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Review

Enteroviruses and Perspectives for Etiotropic Therapy of Enteroviral Infections

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Abstract

Millions of people, mostly children, are infected annually by enteroviruses with a wide variety of clinical manifestations. Most enteroviral infections are inapparent (Modlin *et al*., 2001), i.e. these viruses circulate in the human population without being "spotted". In a number of cases they cause clinically manifested illnesses of varying severity, including diseases affecting the central nervous system, heart, endocrine pancreas, etc. The high morbidity, the mortality rates in children and the high-risk groups (immunocompromised patients and newborns), as well as the lack of effective vaccines (excluding polioviruses) determine chemotherapy as the primary means of control of enteroviral infections.

Up to now, no effective chemotherapeutic agents for specific treatment of such infections have been registered. A multitude of compounds have been tested, but only few of them are active in vivo. The substances that have reached clinical trial stages have adverse side effects, low selectivity, high toxicity or unfavorable pharmacokinetic properties, and are, therefore, not suitable for use in clinical practice.

The main reason for the failure of the anti-enteroviral therapy is that enteroviruses become drug-resistant and even drug-dependent. This is due to the extremely high levels of mutation and recombination in these viruses, leading to heterogeneous mutant populations. One way to combat resistance is to develop approaches for combined application of new or already existing substances with different mechanisms of action. Our team was the first to introduce analysis of the combined effects of selective inhibitors of enterovirus replication with different mechanisms of action (Nikolaeva and Galabov, 1999, 2000, 2011). Furthermore, the process of development of resistance to the most effective enterovirus inhibitors - the WIN compounds - was studied. A panel of phenotype markers was also presented for characterization of drug-dependent mutants (Nikolova and Galabov, 2003; Nikolova *et al*., 2011). In addition, a new highly effective approach has been developed for combined administration of anti-enteroviral substances for chemotherapy of coxsackievirus neuroinfection in newborn mice, which could serve as the basis for new therapeutic strategies. It consists in consecutive, alternating, not concomitant, application of the compounds in the combination (consecutive alternating administration treatment course, CAA course). Double, triple and quadruple regimens with some of the best studied anti-enteroviral inhibitors were tested. The highest activity was manifested by the triple combinations in consecutive administration of inhibitors with different mechanisms of action, applied in a strict order (Vassileva-Pencheva and Galabov, 2010; Stoyanova *et al*., 2015).

Key words: enterovirus, classification, replication, antivirals, drug-resistance

Резюме

Ентеровирусите инфектират милиони хора всяка година и имат разнообразни клинични прояви, като засягат най-вече децата. По-голямата част от ентеровирусните инфекции протичат инапарентно (Modlin *et al*., 2001), т.е. тези вируси циркулират в човешката популация без да бъдат откривани. В редица случаи причиняват клинично изявени заболявания с различна тежест, включително поразяващи ЦНС, сърцето, ендокринния панкреас и други. Високата заболеваемост и смъртността при децата и високо-рисковите групи (имунодефицитни пациенти и новородени), както и липсата на ефективни ваксини (изкл. полиовирусите) определят химиотерапията като основно средство за контрол на ентеровирусните инфекции.

До този момент няма регистрирани ефективни химиотерапевтици за специфично лечение на тези инфекции. Изпитани са множество съединения, но само малка част от тях са активни in vivo. Тези от веществата, достигнали клинични изпитания показват нежелани странични ефекти, ниска избирателност, висока токсичност или неблагоприятна фармакокинетика и поради това не могат да се използват в клиничната практика.

Главната причина за неуспеха на анти-ентеровирусната терапия е развитието на лекарствена резистентност и дори лекарствена зависимост от ентеровирусите. Това се дължи на изключително високите нива на мутации и рекомбинации при тези вируси, водещи до хетерогенни мутантни популации. Един от начините за борба с резистентността е разработването на подходи за комбинирано прилагане на нови или вече известни вещества с различен механизъм на действие. Нашият екип за първи път въведе изследвания върху комбинираните ефекти на селективни инхибитори на ентеровирусната репликация с различен механизъм на действие (Nikolaeva and Galabov, 1999, 2000, 2011). Наред с това бе изследван процесът на развитие на резистентност към най-ефективните инхибитори на ентеровирусите - WIN съединенията. Представен е и панел от фенотипни маркери с цел характеризиране на лекарствените мутанти (Nikolova and Galabov, 2003; Nikolova *et al*., 2011). Също така е разработен нов високоефективен подход за комбинирано прилагане на анти-ентеровирусни вещества за химиотерапия на коксакивирусна невроинфекция в новородени мишки, който може да послужи като основа за нови терапевтични стратегии. Той се състои в последователно, редуващо се, а не едновременно, прилагане на веществата в комбинацията (consecutive alternating administration treatment course, CAA course). По тази схема на прилагане бяха изпитани двойни, тройни и четворни комбинации с едни от най-добре проучените анти-ентеровирусни инхибитори. Най-висока активност показаха тройните комбинации при последователно прилагане на инхибитори с различен механизъм на действие подавани в точно определен ред (Vassileva-Pencheva and Galabov, 2010; Stoyanova *et. al*., 2015).

