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REVIEW

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# Enteroviruses: Classification, Diseases They Cause, and Approaches to Development of Antiviral Drugs

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Received June 13, 2017

**Abstract**—The genus *Enterovirus* combines a portion of small (+)ssRNA-containing viruses and is divided into 10 species of true enteroviruses and three species of rhinoviruses. These viruses are causative agents of the widest spectrum of severe and deadly epidemic diseases of higher vertebrates, including humans. Their ubiquitous distribution and high pathogenicity motivate active search to counteract enterovirus infections. There are no sufficiently effective drugs targeted against enteroviral diseases, thus treatment is reduced to supportive and symptomatic measures. This makes it extremely urgent to develop drugs that directly affect enteroviruses and hinder their development and spread in infected organisms. In this review, we cover the classification of enteroviruses, mention the most common enterovirus infections and their clinical manifestations, and consider the current state of development of anti-enteroviral drugs. One of the most promising targets for such antiviral drugs is the viral Internal Ribosome Entry Site (IRES). The classification of these elements of the viral mRNA translation system is also examined.

DOI: 10.1134/S0006297917130041

**Keywords:** IRES, enteroviruses, Picornaviridae, translation initiation, drug design, taxonomy

*Enterovirus* is a genus of ubiquitous small (with capsids 15–30 nm in diameter) RNA-containing viruses. These viruses infect higher vertebrates and cause a wide spectrum of diseases. These diseases may appear as short-duration sickness or may cause incurable damage in an infected organism, central nervous system diseases, paralysis, swelling, and even death. Exactly these viruses posed (and do so now) a threat of poliomyelitis outbreaks. They are also a cause of other severe deadly diseases such as aseptic meningitis, enteroviral encephalitis, and enteroviral vesicular stomatitis. At the same time, the common cold is also caused by viruses belonging to this genus. Enteroviruses are readily transmitted from person to person through an air and/or via a fecal-oral route. Infection through contaminated objects is also possible. A serious threat is posed by the long asymptomatic virus shedding, which provides the possibility for sudden epidemic bursts of enteroviral infections on different continents and complicates their prediction.

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**Abbreviations:** IRES, internal ribosomal entry sites; ITAF, IRES *trans*-acting factors.

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## INTERNAL TRANSLATION INITIATION OF VIRAL RNAs IN CELL

After virus penetration into a cell, the RNA molecule released from the capsid triggers a cascade of events that result in formation of mature viral progeny and eventually cell death. These events begin with synthesis of viral proteins, i.e. with the translation of viral RNA by the cellular translation system. Expression of viral genes is often regulated at the level of initiation of mRNA translation. At this step, the 40S ribosomal subunit binds to an mRNA and scans it in the 5′-3′ direction until it reaches the start codon, where the 80S ribosome is to be assembled. Various host proteins and *cis*-acting RNA molecules participate in this process. A cap structure is present at the 5′-terminus of most eukaryotic mRNAs, which participates in capturing 40S ribosomal subunits. The scanning mechanism implies that the ribosome initiates translation at the first AUG codon. This is the case for most mRNAs. However, the first AUG codon may be ignored if it is in a non-optimal sequence context. In this case, translation is initiated at the next AUG codon. This initiation mechanism is referred to as leaky scanning. It is

realized in many viruses, which allows saving coding sequence length.

Picornavirus mRNAs ((+)ssRNA viruses) lack the cap structure. In 1988, it was demonstrated that initiation of translation of such uncapped mRNAs is implemented via a structural feature in mRNA molecule, which allows assembly of the translation apparatus near a start codon. These stable secondary structure elements were called internal ribosomal entry sites (IRESs) [1, 2]. Since then, such cap-independent translation initiation pathway has been extensively studied [3]. This discovery overturned a major dogma in translation initiation stating that the eukaryotic ribosome can bind mRNA exclusively at the 5'-terminus. IRESs are usually situated in the 5'-untranslated region and frequently have a complex secondary and tertiary structure. Since these elements were discovered in picornaviruses, they have also been found in several other viral mRNAs. The mechanism of IRES-dependent translation is presumably exploited by some cellular mRNAs. Translation of these mRNAs continues when cap-dependent translation is repressed, which may happen during endoplasmic reticulum stress, hypoxia, starving for nutrients, mitosis, and cell differentiation [4, 5]. In addition to the above-mentioned picornaviruses, initiation of translation at internal sites is utilized in representatives of Flaviviridae [6], Retroviridae, Dicistroviridae [7], Herpesviridae [8], some insect viruses [9], and plants viruses [10], and also retrotransposons in insects and rodents [11].

However, it appeared that, unlike cap-dependent translation initiation (scanning), there is no common mechanism for functioning of all IRESs. Furthermore, the IRESs are very different: no structural element has been found that is shared by all IRESs. Their sequences also lack significant homology [3, 12]. However, it was shown that the majority of viral IRESs have stable secondary and tertiary structure that facilitates their efficient binding to the 40S subunit. Such binding can be either

direct or require participation of additional canonical translation initiation factors along with some other host proteins referred to as ITAF (IRES *trans*-acting factors). It is possible that some ITAF directly participate in specific interaction of mRNA with the 40S subunit, whereas others stabilize specific functionally active IRES conformations [13-15]. Unlike viral mRNAs, existence of IRES-dependent translation of cellular mRNAs is currently being vigorously discussed [4, 5, 16].

