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Direct-acting antivirals and host-targeting strategies to combat enterovirus infections

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Enteroviruses (*e.g.*, poliovirus, enterovirus-A71, coxsackievirus, enterovirus-D68, rhinovirus) include many human pathogens causative of various mild and more severe diseases, especially in young children. Unfortunately, antiviral drugs to treat enterovirus infections have not been approved yet. Over the past decades, several direct-acting inhibitors have been developed, including capsid binders, which block virus entry, and inhibitors of viral enzymes required for genome replication. Capsid binders and protease inhibitors have been clinically evaluated, but failed due to limited efficacy or toxicity issues. As an alternative approach, host-targeting inhibitors with potential broad-spectrum activity have been identified. Furthermore, drug repurposing screens have recently uncovered promising new inhibitors with disparate viral and host targets. Together, these findings raise hope for the development of (broad-range) anti-enteroviral drugs.

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Introduction

The *Picornaviridae* constitutes a large family of non-enveloped, positive-stranded RNA (+RNA) viruses, currently consisting of 31 genera. The genus *Enterovirus*, which is by far the largest genus, comprises many human pathogens, including poliovirus, coxsackie A and B viruses, echoviruses, numbered enteroviruses (*e.g.*, EV-A71 and EV-D68), and rhinoviruses. Infections with non-polio enteroviruses can result in a wide variety of symptoms, including hand-foot-and-mouth disease, conjunctivitis, aseptic meningitis, severe neonatal sepsis-like disease, and acute flaccid paralysis, whereas infections with rhinoviruses cause the common cold as well as exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (reviewed in Ref. [1]). Vaccines are only available against poliovirus and EV-A71. Development of vaccines against all enteroviruses seems unfeasible, given the large number of (sero)types (*i.e.*, >100 non-polio enteroviruses and >150 rhinoviruses). Hence, there is a great need for (broad-acting) antivirals against enteroviruses. Here, we will review recent efforts to develop direct-acting antivirals as well as host factor-targeting inhibitors to treat enterovirus infections (Table 1).