Importance of enteroviruses for human pathology

Human enteroviruses are ubiquitous and can be isolated from sewage, water bodies, as well as from contaminated food. They are primarily transmitted via the fecal-oral route, airborne droplet (respiratory) route, objects and belongings in the patient's immediate environment, contaminated hands, the source of infection being the sick person or the carrier. Enteroviruses normally replicate in the mucous membrane of the gastrointestinal tract or the upper respiratory tract (rhinovirus), wherein the infection may be subclinical or manifest itself as a mild illness. Virus particles are excreted in the feces or saliva even before symptoms appear. The incubation period is 3-10 days, during which time the viruses migrate to the regional lymphoid tissue causing a mild viraemia. They subsequently spread to target organs where they replicate and in some of the cases cause illnesses of various severity. During their replication in the gastrointestinal tract, enteroviruses are subject to high mutation rates, which can lead to long-term excretion and neurovirulence. After a viral illness, long-lasting type-specific immunity develops. Children who have gone through an inapparent form of infection acquire such immunity but remain virus carriers.

Enteroviral infections affect millions of individuals annually, with a high level of hospitalization even in the developed countries (Pallansch, 2011), even though the majority of infections are asymptomatic (Morens and Pallansch, 1995; Pallansch and Roos, 2006; Strauss and Strauss, 2008). They are characterized by high contagiousness, diverse clinical symptoms, and are extremely stable in the environment, often causing outbreaks of nosocomial infections that are particularly severe in neonatal units and nurseries. The main clinical manifestations of the enteroviral infection are fever with or without rashes, inflammation of the upper respiratory tract and nasopharynx (rhinitis, sinusitis, summer flu, acute lymphonodular pharyngitis), rarely gastrointestinal disorders. Rhinoviruses, which are highly similar to enteroviruses, have been shown to be the causative agents of the common cold that contributes to the development of chronic pulmonary diseases (Tan, 2005; Mallia *et al.,* 2007). Representatives of the genus can also cause: meningitis, encephalitis, neonatal sepsis, myocarditis, dilated cardiomyopathy, pericarditis, pleurodynia (Bornholm disease), polio-like illnesses, acute flaccid paralysis, pancreatitis, with subsequent complications or chronic course, such as insulin-dependent diabetes mellitus type I (Hyoty and Taylor, 2002; Galabov and Angelova, 2006), hand-foot-and-mouth disease (HFMD), nonspecific my-

algias and severe multi-organ disease in newborns (Morens and Pallansch, 1995), herpangina, acute haemorrhagic conjunctivitis, uveitis, pneumonia, hepatitis, chronic fatigue syndrome, etc.

Different enteroviral serotypes can present with different symptoms and vice versa, identical clinical manifestations can be caused by different enteroviral serotypes (Strikes *et al.,* 1986). Enteroviral infections occur seasonally during the summer - with peaks from early to late summer (Khesuriarni *et al.,* 2006). Enteroviral infections are rarely fatal, but there is a high risk of complications in newborns and immunocompromised individuals. Persons of all ages can become ill, especially vulnerable children. An epidemiological peculiarity of enteroviruses is their high contagiousness. Part of the affected individuals develop mild non-specific symptoms and syndromes. Clinically ill patients constitute a very small part of the individuals actually infected with enteroviruses. It is therefore considered that both persons with clinical manifestations and those with asymptomatic infection should be treated with effective chemotherapeutic agents (Galabov and Angelova, 2006).

As there is no specific therapy for these infections, the treatment is often symptomatic. In some cases of severe enterovirus infections, immunoglobulins and pleconaril have been used.

Classification and structure of enteroviruses

Enteroviruses belong to the family *Picornaviridae*, which comprises 29 genera of viruses that infect a wide range of vertebrate hosts. The genus *Enterovirus* is subdivided into 12 species on the basis of differences among hosts and the pathogenic potential. Each subtype contains a different number of unique serotypes, which differ in their ability to be neutralized by specific antisera. Human enteroviruses include the three types of poliovirus, coxsackie A and B viruses, rhinoviruses, ECHO viruses and enteroviruses types 68-71.

Enteroviruses are some of the most extensively studied viruses. They are small particles (27-30 nm in diameter) with a relatively simple structure. The virions consist of a capsid without envelope, enclosing positive-sense single-stranded RNA. The capsid has icosahedral symmetric and is composed of 60 capsomers, each of which consists of one copy of the four viral proteins: VP1, VP2, VP3 and VP4 (Hogle *et al*., 1985).

Proteins VP1, VP2 and VP3 constitute the outer surface of the capsid, whose thickness is about 5 nm (Smyth and Martin, 2002). Protein VP4 lies below them and is part of the inner surface of the capsid. The differences in amino acid sequences of VP1, VP2 and VP3 confer certain morphology and antigenicity to each picornavirus.

The icosahedral structure of virions is associated with the minimum energy and maximum stability of the capsid. An icosahedron is composed of 20 equilateral triangles and 12 vertices. VP1 protein surrounds each of the 12 fivefold-symmetry axes, whereas VP2 and VP3 proteins surround each three-fold axis. Viral uncoating during replication is accomplished by destabilization of VP4.

The virion surface is not smooth, but has star-shaped protrusions, or "plateaus", located around the five-fold axes of symmetry and surrounded by deep valleys, or "canyons", which are protrusions around the three-fold axes forming the walls of the icosahedron (Racaniello, 2001).

