, with mutations at positions 127, 173, 187, 193 and 224 of EV-A71 (Figure 3B), at positions E64 and A133 of E-9, and at positions A224, I227 and A229 of CV-B3 (immediately downstream of the Walker C helicase motif) (Figure 3C), conferring resistance. It was also shown in recombinant protein that the ATPase activity of 2C is inhibited by Guanidine-HCl and that resistance mutations confer ATPase activity with improved tolerance to Guanidine-HCl. Remarkably, some resistance mutations have led to the genesis of resistant strains whose *ex vivo* replication is conditioned by the presence of Guanidine-HCl in the culture medium [51]. This dependency mechanism can be explained by the deleterious effect of the resistance mutation on ATPase activity, which is partly restored by the presence of Guanidine-HCl at sub-millimolar concentrations [46]. Guanidines are universally present in the biological environment and can bind to the surface of viral RNA remodeling proteins to alter their conformations, electrostatic states, and protein-protein or protein-RNA interactions, thereby inhibiting these RNA remodeling activities. The development of less toxic and more selective Guanidine-HCl derivatives may lead to the identification of new broad-spectrum DAAs against EVs.

#### ***HBB, MRL-1237 and TBZE-029***

HBB [*1H-benzimidazol-2-yl*(phenyl)methanol] is another compound, identified decades ago, which selectively inhibits viral replication of most EVs (Table 1) [58]. Sequence analysis of HBB-resistant viruses and characterization of E-9, revealed that HBB susceptibility corresponds to the 2C protein, following the identification of mutations at positions I227 and A229 of the target protein [59-60]. Three mutations in the 2C protein of CV-B3, A224V, I227V and A229V, located downstream of the Walker C [46,57,61] confer resistance to HBB, as well as two of its derivatives, namely MRL-1237 [62] and TBZE-029 [63], that share a common benzimidazole motif (Table 1), despite their different antiviral spectra (Figure 3C). A study done by Shimizu *et al.* [62] on PV showed that independent F164Y and I120-Y mutations could be responsible for the reduced susceptibility to MRL-1237 (Figure 3A). At this point, it is important to note that some loci where MRL-1237 resistance mutations are found are close or identical to those mentioned for Guanidine-HCl resistance, and some mutations may induce cross-resistance [62], suggesting the presence of resistance mutation hot-spots in the multimerization domains (Figure 3).

#### ***Metrifudil, N6-Benzyladenosine and derivatives***

In 2008, Arita *et al.* demonstrated that two adenosine receptor agonists, the nucleoside analogs Metrifudil and N6-benzyladenosine, interact with 2C protein and inhibit EV-A71 viral replication [64] (Table 1). Resistance mutants of EV-A71 protein 2C to these two inhibitors were identified at position E325G for resistance to Metrifudil and at positions H118Y and I324M for resistance to N6-benzyladenosine (Figure 3B). The group of S. Mikhailov reported the synthesis of several series of N6-benzyladenosine analogues. Thus, compound **7**, N6-(5-hexene-2-yn-1-yl) adenosine ( $EC_{50} = 4.3 \pm 1.5 \mu\text{M}$ ,  $SI \sim 101$ ) (Table 1) [65], as well as compound **6**, N6-(trans-3-phenyl-2-propen-1-yl)adenosine ( $EC_{50} = 0.66 \pm 0.12 \mu\text{M}$ ,  $SI \sim 645$ ) (Table 1) [66], demonstrated strong selective inhibition on a panel of EV-A71 clinical isolates belonging to different EV-A71 genogroups. More

recently, compound **10**, N6-(3-trifluoromethylbenzyl) adenosine, an analog with a trifluoromethyl moiety on the benzene ring of N6-benzyladenosine, has been shown to be the most promising *ex vivo* candidate ( $EC_{50} = 0.068 \pm 0.01 \mu\text{M}$ ,  $SI \sim 3456$ ) [67].

### ***Formoterol, Zuclopenthixol, Pirlindole, Dibucaine and derivatives***

In 2016, Ulferts *et al.* identified Formoterol, Zuclopenthixol, Pirlindole mesylate and Dibucaine as novel CV-B3 inhibitors through a drug screen in CV-B3 infected cell culture (Table 1) [68]. Formoterol, a bronchodilator known to have antiviral activity against RV-B14 [69], inhibits replication of all EVs and rhinoviruses tested (EV-A71, CV-B3, EV-D68, PV-1, RV-A2 and RV-B14). Zuclopenthixol (an antipsychotic), Pirlindole mesylate (an antidepressant) and Dibucaine (a local anesthetic) exert an inhibitory effect by acting at the early stage of CV-B3 genome replication. Pirlindole and Dibucaine inhibit EV-D68, Dibucaine also inhibits EV-A71, but none of them inhibits the tested rhinoviruses (RV-A2 and RV-B14) and PV-1. Mutations (A224V, I227V, and A229V) in the coding sequence of CV-B3 2C protein have been identified to confer resistance to Dibucaine, Pirlindole, and Zuclopenthixol, suggesting a relationship to inhibition of a 2C protein function, but not to Formoterol, whose mechanism of action remains to be elucidated (Figure 3C).