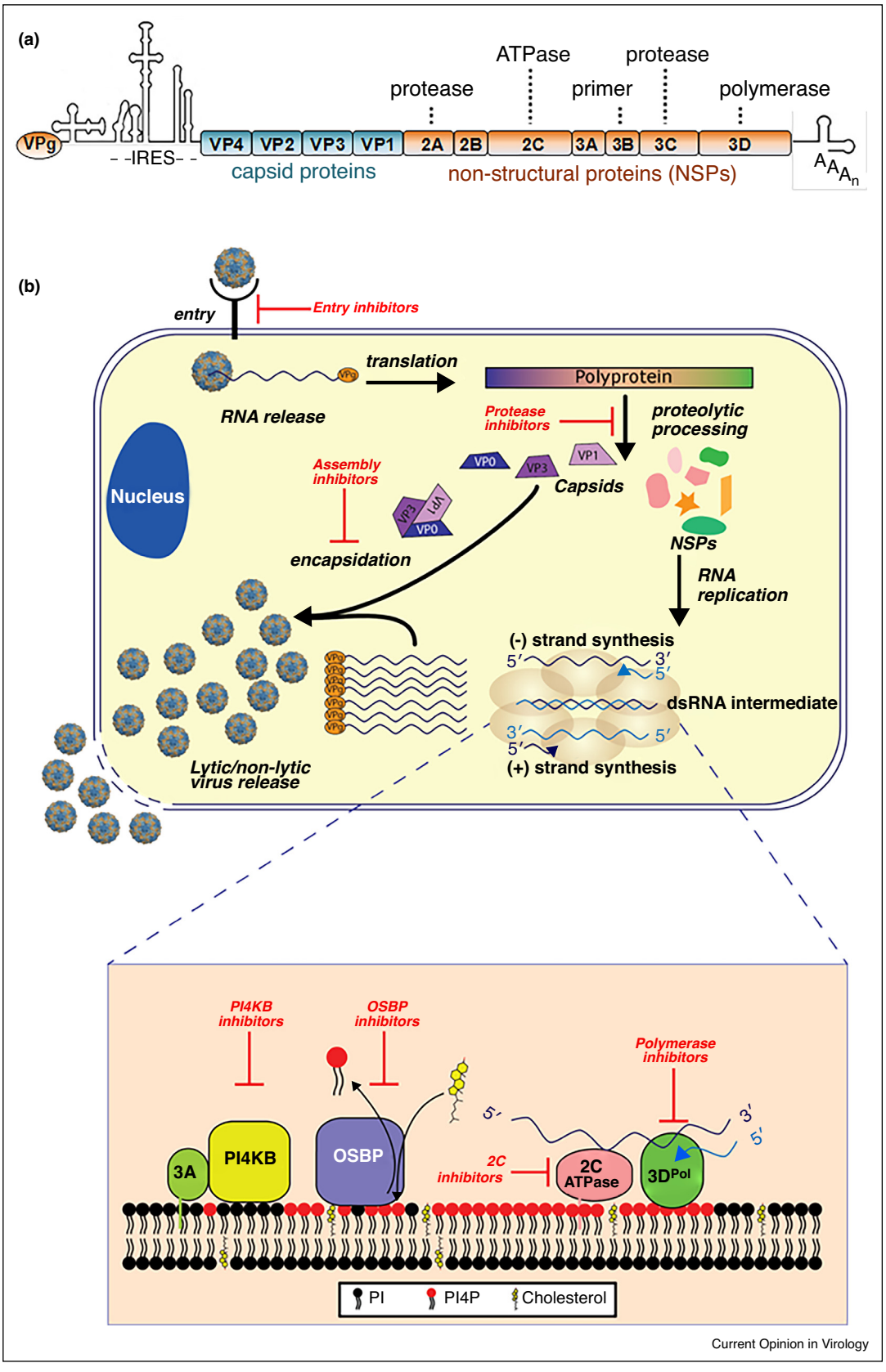
Direct-acting antivirals

Entry inhibitors

Enterovirus capsids are icosahedral (pseudo $T = 3$) structures composed of 60 copies of each of the four capsid proteins (VP1 to VP4). The enterovirus replication cycle (Figure 1b) is initiated by binding of a virion to its receptor. Most enterovirus receptors are protein receptors that belong to the Ig superfamily or the integrin receptor family (reviewed in Ref. [2]). The receptors usually bind in the 'canyon', a depression in the virion surface around the five-fold axes of symmetry [2]. Receptor-binding induces virion destabilization and release of the 'pocket factor', a fatty acid located in a hydrophobic pocket beneath the canyon, to initiate virion uncoating [2].

So-called 'capsid binders' are the most extensively studied class of anti-enteroviral compounds [3,4]. These compounds replace the pocket factor in the canyon and thereby block virion uncoating. Clinical trials for the capsid binders pleconaril, vapendavir (a.k.a. BTA798), and pocapavir (a.k.a. V-073) are currently in progress or have recently been completed, the status of which has been described last year [5]. Since then, another trial with pleconaril was conducted for the treatment of neonates with enterovirus sepsis, which showed greater survival among pleconaril recipients [6]. A major drawback of capsid binders is the rapid emergence of resistance. Indeed, in a clinical trial for the treatment of rhinovirus infections with pleconaril, compound-resistant viruses were isolated [7]. In addition, naturally occurring pleconaril-resistant viruses (*e.g.*, an echovirus 11 strain) have been reported [8]. These resistance issues may complicate the application of capsid binders in the clinic.

Figure 1



Enterovirus genome, replication cycle, and antiviral targets. **(a)** The enterovirus genome encodes four structural proteins (VP1-VP4) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). IRES: internal ribosome entry site. **(b)** The enterovirus life cycle begins with the attachment of the virus particle to a cellular receptor followed by the internalization of the particle into the host cell. The genome is released and directly

Many capsid binders are active against rhinovirus A and B species members [3], but not against members of the rhinovirus C species [9,10]. The recent elucidation of the atomic virion structure of rhinovirus-C15 by cryo-EM revealed a unique 'spiky' structure, vastly different from other enterovirus species. Furthermore, the hydrophobic pocket is filled with bulky hydrophobic residues, thereby not providing sufficient space for a pocket factor or a capsid binder. These features likely explain why rhinovirus C species are not responsive to pocket-binding compounds [11••].