The canyon is a receptor-binding site, where hydrophobic amino acid residues are located (Hogle *et al.,* 1985; Hendry *et al.,* 1999). It surrounds each of the vertices of the icosahedral structure and is located between VP1 on the one side, and

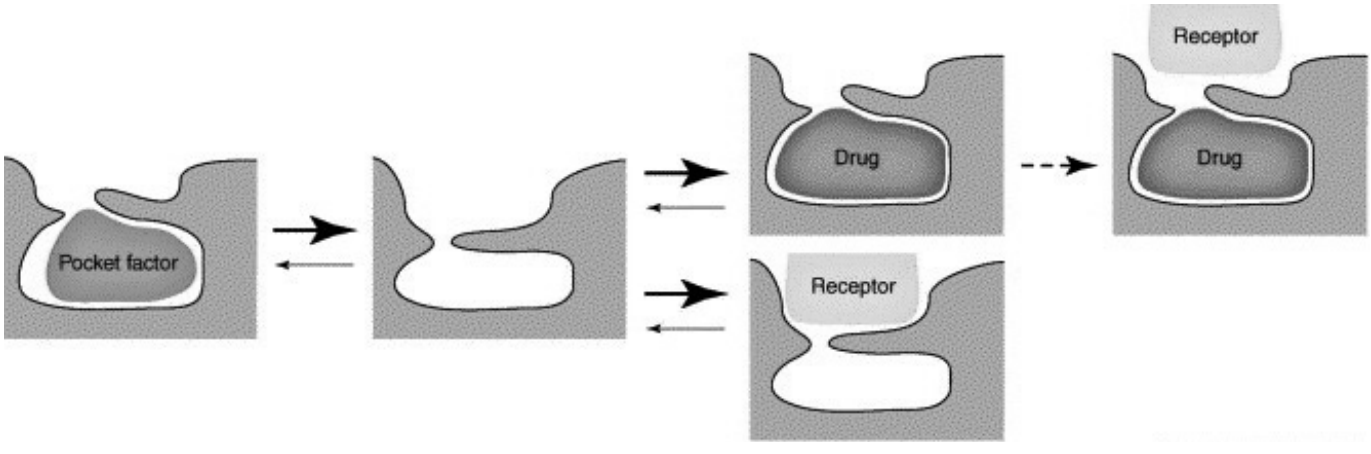

Fig. 1. Diagrammatic representation of the competition between binding of pocket factor or drug into the VP1 binding pocket and receptor into the canyon. (Rossmann *et al*, 2002)

VP2 and VP3 on the other, and its depth is 25Å. Mutations in the amino acid sequence of the proteins that make up the canyon in the poliovirus and rhinovirus may cause a change in the interaction with the cellular receptors. Enteroviruses possess a so-called "hydrophobic pocket" under the base of the canyon (Fig. 1). It is occupied by a higher fatty acid, or so-called pocket factor. It is suggested that the pocket factor regulates the stability of the capsid in the transmission of the virus between hosts. The virus is released from the pocket factor upon binding to its receptor molecule (Rossman and Oliveira, 1993), triggering the uncoating process (Filman *et al.,* 1989; Rossmann, 1994). Certain antiviral agents such as the WIN compounds displace the pocket factor and bind tightly to the hydrophobic pocket, thereby stabilizing the capsid and inhibiting the binding of the virus to the receptor and the subsequent uncoating. According to the so-called "canyon hypothesis", this depression is a strategy to evade immune response. The position of the receptor-binding site in the canyon hampers the recognition of the virus by the antibodies of the host, while binding to the cellular receptor remains possible, because it is narrower than the antibodies (Rossmann *et al.,* 1985; Rossmann и Johnson, 1989; Reisdorph *et al.,* 2003).

Organization of enterovirus genome

The enteroviral genome is monopartite, linear ssRNA(+) of 7.2-8.5 kb in length. Viral genomic RNA has a viral protein (VPg) at its 5′ end instead of a methylated nucleotide cap structure. A single reading frame begins several hundred nucleotides from the 5′ end and terminates several dozen nucleotides from the 3′ end, just upstream from a poly-A tail. The long UTR at the 5′ end contains a type I internal ribosome entry site (IRES). The untranslated sequences at both the 5′ and 3′ ends are involved in viral regulatory activities such as translation and replication. A single polyprotein is translated from the open reading frame (ORF). The P1 region encodes the structural polypeptides. The P2 and P3 regions encode the nonstructural proteins associated with replication. Post-translational modification is accomplished by virus-encoded proteases and results in the generation of the four capsid proteins, as well as the enzymes necessary for replication and translation (Hellen and Wimmer, 1995). Within hours of infecting a host cell, all host cell function