## CLASSIFICATION OF PICORNAVIRUS IRESs

Since the discovery of viral IRESs, difficulties in their classification have arisen due to their dissimilarity. However, extensive studies on viruses, their mRNA, and mechanisms of its translation revealed several common features that may be used to clearly distinguish between the IRES types. The viral IRESs that are now classified according to their sequence and secondary structure are divided into separate families: 1 – intergenic IRESs of dicistroviruses of invertebrates (Dicistroviridae family; for instance, the cricket paralysis virus); 2 – IRES of hepatitis C (HCV) and related viruses of animals (Flaviviridae family), and 3 – IRESs of picornaviruses that are in turn divided into five classes (I-V). Besides, there are polypurine A-rich IRESs (PARS) [17]. An IRES of this type was first discovered in tobamovirus CrTMV [18]. However, we return to reviewing picornavirus IRESs. Picornavirus type I IRESs include IRESs of all representatives of the genus *Enterovirus* and a single representative of the genus *Harkavirus* [19]. Length of such IRES is approximately 450 nucleotides (nt). It comprises domains two to six (Fig. 1); it contains  $Y_n-X_m-AUG$  at the 3'-terminus, where  $Y_n$  – pyrimidine sequence ( $n = 8-10$  nt),  $X$  – a linker between  $Y_n$  and the AUG triplet ( $m = 18-20$  nt) [20-22]. This motif is considered to be a region of ribosome-binding at the 5'-UTR [23]. It is separated from

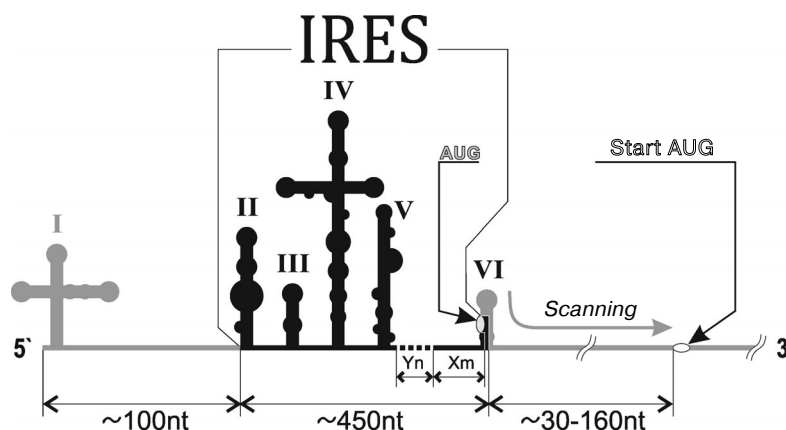


Fig. 1. Scheme of a type I IRES structure.

the start codon with a non-conserved region whose length ranges from <30 nt in rhinoviruses to >150 nt in poliovirus. Conserved functionally important nucleotides are situated at the base of the second domain, in the cruciform fourth domain, and in the fifth domain [12, 19, 24]. Specific binding of the fifth domain to eIF4G and RNA helicase eIF4A promotes binding of the 43S complex to the IRES [12]. Translation initiation on all the studied type I IRESs depends on the presence of ITAF, whose complete list is not yet determined (Fig. 2).

The type II IRES was discovered in representatives of genera *Cardiovirus*, *Aphthovirus*, *Avisivirus*, *Cosavirus*, *Erbovirus*, *Hunnivirus*, *Mischivirus*, *Rosavirus*, *Parechovirus* (*Parechovirus A*, *Parechovirus B*), *Sicinivirus* (<http://www.picornaviridae.com>). This IRES is approximately 450 nt long and contains a *Yn-Xm-AUG* motif at the 3'-terminus. However, in this case the AUG codon can be the start codon. IRESs of this type also contain five domains (H, I, J-K, and L), but they do not resemble domains of the type I IRES, excluding domain I, which like the fourth domain of type I IRES contains a C-rich loop and GNRA tetraloop [19]. Initiation of translation on type II IRES requires specific binding of factors eIF4G and eIF4A with domains J-K [25, 26]. At the same time, such IRESs can function without eIF4E and factors participating in ribosome scanning (eIF1 and eIF1A). Hence, type II IRES differs from type I IRES by the lack of ITAF requirement (with one exception: it has been demonstrated that in certain cases, cellular RNA-binding protein PTB (pyrimidine-binding protein) is required) [25, 26] (Fig. 2).

The type III IRES is only found in hepatitis A virus. It is around 410 nt long [27]. IRESs of this type significantly differ from the first two types both by sequence and structural elements. Efficiency of the translation initiation on this IRES is considerably lower compared to the first two IRES types. The hepatitis A IRES requires the host cap-binding protein eIF4E for functioning, though the exact reason for that is not clear [28] (Fig. 2).

The type IV IRES is found in representatives of genera *Kobuvirus* (*Aichivirus C* (porcine kobuvirus)), *Teschovirus*, *Sapelovirus*, *Senecavirus*, *Tremovirus*, *Limnivirus*, *Megrivirus*, *Parechovirus* (Ferret parechovirus), *Pasivirus*, *Sakobuvirus*, and *Avihepatovirus*. This type of IRES is approximately 330 nt long. It resembles the hepatitis C virus IRES (family Flaviviridae). Viruses with this type of IRES directly bind eIF3 and the 40S subunit facilitating formation of the 48S initiation complex. They do not need factors that are required for binding of the pre-initiator 43S complex to mRNA (eIFs 4A, 4B, 4E, or 4G) or for scanning (eIF1 and eIF1A) [25, 29-31] (Fig. 2).

Recently, the type V IRES was discovered. It was found in representatives of genera *Oscivirus*, *Kobuvirus* (*Aichivirus A*, *Aichivirus B*), and *Salivirus*. This type of IRES is a "hybrid": its central domain is homologous to the fourth domain of the type I IRESs, whereas the next domain that binds to eIF4G is homologous to domain J of the type II

IRESs [32, 33]. The start codon of the viral polyprotein is a part of the *Yn-Xm-AUG* motif and is situated in the stable hairpin of domain L. Translation initiation on these IRESs requires participation of an ATP-dependent RNA helicase DHX29, PTB, and a set of canonical factors (Fig. 2).

The simplest mechanism of translation initiation is a hallmark of an intergenic 180 nt long IRES of representatives of dicistroviruses (for instance, the intergenic IRES of the cricket paralysis virus). Like the hepatitis C IRES, it forms a complex tRNA-like structure, binds directly to the ribosome (P-site), and triggers initiation without involving eukaryotic translation initiation factors [25, 34] (Fig. 2).

#### CLASSIFICATION OF ENTEROVIRUSES HAVING TYPE I IRES

The modern classification of enteroviruses was accepted in 2012 and published as an update to the 9th issue of virus taxonomy from the International Committee on Taxonomy of Viruses. Since then, corrections to this classification have been issued [35-39].

At present, genus *Enterovirus* belonging to family Picornaviridae includes nine enterovirus species (namely, *Enterovirus A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, and *J*) and three rhinovirus species (*Rhinovirus A*, *B*, and *C*). A new enterovirus was discovered in camels in 2015, which is apparently the first representative of a new species, *Enterovirus I* (Fig. 3).