Given the ease of Dibucaine synthesis, a structure-activity relationship (SAR) study conducted in 2019 yielded quinoline analogs, such as compound **12a** ( $EC_{50} < 1 \mu\text{M}$ ,  $SI > 5000$ ) (Table 1), with significantly improved antiviral activity and selectivity index against five different strains of EV-D68 [70]. Another study, carried out by the same team in 2020, yielded quinoline analogues with broad-spectrum antiviral activity (EV-D68, EV-A71, and CV-B3) and favorable *in vitro* pharmacokinetic properties [71]. The best representative, compound **6aw** (Table 1), exhibits submicromolar  $EC_{50}$  values against two strains of EV-D68, two strains of EV-A71 and one strain of CV-B3. Compound **6aw** is stable in mouse microsomes ( $t_{1/2} \sim 115 \text{ min}$ ) and represents a promising broad-spectrum antiviral for the treatment of EV. Meanwhile, another SAR study performed in 2020 by Tang *et al.* identified Dibucaine analogues with improved antiviral activity and cell selectivity index [72]. The most effective compound, **6i** ( $EC_{50} \text{ EV-A71} \sim 1 \mu\text{M}$ ) (Table 1), exhibits *in vivo* antiviral efficacy without significant toxicity in EV-A71 infected mice. However, the pharmacokinetic properties of these compounds have not been reported to date.

### ***Pyrazolopyridine derivatives***

In 2016, the phenotypic screen performed by Zuo *et al.* identified a series of pyrazolopyridine derivatives as antivirals against CV-B3 [73]. These pyrazolopyridine derivatives also exhibit broad-spectrum antiviral activity against EV-A71, PV-1, PV-3, as well as several coxsackieviruses and echoviruses; they significantly reduce viral RNA and viral protein synthesis in infected cells. Sequence analysis of mutants resistant to these compounds suggests that the 2C protein is the viral target.

In 2018, a SAR study led to the synthesis of new, more potent and selective pyrazolopyridine inhibitors, including **JX040** (Table 1) which has the strongest antiviral activity against non-polio EVs ( $EC_{50} \text{ EV-A71} = 0.5 \mu\text{M}$ ,  $EC_{50} \text{ CV-B3} = 0.8 \mu\text{M}$ ,  $EC_{50} \text{ PV-1} > 10 \mu\text{M}$ ) [74].

A most recent SAR study, conducted in 2021, identified a series of pyrazolopyridine analogues with broad-spectrum antiviral activity against EV-D68, EV-A71 and CV-B3 in several cell lines, including RD cells and SH-SY5Y80 neuronal cells [75]. Compound **7d** ( $EC_{50} \text{ EV-A71} = 0.1 \mu\text{M}$ ,  $EC_{50} \text{ CV-B3} = 0.2 \mu\text{M}$ ,  $EC_{50} \text{ EV-D68} = 0.1 \mu\text{M}$ ) (Table 1) was used as a chemical probe for

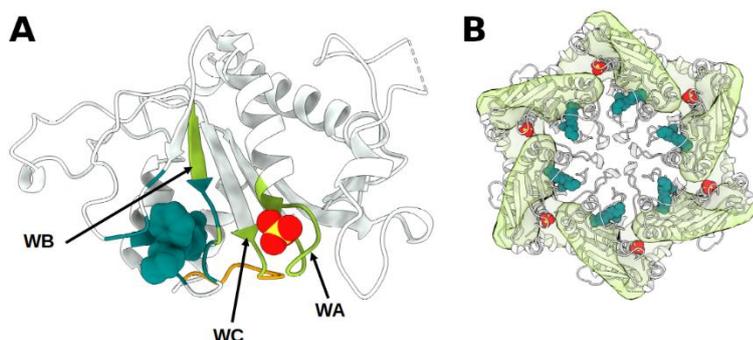
mechanistic studies and revealed that the protein 2C is the viral target for this series of compounds. Resistance mutants of EV-D68 protein 2C to compound **7d** were identified at position D183V and D323G. Although EV-D68 2C-D183V/D323G exhibits reduced sensitivity to compound **7d**, the competitive growth assay showed that its replication capacity is compromised compared to the wild-type virus. Optimization of the pharmacokinetic properties of these compounds could lead to good candidates for *in vivo* studies in animal models as well as chemical probes for *in vivo* validation of drug targets.

### ***Fluoxetine and derivatives***

One of the most studied repositioned drugs targeting 2C is Fluoxetine (Prozac®) (Table 1), a selective serotonin uptake inhibitor antidepressant. In 2012, Zuo *et al.* reported the *in vitro* anti-enteroviral action of Fluoxetine [76], confirmed by a second study that furthermore identified protein 2C as a target of this antidepressant [61]. Fluoxetine interferes with viral RNA replication and selectively suppresses replication of EV-B ( $EC_{50}$  CV-B3 = 3.36  $\mu$ M;  $EC_{50}$  E-9 = 4.58  $\mu$ M) and EV-D ( $EC_{50}$  EV-D68 = 1.35  $\mu$ M), but not with the tested EV-A and EV-C, nor of RV-A or RV-B [61]. The mechanism of antiviral action of Fluoxetine was studied on CV-B3, the amino acids of the 2C protein whose substitution confers resistance to Fluoxetine are located at positions A224, I227 and A229 (short 224AGSINA229 motif C-terminal to the Walker C motif, which is considered to be a "hot-spot" for resistance mutations). This AGSINA motif is conserved in EV-B and EV-D but is not present in other EV [20,61].