The atomic structure of rhinovirus-C also revealed a potential binding site for sialic acids in a sequence-conserved surface depression [11••]. Sialic acids were recently shown to also facilitate entry of EV-D68 [12,13]. Targeting sialic acid (reviewed in Ref. [14]), which has also been applied for influenza virus, could be an approach to inhibit rhinovirus-C and EV-D68 infections. One of the best-described compounds targeting sialic acids is DAS181, a bacterial sialidase that cleaves α2,3- and α2,6-sialic acid linkages, and that has been tested in a phase II clinical trial for (para)influenza infection [15,16]. DAS181 also inhibits EV-D68 replication *in vitro* [17], but it remains to be tested *in vivo*.

Protease inhibitors

The 7.5 kb +RNA genome of enteroviruses encodes a single polyprotein harboring the structural P1 proteins and the non-structural P2 and P3 proteins (Figure 1a). This polyprotein is proteolytically processed into individual proteins by viral proteases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$.

The development of protease inhibitors has focused particularly on $3C^{pro}$, since it is more conserved than $2A^{pro}$. One of the most potent $3C^{pro}$ inhibitors developed over the years is rupintrivir (a.k.a. AG7088). Rupintrivir is a peptidomimetic compound that irreversibly binds to the catalytic site of $3C^{pro}$ [18]. Because it is very active against a broad panel of enteroviruses [3,19–21], this compound was selected for clinical trials, despite its poor oral bioavailability [22,23]. Although the results of rhinovirus challenge trials were promising [24], rupintrivir did not reduce disease severity in naturally infected patients [25], hence the clinical development was halted. However, many rupintrivir derivatives are currently under development [26–28]. Furthermore, non-peptidomimetic small molecule inhibitors are developed to circumvent difficulties with bioavailability [29], but they have yet to be evaluated in clinical trials.

$3D^{pol}$ inhibitors

The viral RNA-dependent RNA polymerase $3D^{pol}$ catalyzes viral RNA synthesis in replication complexes that are associated with so-called replication organelles (ROs, see below). Inhibitors of $3D^{pol}$ can be divided into two classes based on their structure, being nucleoside/nucleotide inhibitors (NIs) and non-nucleoside/nucleotide inhibitors (NNIs).

NIs

Antiviral NIs can act in two ways. They can be incorporated into the viral genome by mimicking nucleotides to induce lethal mutagenesis or terminate elongation of the nascent chain, or inhibit viral replication in an indirect manner by affecting the cellular nucleotide pools (reviewed in Ref. [30]). Until now, few NIs against enteroviruses have been developed, but compounds developed against other viruses offer promising results, such as ribavirin [31,71], which is clinically used for the treatment of hepatitis C virus (HCV) infections [32,33]. Another example is NITD008, which failed in preclinical studies for the treatment of dengue virus infection due to toxicity. However, a 10-fold lower concentration of NITD008 protected mice from a lethal challenge with EV-A71 without showing any cytotoxicity [34].

Drug repurposing – that is, the concept of using compounds developed for a certain disease to treat a different condition – offers an attractive alternative to *de novo* drug development, as profound pharmacological and toxicological profiles are already available allowing a bypass of expensive (pre-)clinical studies. For example, the NI gemcitabine, an anticancer drug, was recently found to exert broad-spectrum anti-enteroviral activity [35,36]. Besides incorporation into nascent viral RNA, gemcitabine has been suggested to block access of nucleotides into the active side of the polymerase. Furthermore, gemcitabine blocks viral genome replication by inhibiting ribonucleotide reductase, the cellular enzyme that catalyzes the conversion of deoxyribonucleotides to ribonucleotides [35]. The dose of gemcitabine needed for antiviral activity is significantly lower than the dose for the anticancer activity, which raises hope for an application without toxic effects that are inherent to many anticancer drugs.