The species *Enterovirus A* includes 25 (sero)types: coxsackievirus A2 (CV-A2), CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, enterovirus A71 (EV-A71), EV-A76, EV-A89, EV-A90, EV-A91, EV-A92, EV-A114, EV-A119, EV-A120, EV-A121, simian enteroviruses SV19, SV43, SV46, and baboon enterovirus A13 (BA13).

The species *Enterovirus B* is one of the most numerous. It consists of 63 (sero)types: coxsackievirus B1, CV-B2 – B6, CV-A9, echovirus 1 (E-1), E-2 – E-7, E-9, E-11 – E-21, E-24 – E-27, E-29 – E-33, enterovirus B69 (EV-B69), EV-B73 – EV-B75, EV-B77 – EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 (from chimpanzee), EV-B111, EV-B112 (from chimpanzee), EV-B113 (from mandrill), and simian enterovirus SA5.

The species *Enterovirus C* includes 23 (sero)types: poliovirus (PV) 1, PV-2, PV-3, coxsackievirus A1 (CV-A1), CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117, and EV-C118.

The species *Enterovirus D* is relatively uncommon; it includes five (sero)types: EV-D68, EV-D70, EV-D94, EV-D111 (from human and chimpanzee), and EV-D120 (from gorilla).

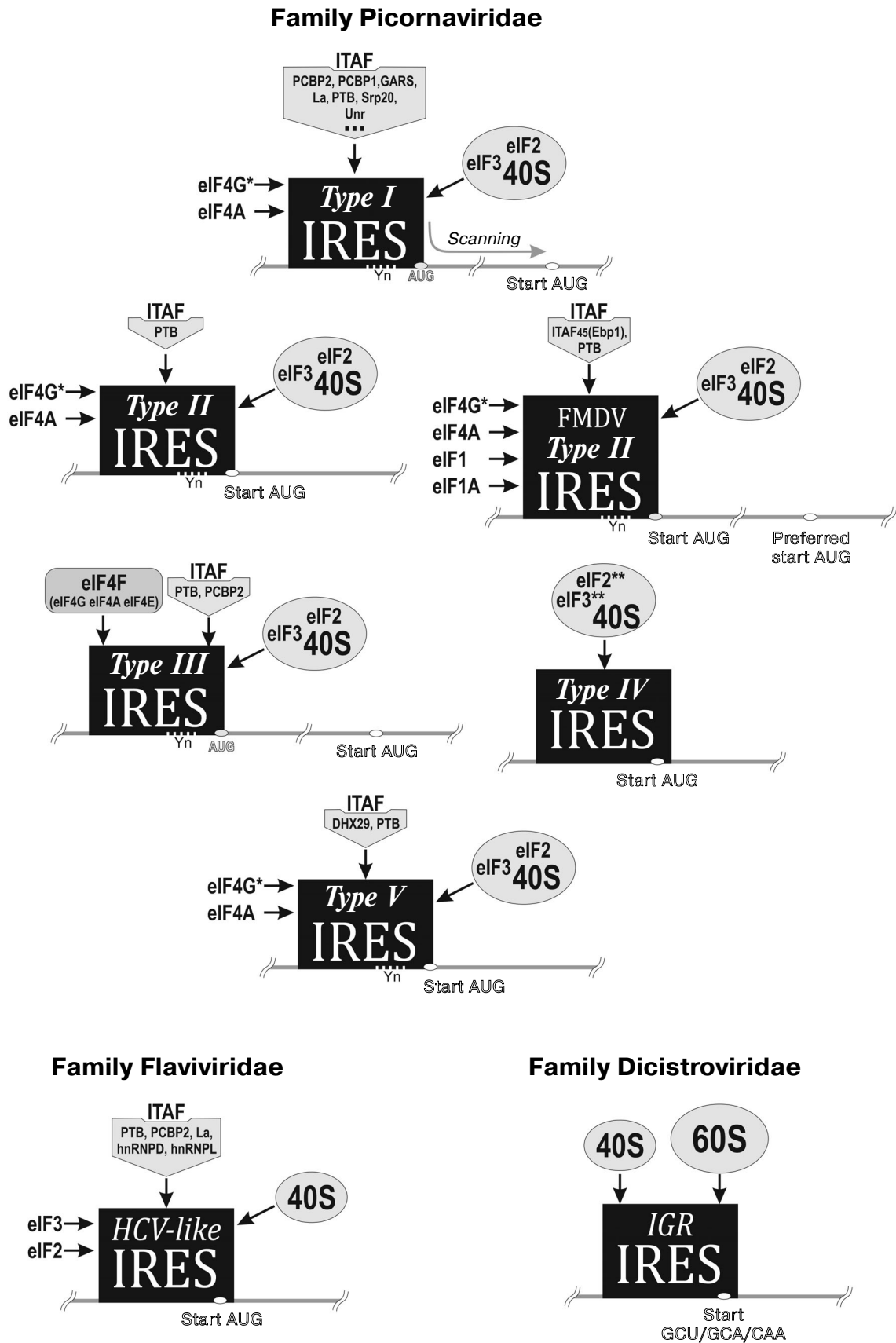


Fig. 2. Main types of classified viral IRESs.

