Perspectives for effective chemotherapy of enterovirus infections

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PERSPECTIVES FOR EFFECTIVE CHEMOTHERAPY OF ENTEROVIRUS INFECTIONS

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Abstract

Human enteroviruses distributed worldwide are causative agents of a broad spectrum of diseases with extremely high morbidity, including a series of severe illnesses of the central nervous system, heart, endocrine pancreas, skeleton muscles, etc., as well as the common cold contributing to the development of chronic respiratory diseases, and the chronic obstructive pulmonary disease. The above diseases along with significantly high morbidity and mortality in children, in the high-risk populations (immunodeficiencies, neonates) definitely formulate the chemotherapy as the main tool for the control of enterovirus infections. At present, clinically effective antivirals for use in the treatment of enteroviral infection do not exist, in spite of the lots of work carried out in this field. The main reason for this is the development of drug resistance.

The monotherapy courses were the only approach used till now. For the first time in the research for anti-enterovirus antivirals our team introduced the testing of combination effect of the selective inhibitors of enterovirus replication with different mode of action. We studied alongside the process of development of resistance to the strongest inhibitors of enteroviruses, WIN compounds (VP1 protein hydrophobic pocket blockers), especially in the models *in vivo*, CVB infections in mice. We introduced the tracing of a panel of phenotypic markers (MIC_{50} value, plaque shape and size, stability at 50° C, pathogenicity in mice) for characterization the drug-mutants (resistant and dependent) as a very important stage in the study of enterovirus inhibitors. Moreover, as a result of VP1 RNA sequence analysis performed on the model of disoxaril mutants of CVB1, we determined the molecular basis of the drug-resistance, two point mutations – M213H and F237L, both in the ligand-binding pocket. The sequence analysis of the drug-dependent mutant showed some reversion to the wild drug-sensitive virus strain.

A large-scale study of the combined effect of a series of anti-enteroviral agents with different modes of action resulted in the selection of a number of very effective *in vitro* double combinations revealing synergistic character of the combination effect and a broad spectrum of sensitive enteroviruses. Two antivirals developed in our previous studies were involved in these studies, PTU-23 and oxoglaucine. The most prospective attainment in our study in this field was the development of a novel scheme for the combined application of anti-enteroviral substances in coxsackievirus B1 neuroinfection in newborn mice. It consisted of a consecutive, alternating and non-simultaneous, administration of the substances in the combination. A triple combination - disoxaril-guanidine.HCl-oxoglaucine (DGO), showing good efficacy was selected. Its effectiveness was expressed in a marked reduction of mortality rate in infected mice as compared both to the placebo group, and to the partner compounds used alone every day, and to the same combination applied simultaneously every day. Studies of the drug sensitivity of viral brain isolates from mice, treated with DGO combination showed not only preserved, but even increased sensitivity to the drugs included in the combination. Obviously, the consecutive alternating administration of anti-enteroviral substances hinders the occurrence of drug-resistance in the course of the experimental enteroviral infections in mice.

Key Words: enteroviruses, antivirals, drug-resistance, combination effects

Резюме

Разпространени повсеместно, ентеровирусите са причинители на широк спектър от заболявания, включващ тежки състояния с патология на ЦНС, сърцето, скелетните мускули и др., а също така и т. нар. настинки, които биха могли да доведат до развитието на хронични респираторни заболявания и хронична обструктивна белодробна болест. Много високата заболеваемост и смъртност при децата, както и при високо-рисковите групи (имунодефицитни пациенти и новородени) категорично определят химиотерапията като основно оръжие за контрол на ентеровирусните инфекции. Независимо от интензивната работа в тази област, на този етап няма антивирусни препарати, които да са клинично ефективни за лечение на тези инфекции. Основната причина за това положение е развитието на лекарствена резистентност.

Изпитването ефекта на монотерапевтични курсове с ентеровирусни инхибито-

ри бе единственият подход използван досега. Нашият екип за първи път въведе изследвания върху комбинираните ефекти на селективни инхибитори на ентеровирусната репликация с различен механизъм на действие. Наред с това, ние изследвахме процеса на развитие на резистентност към най-ефективните инхибитори на ентеровирусите – WIN съединенията (блокиращи хидрофобния джоб в белтъка VP1). Тези изследвания бяха проведени основно *in vivo,* на модели на коксаки В вирусни инфекции в мишки. С цел характеризиране на лекарствените мутанти (резистентни и зависими), което е много важна фаза в изучаването на ентеровирусните ихибитори, ние представихме проследяването на панел от фенотипни маркери ($МИК_{50}$, форма и големина на плаките, стабилност при 50° С, патогенност за мишки). Освен това, като резултат от секвенционен анализ на участъка на РНК, кодираща белтъка VP1, извършен на модел на дизоксариловите мутанти на CVB1, ние установихме молекулната основа на лекарствената резистентност – две точкови мутации (M213H и F237L), намиращи се в лиганд-свързващия джоб. Секвенционният анализ на лекарствено-зависимия мутант показа една реверсия към дивия лекарствено-чувствителен вирусен щам.