In 2019, Bauer *et al.* demonstrated through mechanistic studies that (*S*)-Fluoxetine ( $K_d$  = 9.5  $\mu$ M,  $EC_{50}$  = 0.42  $\mu$ M) binds and inhibits CV-B3 protein 2C with higher affinity than (*R*)-Fluoxetine ( $K_d$  > 200  $\mu$ M) [77]. This stereospecificity is valid for the inhibition of EV-D68, RV-A2 and RV-B14 replication. In contrast, neither the racemic mixture nor (*S*)-Fluoxetine is active against EV-A71 and PV-1 in infected cell culture, at concentrations where (*S*)-Fluoxetine is not toxic *ex vivo*.

The X-ray crystallographic structure of the 2C protein of CV-B3 (117-329) in complex with (*S*)-Fluoxetine was recently solved (PDB ID: 6T3W) [46]. This structure revealed that (*S*)-Fluoxetine binds to an allosteric site on the ATPase domain of CV-B3 protein 2C (Figure 4A). Further biochemical investigation on the mode of action of (*S*)-Fluoxetine demonstrated that it inhibits 2C ATPase activity, which is corroborated by structural data. Indeed, cryo-electron microscopy analysis has refined our knowledge on the mechanism of (*S*)-Fluoxetine and demonstrated that it locks the 2C protein in a hexameric ring-shaped complex (Figure 4B). This structural constraint could either affect ATP binding or limit the conformational changes necessary for its hydrolysis, thus explaining its mode of action. The (*S*)-Fluoxetine resistance mutations are positioned both on the (*S*)-Fluoxetine binding site (C179F, C179Y, and F190L) and on the 224-AGSINA-229 loop (A224V, I227V, and A229V) (Figure 3C). The latter does not interact directly with (*S*)-Fluoxetine, but it has been proposed that the 224-AGSINA-229 loop stabilizes the loop containing the Walker B motif in an "open conformation" allowing (*S*)-Fluoxetine binding.



**Figure 4.** (A) Crystal structure of CV-B3 2C- $\Delta$ 116 in complex with (*S*)-Fluoxetine. The Walker A and B motifs as well as the Walker C motif of the ATPase domain are highlighted in green. An ammonium sulfate molecule binds to the loop of the WA motif delineating the ATP-binding region. The loop highlighted in orange delineates the AGSINA sequence region. (*S*)-Fluoxetine (molecule in duck blue) binds to the WB allosteric pocket and to the direct 2C protein interaction regions (highlighted in the same color as the inhibitor). (B) Positioning of CV-B3 2C- $\Delta$ 116/(*S*)-Fluoxetine monomers in the cryo-EM map of the 2C- $\Delta$ 116 protein hexameric construct. The (*S*)-Fluoxetine binding site as well as the ATP binding site (marked by the ammonium sulfate position) are shown.

Despite convincing *ex vivo* results and an elucidated mechanism of action, Fluoxetine has unfortunately not shown antiviral efficacy in EV-D68 infected mouse models [55,78]. It has been tested in clinical trials for the treatment of patients with proven or presumed EV-D68-associated AFM (acute flaccid myelitis), but has been shown to be ineffective [79-80]. However, Fluoxetine has been used successfully to treat an immunocompromised child with chronic enteroviral encephalitis [81] and a patient with meningoencephalitis due to chronic E-13 infection [82]. These examples highlight the need for further development of Fluoxetine-derived inhibitors with higher efficacy and specificity for protein 2C, especially in the treatment of EV-D68 infection.

With this in mind, a SAR study described by Manganaro *et al.* led to the synthesis of a Fluoxetine analogue, racemic compound **2b** ( $EC_{50}$  CV-B3 = 0.87  $\mu$ M,  $EC_{50}$  ED-68 = 0.56  $\mu$ M,  $EC_{50}$  EV-A71 = not active) (Table 1), in which the methylamine group was substituted with a guanidine moiety, showing similar antiviral activity to (*S*)-Fluoxetine [83]. A chiral separation allowed the isolation of the (*S*)-**2b** enantiomer, which exerts much higher antiviral activity ( $EC_{50}$  CV-B3 = 0.19  $\mu$ M,  $EC_{50}$  ED-68 = 0.11  $\mu$ M) compared to the racemic mixture and confirms the importance of the chiral configuration. The observed resistance mutations (positions A224V, I227V, A229V, C179F) indicate that compound **2b** interacts with viral protein 2C in a similar manner to (*S*)-Fluoxetine. At this point, neither the racemic mixture nor the (*S*)-enantiomer of Fluoxetine and compound **2b** are active against EV-A71 and PV-1 in cell culture, raising the limit of the antiviral spectrum of these inhibitors against EVs.

### *N*-benzylamide derivatives

In this context, Bauer *et al.* optimized new antivirals from a compound previously identified by HTS [73] which is structurally related to Fluoxetine and targets the 2C protein of EV-A71. Synthesis of several substituted amides, derived from *N*-benzylamide, has identified several inhibitors with a broader spectrum than Fluoxetine and that inhibit representative members of all human EV and rhinovirus species [84]. Compound **12b** (Table 1), the most active, inhibits EV-A71,

EV-D68, CV-B3, PV-1, CV-A24, RV-A2, and RV-B14 with  $EC_{50}$  values ranging from 0.0029 to 1.39  $\mu$ M. The structure of complex 2C bound to compound **12b** would allow identification of its binding pocket and elucidation of its mechanism of action.