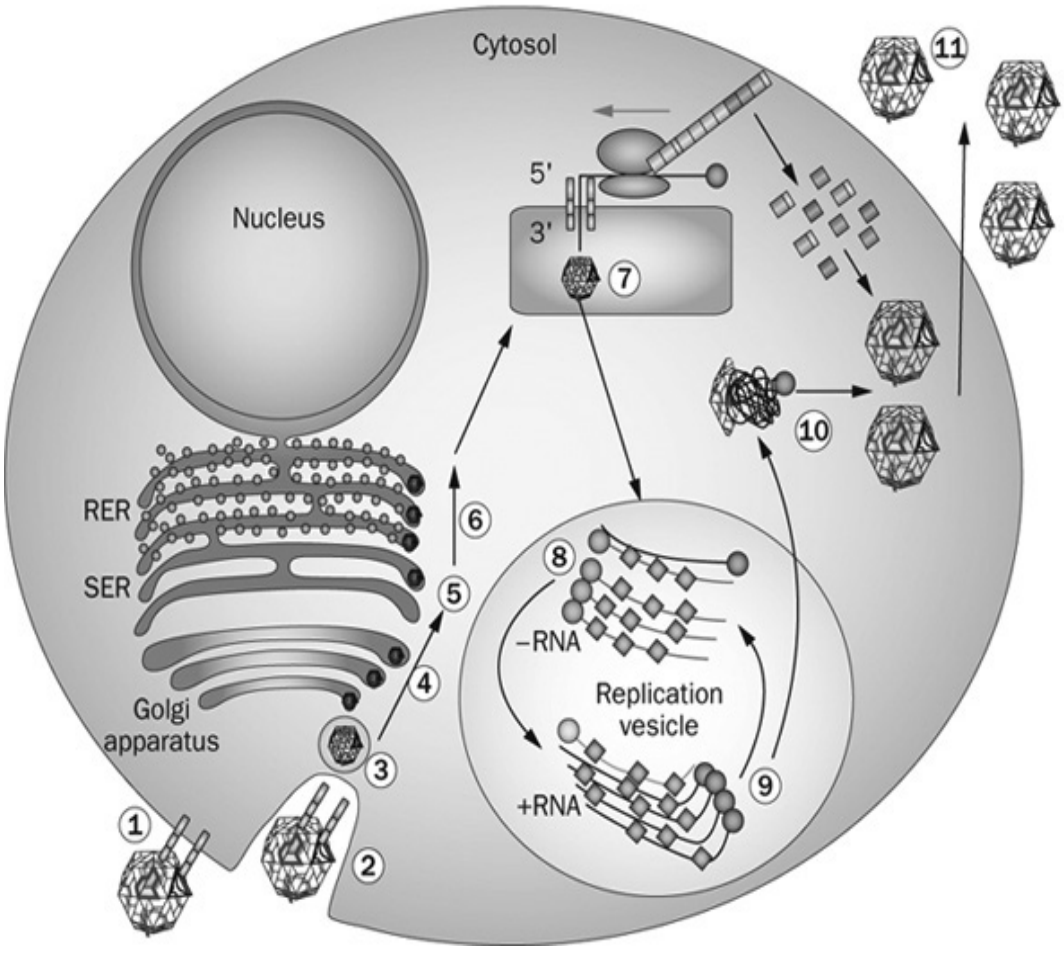

Fig. 2. Enterovirus replication (Hober and Sauter, 2010)

is aborted and the cell becomes a factory for viral replication. Cell death is by lysis, with release of progeny virus into the immediate environment of the cell and the resumption of the viral infection cycle with attachment of new virus particles to neighboring cells (Rotbart, 2000).

Enterovirus replication

The replication cycle of the enteroviruses occurs entirely in the cytoplasm (Fig. 2). The viral particle binds to a specific receptor (1) and triggers its own endocytosis (2). The particle is transported by endocytic vesicles (3) to the Golgi apparatus (4), and then through the smooth endoplasmic reticulum (SER) (5) and rough endoplasmic reticulum (RER) (6). Uncoating occurs and viral RNA is translated at the surface of the RER into a viral polyprotein autocleaved into structural and nonstructural proteins (7). In replication vesicles, positively and negatively stranded viral RNAs are produced with the help of nonstructural proteins (8, 9). Viral RNA encapsidates with structural proteins (10) and new viral particles are extruded (11). A replicative cycle of enteroviruses occurs between 5 and 10 hours depending on the virus, the temperature, pH, multiplicity of infection, and the host cell (Hober and Sauter, 2010).

Inhibitors of enterovirus replication

The combat against enteroviruses follows two main directions. The first them is the development and implementation of effective vaccines. The second direction is the discovery and development of chemotherapeutic agents that selectively inhibit viral replication.

The historical Salk and Sabin vaccines against polio are at present the only ones applied in clinical practice. In 2014, China completed Phase III clinical trials of inactivated EV71 whole-virus vaccines. The results of these tests reveal a high level of protection and good immunogenicity of EV71 vaccine. The protective effect exceeds 90% in EV71-associated HFMD, and over 80% in other EV71-associated diseases, but has no effect on infections caused by other enteroviruses (Li *et al*., 2014; Liang and Wang, 2014). Phase IV clinical trials and registration of the vaccine for clinical use are forthcoming. Despite the breakthrough in the development of a vaccine for EV71, there is still lack of effective vaccines and medications for other non-polio enteroviruses. This requires the search for specific anti-enterovirus chemotherapeutics.

A major obstacle is the extremely high genetic variability of these viruses, due to the high error rate of the viral RNA polymerase and the absence of a repair mechanism. This feature of the RNA viruses underlies the rapid development of drug resistance. Another obstacle to the chemotherapy of enteroviral infections is the high recombination frequency, which also favours the acquisition of drug resistance.

Several groups of anti-enterovirus inhibitors have been described to date. They show good antiviral effects *in vitro*, but unfortunately most fail to manifest a promising effect *in vivo*. This is due to the extremely rapid development of resistance. Nevertheless, a few chemotherapeutic agents have reached clinical trials, while others, though not so promising from a clinical point of view, have contributed to a more detailed understanding of enterovirus replication. Current efforts are directed towards finding highly selective chemotherapeutics and shortening the time of their impact, the aim being to avoid the selection of resistant mutants.