Широкомащабно проучване на комбинирания ефект на серия от анти-ентеровирусни агенти с различен механизъм на действие доведе до подбора на редица много ефективни *in vitro* двойни комбинациии, които показаха синергичен характер на комбинирания ефект при широк спектър от чувствителни ентеровируси. В тези експерименти бяха включени и два ентеровирусни инхибитора, чийто ефект бе доказан за първи път при наши предишни изследвания - PTU-23 и оксоглауцин. Най-перспективното постижение на нашите проучвания бе прилагането на нова схема за комбинирано прилагане на анти-ентеровирусни вещества при коксаки В невроинфекция в новородени мишки. Тази схема се състои в последователно редуващо се, а не едновременно, прилагане на веществата в комбинацията. Бе подбрана една тройна комбинация – оксоглауцин-гванидин HCl-оксоглауцин (DGO), показваща добра антивирусна активност. Нейната ефективност се изразява в отчетлива намаляване на смъртността на заразените животни, при сравнение с плацебо групата, с групите третирани ежедневно с монотерапевтичен курс от партниращите си в комбинацията вещества, както и с комбинацията DGO при ежедневно едновременно прилагане на трите съединения. Изследването на лекарствената чувствителност на вирусни мозъчни изолати, взети от мишки, третирани с тази комбинация показа

не само запазена, но даже и увеличена чувствителност към веществата, включени в комбинацията. Очевидно, последователното алтернираще прилагане на анти-ентеровирусни вещества предотвратява възникването на лекарствена резистентност в хода на екпериментална ентеровирусна инфекция в мишки.

1. Enterovirus infections

Human enteroviruses are members of a large genus in the *Picornaviridae* family including important pathogens like the polioviruses, coxsackieviruses A and B, echoviruses, the newer enteroviruses and the rhinoviruses. These viruses, distributed worldwide, are the most common of human viruses (http://www.cdc.gov/ncidod/dvrd/revb/enterovirus/ non-polio_entero.htm), possibly infecting a billion or more individuals annually and resulting in hundreds of thousands of hospitalizations per year in the developed world (Pallansch 2011). Human rhinoviruses, which comprise a numerous group within the enterovirus genus, cause the development of common cold in individuals of all ages contributing to the development of chronic respiratory diseases, and the chronic obstructive pulmonary disease. In patients with asthma or chronic obstructive pulmonary disease the rhinovirus infection can lead to exacerbations and complications (Tan 2005; Mallia 2007).

The United States have developed a national Enterovirus Surveillance System (NESS), which is a voluntary system monitoring the trends in circulating enteroviruses since 1961 and it records 10-15 million cases each year (Khetsuriani 2006). Enteroviruses are the cause of up to 30% of sepsis like disease in newborns and play important role in infant morbidity and mortality (Lukshev 2010). The disease burden in the developing countries in the tropical regions of the world is poorly estimated, with the exception of poliomyelitis (http://www.cdc.gov/ncidod/dvrd /revb/enterovirus/non-polio_entero. htm). But, it is estimated that between 15% and 30% of young children in Taiwan, for example, may acquire enterovirus infection every year (Chuang 2010). Enteroviruses due to their high stability in the environment can also be the causative agents of nosocomial outbreaks, particularly severe in neonatal units and nurseries (Aitken 2001).