Importantly, the structure of compound **12b**, described by Bauer *et al.* is structurally similar to another 2C inhibitor described by Ma *et al.* in 2020, compound **R523062** (Table 1). Compound **R523062** was identified by a high-throughput phenotypic screen and inhibits multiple strains of EV-D68 ( $EC_{50}$  from 2.3 to 6.4  $\mu$ M) and, less efficiently, strains of EV-A71 ( $EC_{50}$  from 9.6 to 55.7  $\mu$ M) [85]. The 2C-I227L/Q322R mutant virus indicates that these mutations confer significant resistance to **R523062**. Analysis of the *in vitro* interaction between the 2C protein of EV-D68 and the compound **R523062** showed that it binds directly to the wild-type 2C protein and the 2C-Q322R mutant, but not to the 2C-I227L mutated protein. This confirms that the 2C protein is the direct target of **R523062** and that residue I227 is crucial for binding this inhibitor [85].

### **Peptide 2CL**

In this latest study published in 2021, Fang *et al.* design a peptide, named **2CL** (Table 1), based on the structure of the 2C protein of EV-A71 [42]. This peptide effectively alters the oligomerization of the 2C protein of EV-A71 and inhibits the RNA-helicase activities of 2C proteins encoded by EV-A71 and CV-A16 (EV-A), and shows potent antiviral efficacy against EV-A71 ( $EC_{50}$  = 1.50  $\mu$ M) and CV-A16 ( $EC_{50}$  = 2.16  $\mu$ M), CV-B3 ( $EC_{50}$  = 2.31  $\mu$ M) and E-11 ( $EC_{50}$  = 0.37  $\mu$ M)[42]. In addition, **2CL** treatment resulted in strong *in vivo* protective efficacy against EV-A71-related lethal infection. These results demonstrate that targeting the helicase activity of 2C protein with a rationally designed peptide is a promising antiviral strategy against EVs, and that **2CL** peptide is an important target for broad-spectrum drug candidate development against EVs.

### **Conclusion**

Since 1980s, the 2C protein has been identified as a target for the development of antiviral molecules against enteroviruses (EVs). The 2C protein is highly conserved in enteroviruses and potentially the least permissive to the occurrence of resistance mutations that do not affect replicative fitness. In the absence of structural data, understanding the modes of action has long been limited to the characterization of resistance mutations. Intriguingly, many compounds that inhibit viral replication induce cross-resistance mutations in relatively targeted regions of the 2C protein, despite a wide range of structural diversity in the selected compounds. The recent accumulation of structural and functional data has helped to better characterize the role of the protein in the viral cycle, to identify the molecular mechanisms of inhibition for some of these antiviral molecules and to understand the associated resistance mechanisms. It has now been demonstrated that antiviral molecules inducing resistance mutations on the coding sequence of the 2C protein inhibit some of its functions by binding directly to the protein surface. To date, at least two antiviral target sites have been characterized, namely an allosteric site to which Fluoxetine binds and the assembly interface of the 2C hexamer. Given their high sequence conservation and data published so far, these two sites should allow the development of anti-EV molecules targeting a broad spectrum of viruses, that, combined, could limit the risk of mutations emerging in populations.

Future development of antiviral drugs targeting 2C may benefit from structural optimization of guanidine derivatives with better inhibitory effects and less toxicity. Similarly, Fluoxetine, long used to treat major depression and anxiety disorders, has more recently been used as an antiviral treatment for immunocompromised children with chronic EV encephalitis [81] demonstrating its potential for clinical use as an inhibitor of protein 2C functions. However, its pharmacokinetics and side effects restrict its use to the treatment of chronic EV infections [82]. It is therefore important to continue to optimize molecules based on (*S*)-Fluoxetine, to clarify the molecular mechanisms determining inhibitor efficacy, and to evaluate the spectrum of efficacy on a wide panel of EVs, especially in relevant *in vivo* models.

### **Bibliographic references**

1. Simmonds P, Gorbalenya A, Harvala H, Hovi T, Knowles N, Lindberg A, Oberste M, Palmenberg A, Reuter G, Skern T, Tapparel C, Wolthers K, Woo P, and Zell R. Recommendations for the nomenclature of enteroviruses and rhinoviruses. *Arch Virol* 2020; 165: 793-797.
2. Chapman N, and Kim K. Persistent coxsackievirus infection: Enterovirus persistence in chronic myocarditis and dilated cardiomyopathy. *Curr. Top. Microbiol. Immunol.* 2008 ; 323 : 275-292.
3. Huber S. Host immune responses to coxsackievirus B3. *Curr Top Microbiol Immunol* 2008; 323: 199-221.
4. Yi E, Shin Y, Kim J, Kim T, and Chang S. Enterovirus 71 infection and vaccines. *Clin Exp Vaccine Res* 2017; 6: 1-14.
5. Messacar K, Schreiner T, Maloney J, Wallace A, Ludke J, Oberste M, Nix W, Robinson C, Glodé M, Abzug M, and Dominguez S. cluster of acute flaccid paralysis and cranial nerve dysfunction temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. *Lancet* 2015; 385: 1662-1671.
6. Arden K, and Mackay I. Newly identified human rhinoviruses: molecular methods heat up the cold viruses. *Rev Med Virol* 2010; 20: 156-76.
7. Almeida MB De, Zerbinati R, Tateno A, Cristina O, Renata R, Joaquim R, Cláudio P, and Luiz VF da SF. Rhinovirus C and respiratory exacerbations in children with cystic fibrosis. *Emerg Infect Dis* 2010; 16: 996-9.
8. Dougherty W, and Semler B. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. *Microbiol Rev* 1993; 57: 781-822.
9. Gern J. The ABCs of rhinoviruses, wheezing, and asthma. *J Virol* 2010; 84: 7418-26.
10. Pons-Salort M, Parker E, and Grassly N. The epidemiology of non-polio enteroviruses: recent advances and outstanding questions. *Curr Opin Infect Dis* 2015; 28: 479-87.
11. Lindberg A, Andersson P, Savolainen C, Mulders M, and Hovi T. Evolution of the genome of Human enterovirus B: incongruence between phylogenies of the VP1 and 3CD regions indicates frequent recombination within the species. *J Gen Virol* 2003; 84: 1223-1235.
12. Norder H, Bjerregaard L, and Magnius L. Open reading frame sequence of an Asian enterovirus 73 strain reveals that the prototype from California is recombinant. *J Gen Virol*