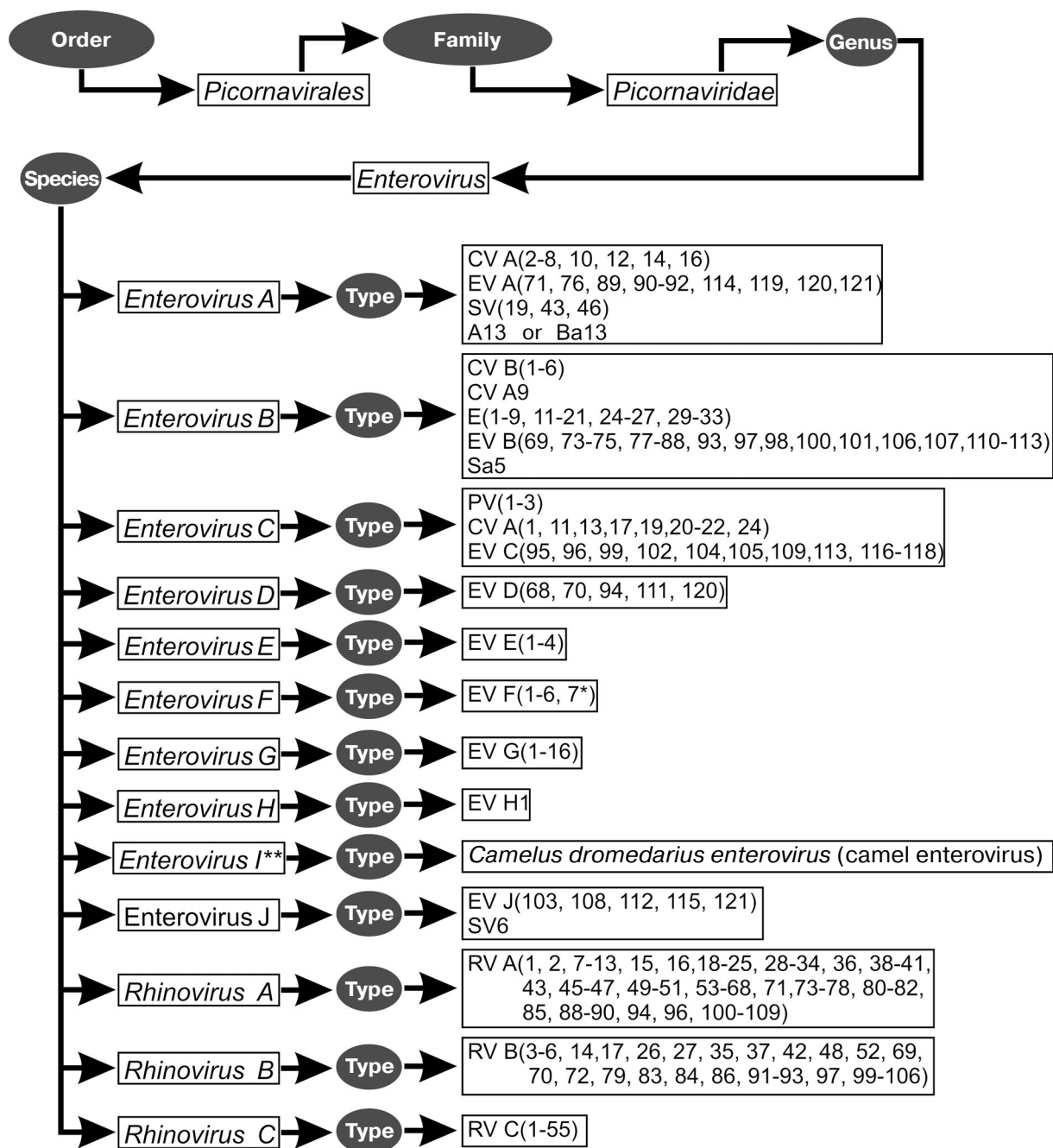


Fig. 3. Taxonomy of enteroviruses.

The species *Enterovirus E* includes bovine enterovirus group A: from EV-E1 to EV-E4.

The species *Enterovirus F* includes bovine enterovirus group B (at present, six types are described): from EV-F1 to EV-F6.

The species *Enterovirus G* consists of 16 (sero)types: from EV-G1 to EV-G16.

The species *Enterovirus H* includes three monkey viruses isolated in 1950 (SV4, SV28, and SA4) and A-2

plaque virus. However, these four viruses were joined into a single (sero)type enterovirus H1 (EV-H1) due to their strong similarity at the molecular level.

The species *Enterovirus J* contains six simian enterovirus species: SV6, EV-J103, EV-J108, EV-J112, EV-J115, and EV-J121.

The species *Rhinovirus A* is the most numerous; it contains 80 (sero)types: rhinovirus (RV) A1, A2, A7-A13, A15, A16, A18, A19-A25, A28-A36, A38-A41, A43, A45-

A47, A49-A51, A53-A68, A71, A73-A78, A80-A82, A85, A88-A90, A94, A96, and A100-A109.

The species *Rhinovirus B* consists of 32 (sero)types: rhinovirus (RV) B3-B6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91-B93, B97, and B99-B106.

As to viruses belonging to the species *Rhinovirus C*, despite its difference from *Rhinovirus A* and *Rhinovirus B*, until recently there were difficulties and misunderstanding in its classification and discriminating the individual types within the species. Some authors on the basis of numerous tests considered these viruses closely related to *Rhinovirus A* and called all of them HRV-A2 [40, 41]. Others preferred to refer to these viruses as HRV-C [42-45] or HRV-X [46]. Since 2010, based on significant phylogenetic clustering of the considered enterovirus species, reasonable suggestions appeared for creating a genetics-based system that would allow discriminating its types similarly to (sero)types of other enterovirus species [47, 48]. By now, this species consists of 55 (sero)types (C1-C55).

In addition, the genus includes several yet unclassified enteroviruses: one monkey enterovirus (SV-47) (it was not assigned to a certain species as its genome is not sequenced) and EV-122 and EV-123, which do not match any of the existing species.

Thus, the genus *Enterovirus* includes many viruses including those highly dangerous for humans. At the same time, these viruses are widespread and highly resistant to the action of physicochemical factors.

#### DISEASES CAUSED BY VIRUSES BELONGING TO GENUS *Enterovirus*

At the beginning, enteroviral infections in humans were classified as acute respiratory diseases caused by intestinal viruses. It was generally accepted to discriminate infections caused by polioviruses (certain (sero)types of *Enterovirus C*) to a separate group called poliomyelitis. We have reviewed polioviruses and their current position in the viral taxonomy. The disease they cause was known already in ancient Egypt [49, 50].

In 1840, the German orthopedist Jacob von Heine discriminated poliomyelitis as a separate disease. In 1890, the Swedish pediatrician O. Medin suggested infectious nature of this disease based on its epidemic dissemination pattern. Poliomyelitis mainly affects children under 5 years old. Unfortunately, there is no antiviral drug for poliomyelitis treatment, only prevention is possible. This disease has several clinical forms.

The abortive form proceeds with no symptoms of nervous system damage. This form of poliomyelitis is called a minor illness as it passes relatively gently, lasts around one week, and ends by recovery [51].

The nonparalytic form is serous meningitis caused by poliovirus. The disease proceeds significantly more

severely than the abortive form with a complete set of meningeal symptoms. However, it also passes favorably and patients make a full recovery [51].