Along with the fact that enteroviruses are very efficient in establishing infection, the majority of them are asymptomatic or mild in their clinical course (Pallansch 2006; Morens 1995; Strauss 2008). The enterovirus contagiousness is such that actually, if an individual from a given group developed the symptoms of an enterovirus infection, it would be

certain that all members of the group were infected (Pallansch 2011). All age groups are infected with enteroviruses, but most primary infections occur during childhood. Usually young children are more susceptible probably due to the lack of cross reacting immunity, caused after repeated exposures (Sawyer 2002). For example, infants develop enteroviral meningitis 5 to 8 times more often as compared to adults (Nicolosi 1986). Curiously, enteroviral meningitis and enteroviral neonatal sepsis occur more commonly in males than in females (Khetsuriani 2006; Morens 1995). Furthermore, serious enteroviral illnesses are, in general, less common in very young children than in older children and adults (Morens 1995). Although the majority of infections are asymptomatic or with a mild clinical course, a wide variety of diverse syndromes and diseases are associated with the enterovirus infection. The most common manifestation of the infection is upper respiratory tract discomfort (Chuang 2010), fever, and, occasionally, mild gastrointestinal symptoms (Morens 1995). The link between enterovirus infection and a clinical manifestation should be discussed with caution, because inapparent infections sometimes are associated with prolonged virus shedding in stools and the mere isolation of an enterovirus from the patient's stools should not be considered as the proof of a definite link between the virus and a disease (Morens 1995).

Typically, a few enterovirus serotypes cause the infections in a given community during a given year, and certain serotypes are more likely to cause disease than are others (Strikas 1986). Usually, the primary site of infection is the epithelial cells of the upper respiratory or the gastrointestinal tract together with the lymphoid follicles of the small intestine. This is followed by the so called minor or primary viremia that can lead to spreading to secondary sites of infection (Pallansch 2006). Enteroviruses may affect various organs and systems including the central nervous system, the respiratory system, the skin, the heart, the pancreas, and the eyes. The most common tissue specific secondary localization of the enteroviral infection is the central nervous system, resulting in aseptic meningitis, or much more rarely, in encephalitis. Some of the enteroviruses may manifest tropism to other definite tissues like the cardiotropic coxsackieviruses or those viruses affecting the pancreatic beta cells, but the tropism is neither unique, nor specific (Archard 1987; Tauriainen 2011). Disseminated infection can lead to exanthema, non-specific myalgias or severe multiorgan disease in neonates (Morens 1995). Individual serotypes can be associated with different clinical manifestations and *vice versa*, a particular clinical manifestation can be caused by several different serotypes (Strauss 2008).

Severe and life-threatening conditions, such as meningitis, encephalitis, neonatal sepsis, myocarditis, pancreatitis, as well as further complications and some chronic diseases later in life, i.g. insulin dependent type 1 diabetes mellitus (Hyoty 2002; Galabov 2006) and dilated cardiomyopathy (Schultheiss 2006) can be attributed to an enterovirus infection, especially in neonates, infants and young children. Symptomatic infections include also the summer cold, herpangina, hand-foot-and-mouth disease, pleurodynia (Bornholm disease), rashes, hemorrhagic conjunctivitis, uveitis, chronic fatigue syndrome.

To survive, enteroviruses have developed a balanced relationship with their human hosts, so that the hosts will continue to maintain virus replication efficiently. Part of the bargain is that enteroviruses must not often harm the host seriously. Almost all of the serious conditions, caused by the enteroviruses, result from the spillover viremia, leading to the secondary sites of infection. An additional aspect is the capacity of each single serotype to contain a heterogeneous population of viruses that constitutes a quasispecies. This provides the raw material for selecting distinct new genetic strains in a single infected host. Thus the enteroviral disease thus not only results from the virulence properties of the agent itself, but also reflects the shaping role of the host (Morens 1995). The disease is the outcome of interactions between the certain agent, the certain host and the certain environment. Hence, the diversity of the manifestations of the enteroviral infection. Furthermore, virus infection is dependent on specific receptors. Five receptors for non-polio enteroviruses have been identified in human cells so far (Pallansch 2006). The diversity of receptors used by enteroviruses for invading the host cells, also contributes to the diversity of the clinical manifestation following viremia.

Once being a nightmare, poliomyelitis, which is caused by the three polioviruses from the *Enterovirus* genus, is nowadays controlled and driven to just a few corners of the world after years of immense efforts, thanks to the mass immunization measures taken by the World Health Organization and its Global Polio Eradication Initiative launched in 1988. Nevertheless, today, when more than 10 years have passed after the initial deadline for eradication of the disease, polioviruses are still endemic in several regions of the world and local epidemics or individual imported cases are announced from time to time (http://www. polioeradication.org /Dataandmonitoring/Poliothisweek.aspx). The WHO has called for a strategy to keep the disease from coming back (Roberts 2004).