Modern antivirus chemotherapy is based on the specific interaction of test substances with viral targets, involved in certain stages of viral replication. There are several steps in the replication cycle of the enteroviruses that are potential targets in antiviral therapy (Rotbart, 2002). Cell susceptibility, viral attachment, viral uncoating, viral RNA replication, viral protein synthesis and host cell factors have all been studied as targets of anti-enteroviral compounds (Table 1).

Inhibitors of attachment, entry, and uncoating

Receptor binding, uncoating and release of the viral RNA into the host cell are the primary steps in the viral life cycle. Hence, antiviral drugs that interfere with any of these processes block the subsequent steps and thus inhibit viral replication. The most extensively studied class of molecules targeting the early events of attachment, entry, and uncoating, are the so-called "WIN compounds" (De Palma *et al*., 2008).

Inhibitors of virus-specific RNA synthesis

The replication of enteroviral RNA occurs within the replication complex, which is associated with the membrane of virus-induced vesicles in the cytoplasm of infected cells. The viral RNA replication complex comprises a variety of viral proteins including 2B, 2C, 2BC, 3A, 3B (VPg), 3AB, 3CD and 3D. The inhibitors of virus-specific RNA synthesis interacting with components of the viral replicative complex such as viral 2C protein (may have two functions during viral RNA synthesis: as

an NTPase and directing replication complexes to cell membranes), 3A protein (ability to serve as a membrane anchor), viral RNA polymerase complex, etc. These drugs preventing the production of the infectious viral RNA of susceptible viruses.

Virus-specific protease inhibitors

The initial step in the translation of the positive stranded genome of enteroviruses is the formation of a single large polyprotein of about 250 kDa. This polyprotein is rapidly processed into mature viral proteins by co- and posttranslational cleavages (Kitamura, 1981), which are executed by the viral 2A and 3C protease. In view of the fact that the $3C^{pro}$ and $2A^{pro}$ proteases are highly conserved and because of its important role, these proteases may be an interesting target for antiviral drug design.

Inhibitors of host cell factors

This group of antiviral drugs includes compounds that interfere with or modulate the function of host factors that play a critical role in the virus replication cycle. Since host factors are unlikely to mutate and develop resistance in response to therapy, they are attractive targets for antiviral drugs. A potential disadvantage of host-targeting compounds is that interference with a cellular target may be associated with toxic side effects (Van der Schaar *et al*., 2013).

Antivirals with other mechanism of action

This group comprises compounds that inhibit viral assembly and release, increase the nucleotide incorporation error rate during RNA viral replication, as well as antiviral agents that target viral RNA in the 5'UTR end, etc.

Table 1. Antivirals Active vs. Enteroviruses (adapted, Rotbart, 2002; Galabov and Angelova, 2006; De Palma *et al.*, 2008; Fechen *et al.*, 2011; Tan *et al.*, 2014; Van der Linden *et al.*, 2015)

Cell susceptibility
- Interferons (IFN-α, Betaferon®)
- Immunoglobulins
Receptor antagonists
- Anti-SCARB2 antibodies
- Anti-PSGL-1 antibodies
- Anti-heparan sulfate peptide
- Bovine lactoferrin
- Human lactoferrin
- SP40 peptide
Viral attachment and binding to host
Antibodies
Soluble receptor analogues (SRA)
- soluble cells ICAM
- soluble DAF-IgG Fc fusion proteins (sDAF-Fc)
- sCAR-Fc
- sCAR-Fc/sDAF-Fc
Molecular decoys
- Recombinant SCARB2
- PSGL-1
- Heparin mimetics (Heparin, Heparan sulphate, Pentosan polysulfate, Dextran sulphate)
- Suramin/NF449
- Kappa carrageenan
Viral uncoating/capsid function
WIN compounds
- Arildone (WIN38020)
- Disoxaril (WIN51711)
- WIN52452
- WIN52084
- WIN54954
- WIN56291

- WIN58768
- WIN61893
- Pleconaril (WIN63843)

Pyridazinamine analogues (R compounds)
- R77975 (Pirodavir)
- R61837
- R78206
- BTA-188
- BTA-39
- BTA-798
- Compound 13

Isoxazole Derivatives
- compound VIa
- compounds 19, 20 and 21

Pyridyl Imidazolidinones
- BPROZ-194
- BPROZ-299
- BPROZ-284
- BPROZ-160
- BPROZ-112
- BPROZ-103
- BPROZ-101
- BPROZ-074
- BPROZ-033
- DBPR-103
- compound 28b

SCH compounds
- SCH 38057
- SCH 47802
- SCH 48973
- Pocapavir (V-073)

SDZ 35-682

SDZ 880-061

Phenoxypyridinecarbonitriles
- DEPC: 6-(3,4-dichlorophenoxy)-3-(ethylthio)-2-pyridinecarbonitrile

Flavane derivatives
- BW 683C: 4',6-dichloroflavan
- BW 4294: 1-anilino-9-benzyl-2-chloropurine
- 3(2H)-isoflavene

Chalcones (Ro compounds)
- Ro 09-0410
- Ro 09-0179
- Ro 09-0696
- Ro 09-0881

Methylthiopyrimidines
- S-7: ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate

Rhodanine (2-thio-4-oxothiazolidine)

44 081 RP: 2-[(1,5,10,10a-tetrahydro-3H-thiazolo[3,4b]isoquinolin-3-ylidene)amino]-4-thiazoleacetic acid