2022; 83: 1721-1728.

13. Domingo E, Martin V, Perales C, and C. E. Coxsackieviruses and quasispecies theory: evolution of enteroviruses. *Curr Top Microbiol Immunol*. 2008 ; 323 : 3-32.
14. Sarhan M, El-Bitar AMH, and Hotta H. Potent virucidal activity of honeybee "Apis mellifera" venom against Hepatitis C Virus. *Toxicon* 2020; 188: 55-64.
15. Lulla V, Dinan A, Hosmillo M, Chaudhry Y, Sherry L, Irigoyen N, Nayak K, Stonehouse N, Zilbauer M, Goodfellow I, and Firth A. An upstream protein-coding region in enteroviruses modulates virus infection in gut epithelial cells. *Nat Microbiol* 2019; 4: 280-292.
16. Bauer L, Lyoo H, Schaar H Van der, Strating J, and Jm van Kuppeveld F. Direct-acting antivirals and host-targeting strategies to combat enterovirus infections. *Curr Opin Virol* 2017; 24: 1-8.
17. Tammaro C, Guida M, Appetecchia F, Biava M, Consalvi S, and Poce G. Direct-Acting Antivirals and Host-Targeting Approaches against Enterovirus B Infections: Recent Advances. *Pharmaceuticals* 2023; 16 .
18. Fleischer R, and Laessig K. Safety and efficacy evaluation of pleconaril for treatment of the common cold. *Clin Infect Dis* 2003; 37: 1722.
19. Palma A De, Vliegen I, Clercq E De, and Neyts J. Selective inhibitors of picornavirus replication. *Med Res Rev* 2008; 28: 823-84.
20. Palma A De, Pürstinger G, Wimmer E, Patick A, Andries K, Rombaut B, Clercq E De, and Neyts J. Potential Use of Antiviral Agents in Polio Eradication. *Emerg Infect Dis* 2008; 14: 545-51.
21. Wang S, Wang K, Zhao K, Hua S, and Du J. The Structure, Function, and Mechanisms of Action of Enterovirus Non-structural Protein 2C. *Front Microbiol* 2020; 14: 615965.
22. Rodríguez P, and Carrasco L. Poliovirus protein 2C has ATPase and GTPase activities. *J Biol Chem* 1993; 268: 8105-10.
23. Xia H, Wang P, Wang G, Yang J, Sun X, Wu W, Qiu Y, Shu T, Zhao X, Yin L, Qin C, Hu Y, and Zhou X. Human Enterovirus Nonstructural Protein 2CATPase Functions as Both an RNA Helicase and ATP-Independent RNA Chaperone. *PLoS Pathog* 2015; 11: e1005067.
24. Li J, and Baltimore D. An intragenic revertant of a poliovirus 2C mutant has an uncoating defect. *J Virol* 1990; 64: 1102-7.
25. Vance L, Moscufo N, Chow M, and Heinz B. Poliovirus 2C region functions during encapsidation of viral RNANo Title. *J Virol* 1997; 71: 8759-65.
26. Verlinden Y, Cuconati A, Wimmer E, and Rombaut B. Cell-free synthesis of poliovirus: 14S subunits are the key intermediates in the encapsidation of poliovirus RNA. *J Gen Virol* 2000; 81: 2751-2754.
27. Wang X, Peng W, Ren J, Hu Z, Xu J, Lou Z, Li X, Yin W, Shen X, Porta C, Walter TS, Evans G, Axford D, Owen R, Rowlands DJ, Wang J, Stuart DI, Fry EE, and Rao Z. A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. *Nat. Publ. Gr.* 2012; 19.
28. Wang H, Xie G, and Duan Z. [Current research on picornavirus 3C protease]. *Bing Du Xue Bao* 2014; 30: 579-86.