2. Development of antiviral chemotherapy and chemoprophylaxis in the strategy of the control of enterovirus infections

Because unusually a great number of the enterovirus serotypes are causative agents of clinical illness, it is evident that the establishment and introduction of vaccination will not be possible in the near future.

The total enormously high morbidity, the development of severe illness, embracing pathology of CNS, heart, beta cells of pancreas, skeleton muscles, etc., significantly high morbidity and mortality in children, in the high-risk populations (immunodeficiencies, neonates) definitely formulate the chemotherapy as the main tool for the control of enterovirus infections. There is no doubt that the establishment of effective chemotherapy would be of great benefit and efforts have to be currently focused on the development of effective antivirals to treat enterovirus infections.

As shown the main factors in the large spread of enterovirus infections in the human population are, as follows: (i) a very high stability of enteroviruses in the environment (including water sources, food preparation procedures at home and in restaurants); (ii) long periods of seasonal spread – "summer viruses" in northern hemisphere climatic zones (Europe, Northern America) and all year without seasonal variation in the tropical climate zones; (iii) the two ways of transmission – sharply predominated fecal-oral rout, but respiratory way has not to be underestimated; (iv) the high prevalence of inapparent cases (more than 85%) in the great number of outbreaks of enteroviral infections in a childcare (nursery schools, kindergarten, primary schools, pediatric hospital units, etc.).

The high prevalence of inapparent cases, is an strong argument for the prevailing role of specific anti-enteroviral chemotherapeutic agents administered during the disease latency period, e.g. their use as urgent prophylaxis. The implication is to start administration of anti-enteroviral chemotherapeutic agents to all children in a childcare unit, immediately after the first case/s of an enterovirus-induced symptomatology been registered. Such approach could reduce the risk of acquired diabetes and other severe forms of enterovirus-induced infections

Another aspect of the treatment of some non-poliovirus induced enterovirus diseases is the formulation of a complex therapy, based on the established pathogenesis of the respective enterovirus illness, e.g. use of anti-enteroviral chemotherapeutic agents in

combination with suitable immunomodulator in the case of insulin-dependent virus-induced diabetes mellitus, etc.

Another indication for the use and development of anti-enteroviral agents is related with some new aspects of the poliomyelitis strategies for control. The striking 50-year-long decline in the incidence of poliomyelitis has stalled in the past 7 years, which has led to calls for an urgent re-assessment of eradication and post-eradication campaign strategies. The current plan of eliminating the circulation of wild poliovirus so that further immunization will be unnecessary does not take into account recent scientific data and political realities that limit the likelihood that this strategy can sustain prevention of the disease. It is crucially important that high levels of population immunity are maintained against the foreseeable future (Chumakov et al. 2007). In general, the new aspects of the poliomyelitis strategies for control are as follows:

(i) Development of fundamentally new vaccines, based on modified Sabin strains that are predicted to have increased genetic stability (Kohara et al. 1993; Agol et al. 1996; Macadam et al. 2006). Introduction of these Sabin strains as vaccinal need enormous size of clinical trials to prove their superiority over the conventional Sabin strains in humans. Attenuating mutations outside the capsid region, no matter how stable, can be readily eliminated by recombination (Chumakov et al. 2007).

(ii) Development of efficacious anti-polio drugs. The Third Meeting of the Advisory Committee on Poliomyelitis Eradication, held in October 2006, proposed the establishment of a "poliovirus antiviral initiative" and the appropriate and possibly essential development of at least two anti-polio drugs for control in the post-eradication era (Conclusions and recommendations of the Advisory Committee on Poliomyelitis Eradication, Geneva 2006). These will be of great benefit for post-exposure prophylaxis and outbreak control (Workshop Report, Committee on Development of a Polio Antiviral and Its Potential Role in Global Poliomyelitis Eradication, 2006; Chumakov et al. 2007).

3. The difficult way from *in vitro* (in cell cultures) screening of inhibitors of enterovirus replication to the clinical trials for chemotherapeutics effective in enterovirus-induced diseases

More than thousand substances products of chemical synthesis or of natural (mainly of plant) origin have been screened and characterized as selective inhibitors of enterovirus

replication as a result of testing in cell culture experiments. Several standardized scheme have been developed and largely applied. The extremely important topic of the methodological aspects merits a special detailed description and consideration.

Here, we would like to stress on the problem of the sharping difference between the antiviral activity established in cell culture (*in vitro*) experiments and testing in *in vivo* experimental models of viral infections (in laboratory animals).