Dibenzofuran

Dibenzosuberol derivatives
- 2-hydroxy-3-dibenzofuran carboxylic acid
- Dibenzosuberenone

3,4-dihydro-2-phenyl-2H-pyrano[2,3-b]pyridines, the 6-substituted 2-(3',4'-dichloropheno-xy)-2H-pyrano[2,3-b]pyridines
- MDL 20,610

- MDL 20,646
- MDL 20,957

Pyridine (compound 71): 2-(3,4-dichlorophenoxy)-5-(methylsulfonyl)

Inhibitors of Virus-Specific RNA Synthesis

Guanidine hydrochloride

PTU-23: N-phenyl-N'-2-hydroxyphenylthiourea

MRL-1237: 1-(4-fluorophenyl)-2-(4-imino-1,4-dihydropyridin-1-yl)methylbenzimidazole hydrochloride

Benzimidazoles

- HBB: 2-α-hydroxybenzyl-benzimidazole
- Enviroxime [2-amino-1-(isopropyl sulfonyl)-6-benzimidazole phenyl ketone oxime]

TBZE-029 [1-(2,6-difluorophenyl)-6-trifluoromethyl-1H,3H-thiazolo[3,4-a]benzimidazole]

Enviroxime-related derivatives (vinyl acetylene benzimidazoles)

- Enviradene: (E)-1-[(1-methyletyl)sulfonyl]-6-(1-phenyl-1-propenyl)-1H-benzimidazole-2-amine

- 2-amino-6-[(E)-1-phenyl-2-(N-methylcarbamoyl)vinyl]-imidazo[1,2-a]pyridines

- AN-12-H5
- AN-23-F6
- TTP-8307

Oxoglaucine

OSW-1: (3β,16β,17α-trihydroxycholest-5-en-22-one16-O-{O-(2-O-(4-methoxybenzoyl)-β-D-xylopyranosyl)-(1→3)-2-O-acetylα-arabinopyranoside

Thiomersal (2-FMC): 2-furylmercury chloride

Phenoxybenzenes, Phenoxypyridines and Related Analogues

- MDL-860 (DNB): 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile
- Compound 13: (3,4-dichlorophenoxy)-(5-methylsulfonyl-2-pyridinyl)-methane
- Compound 21: (2-(3,4-dichlorobenzylamino)-5-methylsulfonylpyridine)

Isothiazole Derivatives

- DID: 5,5'-diphenyl-3,3'-diisothiazole disulphide

Flavonoids

- Ro 09-0179
- Ro 09-0298
- 3-MQ: 3-methylquercetin
- 3-methylkaempferol and 3,4'-dimethylkaempferol
- Compound 4h

Gliotoxin

Nucleoside Analogues

- 2'-C-methylcytidine
- 5-nitro and 5-aminocytidine analogues

Pyrrolidine Dithiocarbamate (PDTC)

Quinacrine

Amantadine

Metrifudil

N_6 benzyladenosine

DTriP-22

Fluoxetine

Virus-Specific Protease Inhibitors

E-64: L-Trans-Epoxysuccinyl-Leucylamido(4-Guanidine)Butane

3C PROTEASE INHIBITORS

Peptidic Inhibitors

- Peptide Aldehydes
- Michael Acceptor-Containing
- Rupintrivir (AG7088): ethyl(2E,4S)-4-(((2R,5S)-2-(4-fluorobenzyl)-6-methyl-5-(((5-methylisoxazol-3-yl)carbonyl)amino)-4-oxoheptanoyl)amino)-5-((3S)-2-xopyrrolidin-3-yl pentenoate

- Azapeptides
- Compound 1 (AG7404)
- Diazomethyl Ketones (DMK)
- Peptidyl Monofluoromethylketones
- Keto-Glutamine Analogues
- S-nitrosothiols
- Peptidyl N-iodoacetamides
- Tripeptidyl a-ketoamides

Non-Peptidic Inhibitors
- 2-Pyridone-Containing Peptidomimetics
- Substituted Benzamide Inhibitors (α,β-unsaturated etobenzamides; 5-substituted benzamides)
- Isatins
- Spiro-Indolinone β-Lactams
- Homophthalimides
- β-Lactones
- Pseudoxazolones
- Low Molecular Weight Non-Peptidic Inhibitors

Compound 10b

Fisetin

Rutin

Microbial Extracts
- naphtoquinonelactol, quinine-like citrinin, radicinin and triterpene sulfates

2A PROTEASE INHIBITORS

Thiol Alkylating Agents
- Iodoacetamide and N-ethylamaleimide

Classic Elastase-Specific Inhibitors
- MCPK: Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone
- Elastatinal

Homophthalimides

Peptide-Based Fluoromethyl Ketones
- zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone)
- zIETD.fmk (benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone)

LVLQTM peptide

Nitric oxide (NO) donors (S-nitrosylation)
- GTN, ISDN
- NO-metoprolol

Inhibitors of Host Cell Factors

PI4KIII β vinhibitors (Phosphatidylinositol-4-Kinase III Beta)
- Enviroxime
- GW5074
- T-00127-HEV1
- AG7404 (compound 1)
- BF738735

OSBP inhibitors (Oxysterol-Binding Protein)
- Itraconazole
- OSW-1
- AN-12-H5
- T-00127-HEV2
- 25-hydroxycholesterol