29. Teterina N, Gorbalenya A, Egger D, Bienz K, and Ehrenfeld E. Poliovirus 2C protein determinants of membrane binding and rearrangements in mammalian cells. *J Virol* 1997; 71: 8962-72.
30. Teterina N, Bienz K, Egger D, Gorbalenya A, and Ehrenfeld E. Induction of intracellular membrane rearrangements by HAV proteins 2C and 2BC. *Virology* 1997; 237: 66-77.
31. Cho M, Teterina N, Egger D, Bienz K, and Ehrenfeld E. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. *Virology* 1994; 202: 129-45.
32. Tang W, Yang S, Wu B, Jheng J, Chen Y, Shih C, Lin K, Lai H, Tang P, and Horng J. Reticulon 3 binds the 2C protein of enterovirus 71 and is required for viral replication. *J Biol Chem* 2007; 282: 5888-98.
33. Liu Y, Wang C, Mueller S, Paul A, Wimmer E, and Jiang P. Direct interaction between two viral proteins, the nonstructural protein 2C and the capsid protein VP3, is required for enterovirus morphogenesis. *PLoS Pathog* 2010; 6: e1001066.
34. Zheng Z, Li H, Zhang Z, Meng J, Mao D, Bai B, Lu B, Mao P, Hu Q, and Wang H. Enterovirus 71 2C protein inhibits TNF- $\alpha$ -mediated activation of NF- $\kappa$ B by suppressing I $\kappa$ B kinase  $\beta$  phosphorylation. *J Immunol* 2011; 187: 2202-12.
35. Li J, Li Y, Zhang S, Ma H, Liu X, Liang Z, Zhang W, Jing H, Du Y, Yang Y, Huo D, Chen L, and Wang Q. Analysis of an Imported Subgenotype C2 Strain of Human Enterovirus 71 in Beijing, China, 2015. *Front Microbiol.* 2018; 9: 2337.
36. Chen P, Wojdyla J, Colasanti O, Li Z, Qin B, Wang M, Lohmann V, and Cui S. Biochemical and structural characterization of hepatitis A virus 2C reveals an unusual ribonuclease activity on single-stranded RNA. *Nucleic Acids Res* 2022; 50: 9470-9489.
37. Liang X, Xiao J, Li X, Liu Y, Lu Y, Wen Y, Li Z, Che X, Ma Y, Zhang X, Zhang Y, Jian D, Wang P, Xuan C, Yu G, Li L, and Zhang H. A C-terminal glutamine recognition mechanism revealed by E3 ligase TRIM7 structures. *Nat Chem Biol* 2022; 18: 1214-1223.
38. Fan W, Mar K, Sari L, Gaszek I, Cheng Q, Evers B, Shelton J, Wight-Carter M, Siegwart D, Lin M, and Schoggins J. TRIM7 inhibits enterovirus replication and promotes emergence of a viral variant with increased pathogenicity. *Cell* 2021; 184: 3410-3425.
39. Xia H, Wang P, Wang G, Yang J, Sun X, Wu W, Qiu Y, Shu T, Zhao X, Yin L, Qin C, Hu Y, and Zhou X. Human Enterovirus Nonstructural Protein 2CATPase Functions as Both an RNA Helicase and ATP-Independent RNA Chaperone. *PLoS Pathog* 2015; 28.
40. Adams P, Kandiah E, Effantin G, Steven A, and Ehrenfeld E. Poliovirus 2C protein forms homo-oligomeric structures required for ATPase activity. *Biol Chem* 2009; 284: 22012-22021.
41. Papageorgiou N, Coutard B, Lantez V, Gautron E, Chauvet O, Baronti C, Norder H, de Lamballerie X, Heresanu V, Ferté N, Veessler S, Gorbalenya AE CB. The 2C putative helicase of echovirus 30 adopts a hexameric ring-shaped structure. *Acta Crystallogr D Biol Crystallogr* 2010; 66: 1116-20.
42. Fang Y, Wang C, Yang R, Bai P, Zhang X, Kong J, Yin L, Qiu Y, and Zhou X. Antiviral Peptides Targeting the Helicase Activity of Enterovirus Nonstructural Protein 2C. *J Virol* 2021; 95: e02324-20.

43. Zhang C, Yang F, Wojdyla J, Qin B, Zhang W, Zheng M, Cao W, Wang M, Gao X, Zheng H, and Cui S. An anti-picornaviral strategy based on the crystal structure of foot-and-mouth disease virus 2C protein. *Cell Rep* 2022; 40: 111030.
44. Guan H, Tian J, Qin B, Wojdyla J, Wang B, Zhao Z, Wang M, and Cui S. Crystal structure of 2C helicase from enterovirus 71. *Sci Adv* 2017; 3: e1602573.
45. Guan H, Tian J, Zhang C, Qin B, and Cui S. Crystal structure of a soluble fragment of poliovirus 2CATPase. *PLoS Pathog* 2018; 14: e1007304.
46. Hurdiss D, Kazzi P El, Bauer L, Papageorgiou N, Ferron F, Donselaar T, Vliet A van, Shamorkina T, Snijder J, Canard B, Decroly E, Brancale A, Zeev-Ben-Mordehai T, Förster F, Kuppeveld F van, and Coutard B. Fluoxetine targets an allosteric site in the enterovirus 2C AAA+ ATPase and stabilizes a hexameric ring-shaped complex. *Sci Adv* 2022; 8.
47. Lambert E. Defects of neuromuscular transmission in syndromes other than myasthenia gravis. *Ann N Y Acad Sci* 1966; 135: 367-84.
48. Pincus S, Diamond D, Emini E, and Wimmer E. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. *J Virol* 1986; 57: 638-46.
49. Tolskaya E, Romanova L, Kolesnikova M, Gmyl A, Gorbalenya A, and Agol V. Genetic studies on the poliovirus 2C protein, an NTPase. A plausible mechanism of guanidine effect on the 2C function and evidence for the importance of 2C oligomerization. *J Mol Biol* 1994; 236: 1310-23.
50. Pfister T, and Wimmer E. Characterization of the nucleoside triphosphatase activity of poliovirus protein 2C reveals a mechanism by which guanidine inhibits poliovirus replication. *J. Biol. Chem.* 1999; 274: 6992-7001.
51. Pincus S, Rohl H, and Wimmer E. Guanidine-dependent mutants of poliovirus: Identification of three classes with different growth requirements. *Virology* 1987; 157: 83-8.
52. Klein M, Hadaschik D, Zimmermann H, Eggers H, and Nelsen-Salz B. The picornavirus replication inhibitors HBB and guanidine in the echovirus-9 system: the significance of viral protein 2C. *J Gen Virol* 2000; 81: 895-901.
53. Sadeghipour S, Bek EJ, and McMinn PC. Selection and characterisation of guanidine-resistant mutants of human enterovirus 71. *Virus Res.* 2012; 169: 72-79.
54. Smee D, Evans W, Nicolaou K, Tarbet E, and Day C. Susceptibilities of enterovirus D68, enterovirus 71, and rhinovirus 87 strains to various antiviral compounds. *Antivir. Res* 2016; 131: 61-5.
55. Hurst B, Evans W, Smee D, Van WA, and Tarbet E. Evaluation of antiviral therapies in respiratory and neurological disease models of Enterovirus D68 infection in mice. *Virology* 2019; 526: 146-154.
56. Barton D, and Flanagan J. Synchronous replication of poliovirus RNA: initiation of negative-strand RNA synthesis requires the guanidine-inhibited activity of protein 2C. *J Virol* 1997; 71: 8482-9.
57. Palma AM De, Heggermont W, Lanke K, Coutard B, Bergmann M, Monforte A-M, Canard B, Clercq E De, Chimirri A, Pürstinger G, Rohayem J, Kuppeveld F van, and Neyts J. The Thiazolobenzimidazole TBZE-029 Inhibits Enterovirus Replication by Targeting a Short Region Immediately Downstream from Motif C in the Nonstructural Protein 2C. *J. Virol.*