The paralytic form of poliomyelitis is the most dangerous [52]. The above-mentioned forms may convert into the paralytic form upon adverse development of the disease. However, it should be mentioned that this form develops in only 1% of patients. The disease may proceed rapidly, and general paralysis may occur within hours due to damage to the central nervous system. The recovery period may last up to 2 years. It is followed by the aftermath stage with stable paralyzes, contractures, and deformations. Irreversible paralysis (usually in legs) occurs in one out of 200 patients. Mortality in this group of patients reaches 10% due to further development of paralysis and its expansion to the respiratory muscles.

In turn, the paralytic form is also divided into several kinds or forms [53].

The spinal form of paralytic poliomyelitis is the most common. It is characterized by lesion of predominantly the lumbar section of the spinal cord. Cervical and other sections are damaged less often. However, lesion of cervical and thoracic sections of the spinal cord is the most severe as it may cause paralysis of the respiratory muscles and thus disturb respiration. The most dangerous in this respect is diaphragmatic paralysis.

The pontine form occurs upon lesion of the bridge of Varolius [54]. It can be isolated or be accompanied by damage to the spinal cord (pontospinal form) or medulla (pontobulbar form). It is characterized by facial muscle paralysis [55]. Most patients make a full recovery, which begins at 10-14 days of the disease.

The bulbar form occurs in 10-15% cases of paralytic poliomyelitis. In this case, bulbar and glossopharyngeal nerves are affected [56]. The disease proceeds rapidly and is characterized by very serious general condition. Fast development of paralysis of corresponding muscle groups is typical. Pharyngeal paralysis (lesion of the palate and the larynx) may develop, leading to disturbance of respiration and upper airway obstruction with saliva, mucus, and sputum. Earlier, this pharyngeal form of poliomyelitis was characterized by high mortality. At present, however, the pharyngeal paralysis may have a good prognosis and with recovery without consequences if it is treated in due time and correctly. In some patients, pharyngeal paralysis is combined with other disorders (spinal, oblongata). Development of collateral laryngeal paralysis (lesion of the larynx and ligaments) is possible. The acute form of this paralysis can cause sudden asphyxiation and cyanosis. The respiratory center may be affected in bulbar poliomyelitis, which results in disturbance in breathing rhythm and frequency and appearance of other breathing pathologies. Breathing disorders are accompanied with vasomotor and vegetative disorders. Early lesion of the vasomotor center may cause death due to sudden decrease in arterial pressure and cardiac arrest. In approximately

half of lethal cases evoked by this form of poliomyelitis, an acute interstitial myocarditis is registered.

A rare encephalitic form of poliomyelitis with high mortality has also been described. This form proceeds rapidly, mental confusion developing very fast and transforming into stupor and coma [53].

Poliomyelitis in pregnant patients is considered separately [57]. During the first half of pregnancy, the disease may cause miscarriage, or preterm birth if infection occurred later. However, most women infected with poliomyelitis during pregnancy give birth in due time without obstetric surgery. Typically, the fetus is affected by intoxication and hypoxia rather than by direct transmission of the infection. Respiratory disorders pose an elevated threat, which remains even after the acute phase of the disease is passed, for pregnant woman as well.

Poliomyelitis outbreaks were widespread from the end of the 19th century. At the middle of the 20th century, anti-poliomyelitis vaccines appeared and were widely used. In 1988, the World Health Organization set the task of elimination of poliomyelitis worldwide by the 2000 [58]. Active prevention measures with wide use of vaccines decreased the incidence level by 99% (as of 1988) [59]. Currently, high risk of poliomyelitis outbreaks remains only in Afghanistan and Pakistan. In 2013, a new strategic plan for elimination of poliomyelitis by 2018 was presented at the global vaccine summit in Abu-Dhabi (United Arab Emirates).

The first reliable mention of the disease caused by non-polio enteroviral infection occurred in 1856. Exactly in this year, there was an outbreak of pleurodynia in Iceland, which was described later in 1874. The first publication devoted to this illness is dated to 1872, when a Norwegian medical journal first published a communication of Dr. A. Daae to Dr. C. Homann titled "Epidemics of acute muscular rheumatism transmitted through the air in Drangedal". The Norwegian name for this illness is "Bamble disease" after the place it first appeared [60]. Later, it was referred to as Bornholm disease after the Danish island Bornholm, whereas it is now known as epidemic myalgia. Normally, the disease is caused by coxsackievirus B infection. More rarely it may be caused by coxsackieviruses A and certain (sero)types of echovirus [61]. It evokes myositis of the upper abdominal muscles and pectoral muscles, fever, and headache. Typically, the prognosis is positive – the patient recovers in 7-8 days. However, there are possible severe complications (including, though rarely, aseptic meningitis) up to lethal outcome.

Viral myopericarditis is a combination of myocarditis and pericarditis, which involves inflammation of both the cardiac muscle and the serous layer of pericardium. In infants, typically, myocarditis is developed, whereas pericarditis is more common in children and adults. Incorrect or late treatment may result in death. The frequent cause of myopericarditis is coxsackievirus B [62, 63] infection

or coinfection with coxsackieviruses A and B [64]. Echoviruses may also evoke this disorder [65, 66]. The disease may proceed without symptoms or be accompanied with chest pains, vertigo, general weakness, arrhythmia, heart failure, fever, diarrhea, and sore throat. Swelling in the hands and legs may also occur. Sometimes, myopericarditis causes sudden loss of consciousness, which may be associated with abnormal heart rhythms. Breathing difficulties may occur in children. Viral myopericarditis may convert into acute myocardial infarction [67].

Acute hemorrhagic conjunctivitis or enteroviral hemorrhagic conjunctivitis is a highly contagious ophthalmic infection that first appeared in 1969-1970 [68, 69]. It proceeds with visible hyperemia, chemosis, eye irritation, photophobia, eye discharge, and subconjunctival hemorrhage. These symptoms appear along with general symptoms (preauricular adenopathy, headache, increased body temperature, tracheobronchitis, etc.) [70]. Recovery occurs in 7-10 days. Causative agents for acute hemorrhagic conjunctivitis are enterovirus 70 and coxsackievirus A24 [71].