Ubiquitin-proteasome system (UPS)
- Pyrrolidine dithiocarbamate (PDTC)
- Curcumin
- MG132
- Lactacystin

Assembly inhibitors
Chaperone Hsp90 Inhibitors
- Geldanamycin
- 17-AAG (17-allyamino-17-demethoxygeldanamycin)
BSO (buthionine sulfoximine) (glutathione synthesis inhibitor)
TP219 (glutathione scavenger)
Antivirals with Other Mechanisms of Action
Ribavirin (1-β-D-ribofuranozyl-1,2,4'triazole-3-carboxamide)
Valopicitabine (2'-C-methylcytidine)
2'-C-methyladenosine
4'-azidocytidine
5-substituted cytidine analogues
- 5-nitrocytidine
N-6-substitiuted purine analogues
Amiloride
Pyrazolo[3,4-d]pyrimidine
Hygromycin B
Hydantoin (5-(3,4-dichlorophenyl) methylhydantoin)
Mycophenolic acid (MPA)
EnteroX
Aziridine and N-dansylaziridine
Antisense Oligonucleotides
Heat-shock protein 90 inhibitor
- Geldanamycin
- 17-AAG
RNA-based therapeutics
- siRNA
- shRNA

Drug resistance - an obstacle to effective chemotherapy

Drug resistance is reduced or absent virus sensitivity to a particular replication inhibitor and is characterized by significantly higher values of 50 (IC_{50}) and 90 (IC_{90}) inhibitory concentration. While drug resistance is a phenotypic feature, a phenotype is determined by the genotype and specific genotype mutations that cause changes in the viral protein targeted by the relevant inhibitor (Heinz and Vance, 1996).

Enterovirus populations display quasispecies dynamics, characterized by high rates of mutation and recombination, followed by competition, selection, and random drift acting on heterogeneous mutant spectra. Direct experimental evidence indicates that high mutation rates and complex mutant spectra can serve for the adaptation of enteroviruses to complex environments. Studies with the RNA-dependent RNA polymerase of picornaviruses suggest that multiple enzyme sites may influence the template-copying fidelity (incorporation of incorrect vs correct nucleotide) during RNA replication. Mutation and recombination are an unavoidable consequence of the molecular mechanisms inherent to the process of viral genome replication and underlie the diversification of enterovirus genomes as they multiply in human and animal hosts.

Enteroviruses, as other RNA viruses, share a potential for adaptation and rapid evolution associated with two key features of their replication: high mutation rates and quasispecies dynamics. One of the first measurements of mutability of an RNA virus was carried out by Eggers and Tamm (Eggers and Tamm, 1965) who calculated a rate of 1×10^{-4} for the transition of coxsackievirus A9 from dependence to independence of 2-(a-hydroxybenzyl)-benzimidazole (HBB). The value is in line with measurements of mutation rates (the rate of occurrence of mutations during genome replication) and mutation frequencies (the frequency of mutations in a genome population) carried out with several RNA viruses (Drake and Holland, 1999). The genome size of RNA viruses is in the range of 3-33 kb. The combined mutation rate and genome size values imply that an average of 0.1-3 mutations per genome are expected to occur every time an RNA template is copied into a complementary RNA or DNA copy. Such template-copying

events may take place hundreds of times in each infected cell from an infected organism.

The diversity of disease manifestations associated with closely related enteroviruses is probably attributable to profound biological effects of some mutations that, because of their limited number, do not necessarily affect the phylogenetic position of the virus. The combination of highly dynamic mutant spectra with unpredictable alterations of biological behaviour by minimal genetic change defies classical classification schemes. The result is the need to update the grouping of enteroviruses quite frequently into genetic and serological types and subtypes.

The application of quasispecies theory to an understanding of the limits of viral genomes to accept mutations, together with an increasingly deeper understanding of the mechanisms of mutagenesis by nucleoside analogs, has paved the way for the application of lethal mutagenesis as a new antiviral strategy (Domingo *et al.,* 2007).

The probability of resistant mutants being generated and their population growing after treatment with antiviral agents is due to four major factors (Richman, 1996): (i) the mutation rate during genome replication; (ii) the inherent ability of the target site of inhibition to mutate; (iii) the selective pressure exerted by the viral inhibitor; (iv) the extent of viral replication.

The enterovirus progeny, resistant to a particular viral inhibitor, grow quite readily and rapidly as a result of the selection of pre-existing mutants with mutations in the ligand-binding domain (for the antiviral substance) of the virus-specific target protein. Such mutants always exist in the virus population, which consists of a large number pseudo-species (Loddo, 1980; Nikolova and Galabov, 2003). The selective pressure exerted by a certain antiviral agent leads to an increase in the population of mutant viruses (Cuevas *et al.,* 2005), since they are the fittest in the presence of this compound (Zambon and Pillay, 1998). The hypothesis accepted today is that of Herrmann and Herrmann (1977), according to which the selection of resistant viral progeny is an indicator of specific antiviral activity.

A great number of cases have been described of laboratory selection of viral strains resistant to guanidine (Melnick *et al.,* 1961), to HBB (Loddo, 1963), to arildon (Egger and Rosenwirth, 1988), to disoxaril (Nikolaeva and Galabov, 2003), to oxoglaucine (Nikolaeva-Glomb and Galabov, 2008), etc.