(Figure 1 Legend Continued) translated into a polyprotein, which is processed by virally encoded proteases into the individual viral proteins. Non-structural proteins rewire host cell membranes and generate replication organelles (ROs) for viral RNA replication. Several host proteins, such as PI4KB (phosphatidylinositol 4-kinase III beta) and OSBP (oxysterol-binding protein), are recruited to ROs by viral 3A protein, which results in ROs with a unique lipid composition. Genome replication starts with synthesis of complementary negative-stranded RNA, which is used as template for the synthesis of a large number of +RNA molecules. Newly synthesized +RNAs either enter a new round of genome replication or are packaged into capsid proteins to build infectious particles. Viruses are released by a non-lytic mechanism as well as upon cell lysis. Inhibitors of the different stages in the replication cycle are depicted in red.

Table 1

Overview of direct-acting or host-targeting inhibitors discussed in this review

Type of inhibitor		Compounds
Capsid binder		Pirodavir [5], Pleconaril[b] [5], Pocapavir (V-073)[b] [5], Vapendavir (BTA798)[b] [5]
$3C^{pro}$ inhibitor	peptidic mimetic	Rupintrivir (AG7088)[b] [18] and its analogs (eg Compound 1[a]) [26–28]
	non-peptidic mimetic	DC07090 [29]
$3D^{pol}$ inhibitor	nucleoside/nucleotide analog	Gemcitabine [35], NITD008 [34], Ribavirin [31,71]
	non-nucleoside/nucleotide analog	Amiloride [5], Aurintricarboxilic acid [5], BPR-3P0128 [5], DTrip-22 [5], Gliotoxin [5], GPC-N114 [37••]
$2C^{ATPase}$ inhibitor		Dibucaine [72], Fluoxetine [41], Guanidine hydrochloride [5], HBB [5], MRL-1237 [5], Pirlindole [72], TBZE-029 [5], Zuclopenthixol [72]
Host factor inhibitor	HSP90	Geldanamycin (analog 17-AAG) [51]
	PI4KB	BF738735 [5], Enviroxime[b] [5], GW5074 [5], PIK93 [5], T-00127-HEV1 [5]
	OSBP	25-hydroxycholesterol [5], AN-12-H5 [64], Itraconazole [61], OSW-1 [63], T-00127-HEV2 [64], TTP-8307 [65]
	Cyclophilins	Cyclosporin A [67•], HL05100P2 [67•], NIM-811 [69]
	Glutathione	Buthionine sulfoximine (BSO) [46•], TP219 [47•]

[a] Phase 1 clinical trial.
[b] Phase 2 clinical trial or completed.

NNIs

Several NNIs of $3D^{pol}$ have been identified (*e.g.*, gliotoxin, DTrip-22, aurincarboxilic acid, BPR-3P0128, and GPC-N114), but their mechanism of action is poorly understood, except for amiloride, which decreases the polymerase fidelity (reviewed in Ref. [5]). GPC-N114 was identified as a novel broad-range enterovirus inhibitor that targets the RNA template-primer site in the core of $3D^{pol}$, making it the first anti-enteroviral compound with this mechanism of action [37••]. Unfortunately, the efficacy of GPC-N114 in animal models remains to be tested due to problems with formulating the compound for *in vivo* use. Alternative strategies for $3D^{pol}$ inhibition, although thus far unexplored, may be to interfere with posttranslational modifications of $3D^{pol}$ like sumoylation and ubiquitination, both of which are important for $3D^{pol}$ activity [38].