2008 ; 82 : 4720-4730.

58. Eggers H, and Tamm I. Synergistic effect of 2-(alpha-hydroxybenzyl)-benzimidazole and guanidine on picornavirus reproduction. *Nature* 1963; 199:513.
59. Hadaschik D, Klein M, Zimmermann H, Eggers HJ, and Nelsen-Salz B. Dependence of Echovirus 9 on the Enterovirus RNA Replication Inhibitor 2-( $\alpha$ -Hydroxybenzyl)-Benzimidazole Maps to Nonstructural Protein 2C. *J. Virol.* 1999 ; 73 : 10536-10539.
60. Klein M, Eggers H, and Nelsen-Salz B. Echovirus-9 protein 2C binds single-stranded RNA unspecifically. *J Gen Virol* 2000; 81: 2481-2484.
61. Ulferts R, Linden L Van Der, Thibaut HJ, Lanke KHW, Leyssen P, Coutard B, Palma AM De, Canard B, Neyts J, and Kuppeveld FJM Van. Selective serotonin reuptake inhibitor fluoxetine inhibits replication of human enteroviruses B and D by targeting viral protein 2C. *Antimicrob. Agents Chemother.* 2013; 57: 1952-1956.
62. Shimizu H, Agoh M, Agoh Y, Yoshida H, Yoshii K, Yoneyama T, Hagiwara A, and Miyamura T. Mutations in the 2C Region of Poliovirus Responsible for Altered Sensitivity to Benzimidazole Derivatives. *J. Virol.* 2000 ; 74 : 4146-4154.
63. Eggers H. Successful treatment of enterovirus-infected mice by 2-(alpha-hydroxybenzyl)-benzimidazole and guanidine. *J Exp Med* 1976; 143: 1367-81.
64. Arita M, Wakita T, and Shimizu H. Characterization of pharmacologically active compounds that inhibit poliovirus and enterovirus 71 infectivity. *J. Gen. Virol.* 2008 ; 89 : 2518-2530.
65. Tararov V, Tijsma A, Kolyachkina S, Oslovsky V, Neyts J, Drenichev M, Leyssen P, and Mikhailov S. doi: 10.1016/j.ejmech.2014.11.048. Epub 2014 Nov 25. Chemical modification of the plant isoprenoid cytokinin N(6)-isopentenyladenosine yields a selective inhibitor of human enterovirus 71 replication. *Eur J Med Chem* 2015; 90: 406-13.
66. Drenichev M, Oslovsky V, Sun L, Tijsma A, Kurochkin N, Tararov V, Chizhov A, Neyts J, Pannecouque C, Leyssen P, and Mikhailov S. Modification of the length and structure of the linker of N(6)-benzyladenosine modulates its selective antiviral activity against enterovirus 71. *Eur J Med Chem* 2016; 111: 84-94.
67. Oslovsky V, Drenichev M, Sun L, Kurochkin N, Kunetsky V, Mirabelli C, Neyts J, Leyssen P, and Mikhailov S. Fluorination of Naturally Occurring N<sup>6</sup>-Benzyladenosine Remarkably Increased Its Antiviral Activity and Selectivity. *Molecules* 2017; 22: 1219.
68. Ulferts R, Boer SM De, Linden L Van Der, Bauer L, Lyoo HR, Maté MJ, Lichière J, Canard B, Lelieveld D, Omta W, Egan D, Coutard B, and Kuppeveld FJM Van. Screening of a library of FDA-approved drugs identifies several enterovirus replication inhibitors that target viral protein 2C. *Antimicrob. Agents Chemother.* 2016; 60: 2627-2638.
69. Yamaya M, Nishimura H, Nadine L, Kubo H, and Nagatomi R. Formoterol and budesonide inhibit rhinovirus infection and cytokine production in primary cultures of human tracheal epithelial cells. *Respir Investig* 2014; 52: 251-60.
70. Musharrafieh R, Zhang J, Tuohy P, Kitamura N, Bellampalli S, Hu Y, Khanna R, and Wang J. Discovery of Quinoline Analogues as Potent Antivirals against Enterovirus D68 (EV-D68). *J Med Chem* 2019; 62: 4074-4090.
71. Musharrafieh R, Kitamura N, Hu Y, and Wang J. Development of broad-spectrum enterovirus antivirals based on quinoline scaffold. *Bioorg Chem* 2020; 101: 103981.