Combination chemotherapy

Although many substances have been developed and their effect has been studied both in animal models and in humans, to date there are no registered products for specific treatment of enteroviral diseases. More than 1 000 substances have been selected as enteroviruses inhibitors in experiments *in vitro*, but less than 20 of them have manifested activity *in vivo*. The practical implementation of inhibitors is limited by the fact that these viruses have a very short replicative cycle and undergo multiple such cycles in the presence of the substances under conditions of experimental infection *in vivo*, resulting in decreased sensitivity to the inhibitor and development of resistance. The huge serotype diversity in the family and the possible toxicity of the medications used also contribute to the limited practical use.

These disadvantages can be avoided using combination therapy for treatment of viral infections in clinical practice. When applied in appropriate combinations, viral inhibitors have a higher efficacy than when used independently. One of the advantages of combination chemotherapy is that it restricts the selection of resistant and dependent mutants. Even in the case of prior resistance of a particular strain to one of the partners in the combination, most likely it will be sensitive to the effect of the other partner. The possibility of dual resistance to both partners in the combination is minimal, especially if the partners in the combination have different mechanisms of action.

Another advantage of the combination chemotherapy is that by using synergistic combinations, a more potent antiviral effect can be achieved at lower concentrations of the relevant inhibitors as compared to their independent administration, thereby reducing the so-called "dosage pressure" favouring the selection of resistant mutants, and solving the problem of toxicity.

To be considered effective and clinically promising, antiviral combinations must meet one the following conditions (Schinazi, 1991): to have at least an additive effect; to suppress or at least restrict the possibility of emergence of dependent and resistant mutants to each of the partners in the combination; to possess a therapeutic index which is higher than that of the two substances taken separately, i.e. the combination should not have enhanced toxicity.

The study of combined effects of the replication inhibitors of picornavirus, enterovirus in particular, is necessary because of the need for ef-

fective chemotherapy of enteroviral infections and better understanding of the mechanism of action of the combination partners, as well as to clarify the different stages of the replicative cycle of viruses.

The Laboratory of Experimental Chemotherapy of enteroviral infections at the Stephan Angeloff Institute of Microbiology has designed a model scheme for implementation in experimental infections *in vivo*, in which the approach to the application of substances differs from the standard simultaneous administration of the partner substances in the combination. The new approach for combined administration of antivirals is by sequentially alternating, non-simultaneous, application of the combination (CAA) of enterovirus inhibitors with different mechanisms of action. Initially, this scheme tested various double, triple and quadruple combinations against an experimental neurotropic infection with coxsackieviruses B1 in newborn mice. In these pilot studies, the best effect was achieved with the triple combination disoxaril/guanidine hydrochloride /oxoglaucine (DGO) (Vassileva-Pencheva and Galabov, 2010). Later on, two new triple combinations were tested on the same experimental model: pleconaril/guanidine hydrochloride/oxoglaucine (PGO) and pleconaril/MDL-860/oxoglaucine (PMO), where two of the partner compounds were replaced with more active ones (Stoyanova *et al.,* 2015; unpublished data). All three combinations showed distinct activities - a decrease in lethality and prolonged mean survival time in the experimental animals. The course with the triple combinations administered simultaneously led to high lethality and resistance to each of the partners. In contrast to this result, CAA courses with DGO, PGO and PMO led to a significant reduction of the viral content in the brain of animals. The sensitivity to each partner inhibitor was not only preserved but even increased in all three combinations. The way the compounds are arranged in the combination is of key importance for its performance. This course of treatment has confirmed its efficacy in other experimental models of coxsackie B virus infection *in vivo* (Vassileva-Pencheva and Galabov, unpublished data). The effectiveness of this new approach has been proven and can serve as the basis for developing other combinations and novel therapeutic strategies (Vassileva-Pencheva and Galabov, 2008, 2010, Stoyanova *et al.,* 2015). The traceability of phenotypic markers has been introduced for characterization of drug mutants (resistant and dependent) as an essential stage in the study of enteroviral inhibitors (Nikolova and Galabov, 2003).

In recent years, the studies on the antiviral activity of small interfering RNAs (siRNAs) have been gaining more and more attention. Once they have been inserted into the cell, siRNAs induce RNA interference (RNAi). RNA interference is a mechanism in which, in the presence of a double-stranded RNA molecule homologous to a given mRNA (or viral genome), the expression of the latter ceases. Since a gene has been made inoperative, the mechanism is referred to as post-transcriptional gene silencing. This mechanism may be used with certain viruses, where the goal is to inhibit their replication. The possibility of producing siRNAs that are effective against many closely related enteroviruses makes this approach particularly attractive. A team of the Department of Virology has studied the *in vitro* effect of siRNAs on the replication of coxsackieviruses B1 and B3 by a new technology for generation of a pool of siRNAs targeting a specific genetic region, overlapping and silencing the target sequence, irrespective of whether mutation or recombination has occurred in it (Petrov and Galabov, 2012, 2015). The possibility of producing siRNAs that are effective against many closely related enteroviruses makes this approach particularly attractive. As with all approaches, further testing is necessary with different enterovirus infections first in animals, then in humans, including the development of an efficient and convenient system for delivery of siRNA.

Due to the rapid development of resistance in enteroviruses, successful chemotherapy and prophylaxis of infections caused by them will probably involve a combination of powerful antiviral medications or a combination of an antiviral drug and an immunomodulator. Another no less promising solution is the combination therapy through sequentially alternating administration of antivirals with different mechanisms of action, unlike the standard simultaneous administration of the partner substances in the combination.

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