The studies followed in cell culture experiments of the selected in the screenings substances manifesting some antiviral activity includes two very important steps: (i) determination of the main parameters of antiviral effects - the values of IC_{50}, CC_{50} and the selectivity ratio (selectivity index, SI); (ii) elucidation of the mode of action – the target in the viral replication cycle attacked by the active substance. A very important requirement for the anti-enteroviral substances is the large spectrum of susceptible viruses within *Enterovirus* genus.

Here is presented the list of antivirals, inhibitors of enterovirus replication grouped and classified according to their mechanism of action (Galabov and Angelova 2006; De Palma et al. 2008).

Antivirals active vs. enteroviruses *in vitro*

A. Inhibitors of early replication stages (mainly capsid-binding compounds)

- WIN compounds *(Sterling-Winthrop)*: arildone [4-[6-(2-chloro-4-methoxy-phenoxy) hexyl] 3,5-heptadion] (WIN38020); disoxaril [5-[7-[4(4,5-dihydro-2-oxa-zolyl)phenoxy] heptyl]-3-methyl-izoxazole] (WIN51711); WIN54954; pleconaril [3-3,5-dimethyl-4-[(3-methyl- 5-isoxazolyl)propyl]-5- (trifluoromethyl)-2,4-oxadiazole] (WIN61893).
- Pyridazinamine analogues (R compounds) (*Janssen Research Foundation*): R 77975 (Pirodavir) [ethyl p-(2-(1-(6-methyl-3-pyridazinyl)-4-piperidyl)ethoxy) benzoate]; R 61837;

R 78206; BTA-39; BTA-188; BTA-798 *(Biota)*.

- Imidazole derivatives *(Schering-Plough)*: SCH 38057 [1-[6-(2-chloro-4-meth-oxy)-hexyl]imidazol hydrochloride]; SCH 47802; SCH 48973.
- Isoxazole derivatives: compounds VIa,19, 20, 21.
- Pyridil imidazolidinones: BPROZ-194 [1-[5-(4-bromophenoxy)pentyl]-3-(4-pyridyl)-2-imidazolidinone]; DBPR 103; compounds 8b,14a, 28b.

- Phenoxybenzenes, phenoxypyridines and related analogues *(Merrell Dow)*: MDL-860 [2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile] (DNB)*; compounds 71, 13, 21; DEPC [6-(3,4-dichlorophenoxy)-3-(ethylthio)-2-pyridine carbonitrile].
- Pyranopyridines *(Merrell Dow)*: MDL 20,610 [6-substituted 2-(3,'4'dichloro-phenoxy) -2H-pyrano[2,3-b]pyridines]; MDL 20,646; MDL 20,957.
- Flavine derivatives: BW 683C (4,6-dichloroflavane); BW 4294 (1-anilino-9-benzyl-2-chloropurine); 4',6-dicyanoflavan; 3(2H)-isoflavene.
- Chalcones (Ro compounds) *(Roche)*: Ro 09-0410 [4'-ethoxy-2'-hydroxy-4,6'-dimeth-oxychalcone]; Ro 09-0696; Ro 09-0881.
- Methylthiopyrimidines: S-7: Ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate.
- Rhodanine [2-thio-4-oxothiazolidine; ethylene thiourea].
- 44 081 [2-[(1,5,10,10a-tetrahydro-3H-thiazolo[3,4b]isoquinolin-3-ylidene) amino]-4-thiazoleacetic acid].
- Dibenzofuran and related compounds.

B. Inhibitors of virus-specific RNA synthesis

- Guanidine hydrochloride.
- N,N'-Diphenylthioureas: PTU-23 [N-phenyl-N'-2-hydroxyphenylthiourea]; PTU-20; PTU-24.
- Benzimidazoles: HBB [2-α-hydroxybenzyl-benzimidazole]; enviroxime [2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketonoxim (LY 122771-72); enviroxime-related derivatives (vinylacetylene benzimidazoles); enviradene: (E)-1-[(1-methyletyl)sulfonyl]-6-(1-phenyl-1-propenyl)-1H-benzimidazole-2-amine; compound 12; C2-analogue with 2-amino substitution; imidazo[1,2-*b*]pyridazine analogues; imidazo[1,2-*a*]pyridines; MRL-1237 [1-(4-fluorophenyl)-2-(4-imino-1,4-dihydropyridin-1-yl)methylbenimidazole hydrochloride]; TBZE-029 [1-(2,6-difluorophenyl)-6-trifluoromethyl-1H,3H-thiazolo[3,4-a]benimidazole].
- Gliotoxin.
- Isothiazole derivatives: DID [5,5'-diphenyl-3,3'-diisothiazole disulfide].
- Flavones: 3-methylquercetin (3-MQ); Ro 09-0179 [4',5'-dihydroxy-3,3', 7-trimeth-oxyflavone]; 3-methylkaempferol; 3,4'-dimethylkaempferol;

*Inhibitor of an early event in the virus replication after initial uncoating

2-styrylchromones vinylogues flavones.