The most frequent manifestation of enteroviral infections is a nonspecific febrile illness. Usually, these infections are well-tolerated and pass within a week. The disease may proceed in two phases [72]. Acute respiratory viral infections (ARVI) are also ascribed to low-hazard enteroviral infections caused by rhinoviruses and known as nasopharyngitis, rhinopharyngitis, rhinovirus infection, rhinonasopharyngitis, epipharyngitis, and the common cold [73]. However, some respiratory enteroviral infections (for instance, those caused by enterovirus 68) may lead to serious consequences resulting in severe complications such as pneumonia [74].

Aseptic meningitis is a viral infectious disease that affects humans of all ages. However, individuals under 30 years old are more susceptible. The most common cause for this disease is non-polio enteroviruses [75], namely coxsackieviruses A and B, echoviruses, and enteroviruses 69 and 73 [76, 77]. During infection, the meninges are affected. Patients suffer from headache, fever, muscle aches, stomach aches, and stiff neck. Other possible symptoms are light sensitivity, rash, nausea, diarrhea, sore throat, and cough. As a rule, this illness has good prognosis and passes without consequences in 7-10 days. However, especially in newborns, the infection may develop to symptoms of encephalitis with focal neurologic signs and cramps. In this case, prognosis may be very poor up to lethal outcome caused by heart failure or liver damage [78]. Such infectious damage of the central nervous system in children may be associated also with enterovirus A71. In this case, the disease proceeds in more severe form and may evoke paresis and brainstem encephalitis [79].

Herpangina is an acute infectious disease caused mainly by coxsackieviruses A, which affects 3-10 years

old children. This disease may be caused also by enterovirus A71 [80-82]. Distinctive symptom of herpangina is appearance of vesicles with serous contents on the soft palate, tonsils, and back of throat. They are small and resemble herpetic damage. Usually, vesicles open rapidly, dry up with a crust formation, and then heal. Upon bacterial coinfection, they may suppurate or ulcerate. The disease proceeds with general symptoms such as fever, headache, rhinitis, hypersalivation, severe sore throat, hyperemia, and pain during palpation of regional lymph nodes. The disease usually passes in a few days.

Enteroviral vesicular stomatitis (hand, foot, and mouth disease, HFMD) is an acute disease caused by coxsackieviruses A, B, and enterovirus A71. It predominantly occurs in children under 10 years old. However, it may also affect adults [83, 84].

Incubation period lasts approximately 3-6 days. During the prodromal period (12 to 36 h), patients experience such symptoms as cough, sore throat, general illness, and loss of appetite. After that, vesicular rash appears on hands, legs, and oral cavity. If the disease goes on auspiciously, it ends in 5-7 days. However, sometimes (especially if infection is caused by enterovirus A71) it may lead to severe neurological complications such as encephalitis, meningitis, and paralyzes like those caused by poliovirus. This form is highly severe and features high mortality. From 2008 to 2012, over seven million cases were registered in China. It was fatal in 2457 cases [85].

Enteroviral encephalitis amounts to approximately 5% of cases of enteroviral infections [86]. The main cause of this severe neurological disease is coxsackieviruses A and B, echoviruses [87], and enterovirus A71 [86]. The disease involves inflammation of the brain. It is accompanied by fever, vomiting, headache, and weakness. Disorders of consciousness, cramps, behavior disorder, and paresis may occur. Severe disease may result in coma. Acute cerebellar ataxia, drop attacks, and hemichorea may occur in children. Several clinical types of enteroviral encephalitis are discriminated according to localization of inflammation: brainstem, cerebellar, hemispheric. The cerebellar form is the most auspicious, which ends with full recovery [88]. However, enteroviral encephalitis is a deadly disease [89]. Encephalitis caused by enterovirus A71 infection usually has brainstem clinical features and high mortality [86, 90].

Polio-like illnesses, acute flaccid paralysis and acute paralytic poliomyelitis of non-polio etiology, are diseases having symptoms similar to those of poliomyelitis but caused by other viruses, namely enteroviruses 68-71, coxsackieviruses, and echoviruses [91, 92]. These diseases affect predominantly children. The most severe forms are commonly caused by enterovirus A71 [93, 94]. Lesion of the central nervous system occurring during development of severe forms of the diseases, similarly to poliomyelitis, may evoke very serious consequences including fatal outcome [95].

Enteroviral infections are dangerous not only for humans. Many animal species are susceptible to these viruses, especially higher mammals. Enteroviral infections of animals may worsen life of pets and even cause significant damage to entire branches of agriculture associated with livestock farming. Cases of severe gastroenteritis caused by enteroviral infections leading to 50% mortality in young stock were registered in poultry farm birds [96]. Livestock farming suffers from outbreaks of enteroviral infections causing high mortality in farm animals (for instance, pigs) [97]. In addition, entire populations of rare or endangered species become victims of these infectious diseases. Even dolphins are susceptible to these infections [98]. Thus, counteraction to spread of these diseases is of great importance.

Early diagnosis of enteroviral infection followed by antiviral therapy may prevent occurrence of severe complications in patients. However, now there are no highly efficient and widely used anti-enteroviral preparations. Therefore, treatment of enteroviral infections is limited to a complex of procedures for relieving the general condition of patients, counteracting concomitant bacterial infections, and minimizing possible complications. Viral infection as such must be dealt with by the immune system of the patient. Therefore, there is a great need for development of highly efficient antiviral agents for treatment of enteroviral infections.

#### APPROACHES TO DEVELOPING ANTIVIRAL DRUGS

The life cycle of enteroviruses includes virus adsorption, release of genetic material from the envelope, RNA translation, maturation of viral proteins, replication of viral RNA, and virus assembly. Any of these stages can be a target for antiviral agents.

The enterovirus envelope consists of four viral proteins (VP1-VP4). VP1 is one of the most frequently used targets in counteracting enteroviral infections. A great number of chemical compounds have antiviral properties *in vitro* through interaction with VP1 and prevention of virus adsorption or release of viral RNA from the envelope. One of the most successful experiences in designing antiviral preparation that interacted with the viral envelope was pleconaril [99]. It inhibited replication of several enteroviral (sero)types by 50% (though it did not affect EV-A71) [100]. It was demonstrated that intake of this drug alleviated disease passage [101]. Although the preparation has numerous negative side effects and it did not pass clinical trials yet, it served as a basis for developing other more efficient and less toxic antiviral drugs [102]. Preparations based on other drugs that interact with viral capsid, but have not passed clinical trials due to side effects, are also being developed [103].