$2C^{ATPase}$ inhibitors

The highly conserved viral protein 2C, an ATPase, is an attractive target for broad-spectrum enterovirus inhibitors. $2C^{ATPase}$ has several functions in genome replication (reviewed in Ref. [5]). Several structurally disparate $2C^{ATPase}$ inhibitors have been identified, such as guanidine hydrochloride, HBB, MRL-1237 and TBZE-029 [3]. In addition, drug repurposing screens have recently uncovered a number of FDA-approved drugs (fluoxetine, pirlindole, dibucaine, zuclopenthixol) that inhibit replication of enterovirus species B and D members [39–41]. Since mutations in $2C^{ATPase}$ provide resistance to these compounds, they are considered to target $2C^{ATPase}$. Indeed, fluoxetine (*i.e.*, Prozac) was shown to interfere with the ATPase activity of $2C^{ATPase}$, but the mechanism of inhibition of the other drugs has yet to be unraveled [40]. Recent *in vitro* experiments have confirmed the long-presumed ATP-dependent RNA helicase activity and ATPase-independent RNA chaperone functions of $2C^{ATPase}$ [42••], paving the way for studies to elucidate the mechanism of action of $2C^{ATPase}$ inhibitors in more detail. So far, $2C^{ATPase}$ inhibitors have not been tested in clinical trials. However, fluoxetine was effective in an immunocompromised child with chronic enterovirus encephalitis [43•], implying that $2C^{ATPase}$ inhibitors have potential for clinical use.

Assembly inhibitors

Virion morphogenesis is a poorly understood, step-wise process [44]. The first step is the liberation of P1 from the polyprotein. Assisted by the chaperone heat shock protein 90 (Hsp90) [44,45], P1 is processed into VP0 (*i.e.*, the precursor of VP4 and VP2), VP1, and VP3, which spontaneously form a protomer. Five protomers subsequently assemble into a pentamer, twelve of which in turn form an empty capsid (a.k.a. procapsid). Assembly of pentamers and procapsids is supported by glutathione by an as yet unidentified mechanism [46•,47•]. Governed by interactions between VP1/VP3 and $2C^{ATPase}$ [44,48–50], actively replicating viral RNA is included in the procapsid to form a provirion. The final step in virion morphogenesis is the cleavage of VP0 into VP4 and VP2 to form a stable icosahedral particle.

Only a few assembly inhibitors have been identified so far. Geldanamycin and its analog 17-AAG target Hsp90 to inhibit the processing of P1 [51]. Buthionine sulfoximine, an inhibitor of glutathione synthesis, and TP219, a small molecule that covalently binds to glutathione, both impede the role of glutathione in morphogenesis [46•,47•]. Yet, not all enteroviruses rely on glutathione [47•], thereby precluding glutathione as an important target for broad-spectrum inhibitors.

Inhibitors of host factors

Viruses critically depend on specific host factors. In recent years, several host factors required for enterovirus

replication have been discovered, spurring host-directed drug development. Since some host factors appear to be used by many – or even all – enteroviruses, inhibitors of these cellular factors may have broad-spectrum activity.

PI4KB

Enteroviruses, like all +RNA viruses, induce specific alterations in intracellular membranes and lipid homeostasis to form replication organelles (ROs). The formation of enterovirus ROs is mediated by the concerted actions of viral proteins 2B, 2C, and 3A, as well as a selected set of hijacked host factors (recently reviewed in Ref. [52]). One of these pivotal host factors is phosphatidylinositol 4-kinase type IIIβ (PI4KB) [52–54]. It is recruited to membranes by the viral protein 3A and enriches ROs in phosphatidylinositol 4-phosphate (PI4P) lipids, which is essential for genomic RNA replication [53••]. As PI4KB is essential for all enteroviruses, inhibitors of this enzyme (*e.g.*, PIK93, GW5074, T-00127-HEV1 and BF738735) (reviewed in Ref. [5]) have broad-spectrum activity [53••,54–56]. However, some PI4KB inhibitors showed lethal toxicity in mice and affected lymphocyte function *in vitro*, which has stalled the development of PI4KB inhibitors [57]. Besides the fact that PI4KB inhibitors may be toxic, their activity may also be overcome by some mutations in the viral 3A, as recently published [58,59].