72. Tang Q, Xu Z, Jin M, Shu T, Chen Y, Feng L, Zhang Q, Lan K, Wu S, and Zhou H. Identification of dibucaine derivatives as novel potent enterovirus 2C helicase inhibitors: In vitro, in vivo, and combination therapy study. *Eur J Med Chem* 2020; 202: 112310.
73. Zuo J, Kye S, Quinn KK, Cooper P, Damoiseaux R, and Krogstad P. Discovery of structurally diverse small-molecule compounds with broad antiviral activity against enteroviruses. *Antimicrob. Agents Chemother.* 2016; 60: 1615-1626.
74. Xing Y, Zuo J, Krogstad P, and Jung ME. Synthesis and Structure-Activity Relationship (SAR) Studies of Novel Pyrazolopyridine Derivatives as Inhibitors of Enterovirus Replication. *J. Med. Chem* 2018; 61: 1688-1703.
75. Hu Y, Kitamura N, Musharrafieh R, and Wang J. Discovery of Potent and Broad-Spectrum Pyrazolopyridine-Containing Antivirals against Enteroviruses D68, A71, and Coxsackievirus B3 by Targeting the Viral 2C Protein. *J. Med. Chem.* 2021; 64: 8755-8774.
76. Zuo J, Quinn K, Kye S, Cooper P, Damoiseaux R, and Krogstad P. Fluoxetine is a potent inhibitor of coxsackievirus replication. *Antimicrob Agents Chemother* 2012; 56: 4838-44.
77. Bauer L, Manganaro R, Zonsics B, Strating JRPM, Kazzi P El, Lorenzo Lopez, Moira Ulferts R, Hoey C van, Maté MJ, Langer T, Coutard B, Brancale A, and Kuppeveld FJM van. Fluoxetine Inhibits Enterovirus Replication by Targeting the Viral 2C Protein in a Stereospecific Manner. *ACS Infect Dis* 2019; 5: 1609-1623.
78. Hixon A, Clarke P, and Tyler K. Evaluating Treatment Efficacy in a Mouse Model of Enterovirus D68-Associated Paralytic Myelitis. *J Infect Dis.* 2017; 216: 1245-1253.
79. Tyler KL. Rationale for the evaluation of fluoxetine in the treatment of enterovirus D68-associated acute flaccid myelitis. *JAMA Neurol* 2015; 72: 493-4..
80. Messacar K, Sillau S, Hopkins S, Otten C, Wilson-Murphy M, Wong B, Santoro J, Treister A, Bains H, Torres A, Zabrocki L, Glanternik J, Hurst A, Martin J, Schreiner T, Makhani N, DeBiasi R, Kruer M, Tremoulet A, Van HK, Desai J, Benson L, Gorman M, Abzug M, Tyler K, and Dominguez S. Safety, tolerability, and efficacy of fluoxetine as an antiviral for acute flaccid myelitis. *Neurology* 2019; 92: e2118-e2126.
81. Gofshteyn J, Cárdenas A, and Bearden D. Treatment of Chronic Enterovirus Encephalitis With Fluoxetine in a Patient With X-Linked Agammaglobulinemia. *Pediatr Neurol* 2016; 64: 94-98.
82. Vermeersch G, Laenen L, Lens G, Elst K Van der, Thal D, Jentjens S, Demaerel P, Nieuwenhuysse T Van, Wollants E, Boeckx N, Verhaert N, Dubois B, Kuppeveld F, and Woei-A-Jin F. Antiviral treatment with fluoxetine for rituximab-associated chronic echovirus 13 meningoencephalitis and myofasciitis. *Eur J Neurol.* 2022; 29: 3117-3123.
83. Manganaro R, Zonsics B, Bauer L, Lopez, Moira Lorenzo Donselaar T, Zwaagstra M, Saporito F, Ferla S, Strating J, Coutard B, Hurdiss D, Kuppeveld F, and Brancale A. Synthesis and antiviral effect of novel fluoxetine analogues as enterovirus 2C inhibitors. *Antivir. Res* 2020; 178: 104781.
84. Bauer L, Manganaro R, Zonsics B, Hurdiss D, Zwaagstra M, Donselaar T, Welter N, Kleef R van, Lopez M, Bevilacqua F, Raman T, Ferla S, Bassetto M, Neyts J, Strating J, Westerink R, Brancale A, and Van KF. Rational design of highly potent broad-spectrum enterovirus inhibitors targeting the nonstructural protein 2C. *PLoS Biol* 2020; 18: e3000904.
85. Ma C, Hu Y, Zhang J, and Wang J. Pharmacological Characterization of the Mechanism of

Action of R523062, a Promising Antiviral for Enterovirus D68. *ACS Infect Dis* 2020; 6: 2260-2270.