Viral proteases are also targets in drug design. Proteins 2A and 3C are proteases of picornaviruses that play an important role in the processing of the viral polyprotein. In addition, they affect host cap-dependent protein synthesis by cleaving elongation factor eIF4GI/II [104], disturb nuclear transport [105], and impair cellular splicing and transcription [106]. Successful propagation of many viruses depends on correct processing of the viral polyprotein. For instance, polyprotein EV-A71 is cleaved by viral proteases, giving rise to four structural envelope proteins (VP1-VP4) and seven nonstructural proteins (2Apro, 2B, 2C, 3A, 3B, 3Cpro, and 3Dpol), which are required for virus replication [107]. During translation, 2A protease cleaves its own N-terminus from C-terminus of VP1, thus separating capsid protein precursor from precursor of replicative proteins. However, 3C protease is considered the main viral protease, as it is responsible for cleaving the other linkers joining proteins in the viral polyprotein [108, 109].

It was shown that alkylating agents (iodoacetamide and N-ethylmaleimide) reduce activity of 2A protease [110]. Caspase inhibitors also may block 2A protease of rhinoviruses and coxsackievirus 2A both *in vitro* and *in vivo* [111]. Some antiviral agents affecting 3C protease were obtained based on the substrate of this protease. Many of them are peptides comprising 3-5 amino acid residues (a.a.), aldehyde groups of which are used as electrophilic anchors [112]. Some peptide inhibitors of the 3C protease were modified so that they could form irreversible covalent bonds with the protease [113]. Such agents possess high antiviral activity toward rhinoviruses CV-A21, CV-B3, and EV-A70, and echovirus 11.

It is known that some alkaloids have antiviral properties. So, lycorine (an alkaloid of the family Amaryllidaceae) having a broad spectrum of biological activities inhibits development of polioviruses and EV-A71 by affecting, in particular, 2A protease [114].

Rupintrivir was initially designed as an inhibitor of rhinoviral 3C protease. Later it demonstrated antiviral activity toward other representatives of the family Picornaviridae. Derivatives of this preparation were also able to inhibit enteroviruses EV-A71 and CV-A16 [115].

The following group includes antiviral drugs affecting viral proteins involved in replication of viral RNA, or cell systems that are used by viruses for RNA replication. Replication of viral RNA occurs with the participation of replicative complex comprising various viral proteins: 2B, 2C, 2BC, 3A, 3B, 3AB, 3CD, and 3D. Some of these were tested as targets for antiviral agents. The preparations obtained featured a narrow activity spectrum and also had side effects [116]. For example, 5-(3,4-dichlorophenyl)methylhydantoin (a hydantoin derivative) inhibits replication of EV-A71 RNA. The exact mechanism of this effect is to be studied, but, apparently, the process of viral RNA replication is disturbed due to interaction of the preparation with the capsid protein

VP3, thus blocking activity of 2C protein, which also directly interacts with VP3 [117]. Compound BPR-3P012 (6-bromo-2-[1-(2,5-dimethylphenyl)-5-methyl-1H-pyrazol-4-yl]quinoline-4-carboxylic acid) interacts with viral RNA-dependent RNA polymerase (3D) and inhibits translation of EV-A71 RNA [118]. Already known drugs frequently possess antiviral activity by affecting replication of viral RNA. For instance, isoflavone formononetin, which is commonly obtained from red clover [119], or antifungal broad-spectrum preparation itraconazole [120]. Besides, experiments are carried out for discovery of antiviral preparations based on noncoding regulatory microRNAs, which are also used for vaccine preparation [121].

#### IRESs OF VIRAL RNAs AS A PHARMACEUTICAL TARGET

A promising target for antiviral drugs is the internal ribosome entry site (IRES) on viral mRNA. A region of the 5'-UTR of viral mRNA, on which the preinitiation complex assembles, plays a pivotal role in regulation of its translation [122]. Viral IRESs differ from cellular IRESs by the presence of highly ordered secondary structures, a set of factors used for the translation initiation, and requirement for IRES *trans*-acting factors (ITAF). Therefore, the process of translation initiation of viral mRNA is a promising target for pharmacological action. Besides, it was demonstrated that mutations in IRES affecting interaction of ITAF with viral mRNA also affect viral tissue tropism [123, 124]. IRESs of some viruses may act as chaperones influencing development of viral infection not only during the initiation of translation of viral mRNA [125]. As soon as viral IRESs were discovered, attempts were made to use them for therapy [126-129]. The main efforts were focused on developing a compound that would be able to modify IRES structure to make it ineligible for initiation of protein synthesis, or disturbing its interaction with the ribosome, translation initiation factors, and ITAF [126-128, 130-133].

Approaches associated with designing or searching for antiviral agents whose action is directed against IRESs are being extensively developed. The following compounds are considered as such preparations or a basis for their development: complementary oligonucleotides [131], peptide nucleic acids [130], locked nucleic acids [130], morpholines [134, 135], short RNA hairpins [133, 136, 137], small interfering RNAs [133, 136, 137], RNA aptamers, ribozymes [138, 139], DNazymes [140, 141], peptides [142, 143], and low molecular weight inhibitors [141-148].

Historically, the first agents directed against IRESs were complementary oligonucleotides. The majority of early attempts were made to prevent hepatitis C virus gene expression [149-151].

Two approaches were applied in these works. One approach used complementary DNA oligonucleotides as a target for RNase H cleavage. The second approach consisted in designing DNA oligonucleotides that would block interaction between IRES and the ribosome. Unfortunately, the latter approach has several typical disadvantages associated with efficiency of transport of DNA oligonucleotides, their intracellular stability, and in certain cases negative side effects. To improve stability and affinity of complementary DNA oligonucleotides, their modified analogs were developed, which consisted of peptide nucleic acids and locked nucleic acids [151-153]. However, it did not solve the problem of delivery, intracellular transport, and toxicity of these compounds.