OSBP

Itraconazole, a clinically used antifungal drug that also has anti-cancer properties, was identified in drug repurposing screens as a broad-spectrum enterovirus inhibitor [36,60••,61]. We identified the oxysterol-binding protein (OSBP) as a novel target of itraconazole responsible for the antiviral effects [60••]. OSBP is a PI4P-binding protein that shuttles cholesterol and PI4P at ER-Golgi membrane contact sites [62]. OSBP is recruited to ROs through the PI4KB-mediated increase in PI4P and its lipid shuttling activity is essential for viral genome replication. Other OSBP inhibitors (*e.g.*, 25-hydroxycholesterol, AN-12-H5, T-00127-HEV2, TTP-8307, and the natural compound OSW-1) also impaired enterovirus replication [56,63–65]. In a rhinovirus mouse model, prophylactic intranasal treatment with itraconazole reduced viral titers and pathology, raising expectations for topically applied itraconazole to prevent or treat common colds [66].

Cyclophilins

Cyclophilin A plays a role during the uncoating process of EV-A71 [67•]. In line with this, cyclophilin A inhibitors HL05100P2 and cyclosporine A block EV-A71 replication [67•]. Cyclophilins facilitate protein folding by catalyzing peptide bond isomerization and also play a role in the replication of other +RNA viruses, including HCV and coronaviruses (reviewed in Ref. [68]). Because cyclophilin inhibitors like cyclosporine A have an immunosuppressive effect, non-immunosuppressive inhibitors (*e.g.*, NIM-811 [69]) were developed and are currently in clinical trials for antiviral activity (*e.g.*, alisporivir, a.k.a. Debio025, for HCV treatment). It remains to be established whether uncoating of other enteroviruses also relies on cyclophilins. Hence, the spectrum of anti-enteroviral activity of cyclophilin inhibitors remains to be explored.

Outlook

Currently, there are no antiviral drugs available for the treatment of enterovirus infections, while several potent antivirals are available against HCV, a +RNA virus with a similar replication strategy. Possibly, the small market for anti-enteroviral drugs impedes extensive (industrial) efforts to develop enterovirus inhibitors. Yet, antiviral drugs are urgently needed as enterovirus infections can be life-threatening especially in young children. Furthermore, antiviral drugs are expected to play a crucial role in poliovirus eradication and the post-eradication era.

Capsid binders are currently most advanced in clinical trials, but the inherent problem of rapid resistance development raises concerns. The development of protease inhibitors requires the synthesis of relatively complex peptidomimetic molecules. $2C^{ATPase}$ and $3D^{pol}$ may be more promising targets for direct-acting antiviral drugs as they can be inhibited by small molecules, and several inhibitors of these factors were found to have broad-range anti-enteroviral activity. Other targets for broad-spectrum antiviral drugs are host factors, as many host factors are shared by enteroviruses, but a possible downside is the chance of adverse effects and toxicity, as exemplified by PI4KB inhibitors. Lately, several enterovirus inhibitors have been discovered in drug repurposing screens. These compounds are already available or at least quite advanced in (pre-)clinical development for their respective conditions, thus shortening the development process. Importantly, several of those inhibitors have broad-range, sometimes even pan-enterovirus activity.

Besides targeting individual viral or cellular proteins, an emerging concept in drug design is to interfere with essential protein–protein interactions. In the case of enterovirus infections, one may pharmaceutically disrupt essential interactions between viral proteins and host factors, which likely hampers virus replication without causing the overt toxicity issues that may be associated with inhibition of host proteins. Unfortunately, most protein–protein interactions cannot be addressed by current drug formats, including small molecules. The recent development of small cell-permeating, synthetic protein scaffolds (*e.g.*, Alphabodies) may potentially lead to a novel approach to target protein–protein interactions in (entero)virus-infected cells [70].

Since targeted drug discovery depends heavily on basic knowledge of virus replication, fundamental research on the role of viral enzymes as well as essential host factors

for enterovirus replication remains needed for the development of broad-range antiviral drugs against these important pathogens.

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