During development of this approach, attention of researchers was drawn to morpholines (single-stranded DNA-like cell-penetrating complementary agents able to decrease levels of gene expression by blocking complementary RNA sequences). They represent a third generation of complementary oligonucleotides and feature acceptable toxicity and resistance to nucleases [154]. Morpholino-RNA duplexes are significantly more stable than similar DNA-RNA duplexes. Morpholines sterically block target RNA. They are widely used for modulating expression of genes of certain organisms (such as frogs and zebrafish) [154]. A set of peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) was designed that were complementary to conserved type I IRES regions of RNA viruses (rhinovirus B14, coxsackievirus B2, and poliovirus type 2) [155]. These compounds are soluble in water and resistant to the action of nucleases. They efficiently penetrate cells and inhibit virus replication through forming a duplex with complementary viral mRNA. In cell culture, they reduce virus titer by several orders of magnitude. Application of PPMO increases survivability in mice infected by poliovirus, coxsackievirus B3, Ebola virus, and influenza virus [155, 156].

Octa-guanidine conjugated to morpholines (Vivo-morpholinos, vPMOs) are also single-stranded DNA-like complementary agents. It was shown that these compounds reduced RNA replication and expression of a capsid protein of EV-A71 virus. Besides, these compounds inhibited development of poliovirus and coxsackievirus A16 [157].

Single-stranded mRNA regions potentially available for binding are preferable targets when designing a complementary antiviral agent using the above-mentioned technologies. These regions are usually located within apical loops of various hairpins, in bulges, and in other elements of RNA secondary structures [151, 152, 158].

Another kind of antiviral drugs is RNA hairpins or small interfering RNAs (siRNAs) [159]. Upon transfection of poliovirus-infected murine fibroblasts with siRNAs, strong inhibition of poliovirus replication occurs [160]. Though siRNAs might be used as a basis for devel-

opment of medications, they suffer the same cell-transport problems as the above-mentioned drugs. Furthermore, these RNA agents carry net negative charge and they are less stable, which impedes their delivery into the cell. To overcome these problems, liposomes and polymeric nanoparticles are used as drug delivery vehicles [161]. Besides, siRNAs suffer a significant disadvantage: they can activate protein kinase K, which inhibits translation of cellular proteins due to phosphorylation of the  $\alpha$ -subunit of translation initiation factor 2 [13, 162]. Therefore, approaches based on use of DNA oligonucleotide agents are currently considered more promising as these agents feature higher intracellular stability and increased affinity toward target viral RNA.

The next kind of antiviral drugs are ribozymes, DNazymes, or ribozyme-conjugated RNA aptamers. DNazymes are catalytic DNAs that can cleave the phosphodiester bond in an RNA molecule [163]. They can be obtained more easily than synthetic ribozymes, and they are more stable. Unlike siRNAs, DNazymes do not activate protein kinase K [164]. As ribozymes and DNazymes can specifically inhibit viral IRESs, they represent a reasonable basis for developing therapeutic preparations directed against viruses that use IRES-dependent translation initiation [164-167].

Peptide-inhibitors and small molecules are extensively used in medicine [168, 169]. These peptides usually consist of 5-40 a.a. They mimic functionally active regions of intact proteins that serve as the basis for their design. Due to their small size, they can specifically bind target RNA, disrupting functional complexes that were formed already [168, 170]. Several such peptides were designed to block an IRES of hepatitis C virus [133, 142, 144]. They are based on an RNA-recognition motif of La autoantigen and prevent binding of La to the IRES of hepatitis C virus [142]. However, La is also an ITAF for numerous viral and cellular IRESs [4, 5, 171, 172]. Therefore, such peptides are not specific to hepatitis C virus.

To solve the problem of intracellular peptide resistance against proteolysis, unnatural amino acids are introduced into them (the corresponding compounds are referred to as peptidomimetics). Liposomes and polymeric nanoparticles are used for delivery of these peptides to cells. Also, a peptide can be linked to a protein domain that assists its transmembrane transport. Alternatively, they may be synthesized in cells upon transduction with viral vectors during gene therapy [168].

Currently, small molecules are preferred drugs [169]. There are new approaches directed toward generation of libraries of small molecules with desired properties [169]. Using combinatorial chemistry, large libraries are generated of closely related structural analogs that are further tested in biological screening. Numerous attempts are made to of small molecules able to deactivate viral IRESs [173-176]. As a result, some potential low molecular weight antiviral agents were obtained [173, 176]. For

instance, it was shown that the 9-aminoacridine derivative quinacrine inhibits translation of poliovirus in a cell-free system and in infected HeLa cells, which makes it prospective for further studies [174]. Only a few molecules become subject to clinical trials. However, despite these failures, such approach is still considered one of the most prospective ones

Summarizing the above-said, it should be noted that despite several promising direct-action antiviral preparations including the ones already approved for medical application [177], there is no worldwide certified and commonly recognized antiviral drug for treatment of diseases caused by enteroviruses. Physicians are forced to counteract consequences of disease rather than its cause. Thus, valuable time is lost, which increases risks of irreversible damage in patients. Currently, the only very efficient remedy against enteroviral infections is prevention. Joint global efforts in this direction may provide great outcome like that reached in fighting poliomyelitis [59]. For this reason, vaccines against other dangerous representatives of the genus *Enterovirus* are being developed extensively [85]. Nevertheless, despite 99% reduction in infection cases because of global efforts toward elimination of poliomyelitis, this disease persists. At the same time, one should clearly recognize that if there is still a single poliovirus-infected individual, unvaccinated people worldwide are at risk of infection.

It should be mentioned that development of antiviral drugs is associated with significant difficulties, not only scientific ones, but also organizational and financial difficulties on a global scale. To be efficient, prevention must be ubiquitous and constant, which is still a problem. Besides, vaccine usually is highly specific, whereas enteroviruses are very diverse. Furthermore, under certain conditions vaccination may lead to negative events, such as vaccine-borne virus infection as occurs during overall successful fighting against poliomyelitis. A main point is that prevention does not help those who are already infected. They may only rely on their own immune system and symptomatic treatment. Therefore, the urgent need for designing drugs that directly affect enteroviruses causing numerous dangerous diseases is clear. Drugs with wide spectrum of specificity are especially required, which would allow suppressing enterovirus epidemics at the beginning, before they spread. Development of such preparations is being carried out worldwide.

#### Acknowledgments

We express our gratitude to A. P. Korepanov for careful reading and valuable critical remarks.

This work was supported by the Russian Science Foundation (project No. 15-14-00028).

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