- Pyrrolidine dithiocarbamate (PDTC).

C. Virus-specific protease inhibitors (attacking virus specific proteases 2A and 3C):

- Peptidic inhibitors: Peptide aldehydes: tripeptyde aldehyde; 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-oxopyrrolidin-3-yl pent - 2- enolate *(Pfizer)*;diazomethyl ketones (DMK): tripeptidyl-DMK; peptidyl monofluoromethyl ketones; peptidyl N-iodoacetamides.
- Non-peptidic inhibitors: 2-pyridone-containing peptidomimetics (compounds XIX, XX, XXI); substituted benzmides (compound XXIII); homophthalamides (compound XXVI).
- Elastase-specific inhibitors: methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MCPK); elastinal.
- Homophthalamides: compound XV.

D. Antivirals with other mechanism of action

- Ribavirin: 1-β-D-ribofuranozyl-1,2,4-triazole-3-carboxamide.
- Hydantoin [5-(3,4-dichlorophemyl)methylhydantoin.
- Hydrophyllic compounds: hygromycin B.
- Aziridine and N-dansylaziridine.
- Oxoglaucine.

The following Table 1 summarizes the effects of those substances showed above which demonstrated an antiviral activity in experimental enterovirus infections in laboratory animals (in vivo). As seen, their number is drastically decreased.

Table 1
Inhibitors of entero/picornavirus replication effective in experimental enterovirus infections in laboratory animals (*in vivo* models)

Compound	Virus	Lab animal	Route of administration (Dose)	References
Inhibitors of attachment, entry, and uncoating				
WIN compounds				
Aryldone	poliovirus	mice		McKinley et al. (1982)
Disoxaril	poliovirus 2	mice	per os	McKinley et al. (1986a,b); Jubelt et al. (1989)
	echovirus 9	mice	per os	
WIN54954	CVB4/diabetogenic	mice	per os (50 mg/kg)	See, Tiles (1993)
	CVA9/miocarditis	mice	per os (50 mg/kg)	See, Tiles (1992)
Pleconaril	CVA9	mice	i.p. (200 mg/kg)	Pevear et al. (1999); Florea et al. (2003)
	CVA21	mice	per os (19-75 mg/kg)	
	CVB4/miocarditis	mice	per os (20-200 mg/kg)	
SCH				
38057	CVB3	mice	per os (60 mg/kg)	Rozhon et al. (1993)
	echovirus 9	mice	s.c. (20 mg/kg)	
47802	poliovirus	mice	per os (60 mg/kg)	Cox et al. (1996)
48973	poliovirus	mice	per os (3-20 mg/kg)	Cox et al. (1996)
SDZ				
35-682	echovirus 9	mice	s.c. (36-126 mg/kg)	Rosenwirth et al. (1995)
Phenoxybenzenes				
MDL-860	CVB3/myocarditis	mice	s.c. (250 mg/kg)	Markley et al. (1986); Padalko et al. (2004)
	CVA21	mice	s.c. (250 mg/kg)	
Compound 21	CVA21	mice	s.c. (250 mg/kg)	Markley et al. (1986)
Replication inhibitors				
HBB				
HBB + guanidine.HCl	CVA9	mice	s.c. (10 mM HBB +100 mM Gua)	Eggers (1976)
	echovirus 9	mice		
N,N'Diphenylthioureas				
PTU-23	CVA7-IV	cotton rats	s.c. (400 mg/kg)	Galabov (1978)
	CVA6	mice	s.c. (130 mg/kg)	Galabov, Velichkova (1974); Galabov (1979)
	CVA7	mice	s.c. (260 mg/kg)	
	CVB1	mice	s.c. (130 mg/kg)	
	CVB3	mice	s.c. (173.2mg/kg)	
PTU-20	CVA7	mice	s.c. (346.5 mg/kg)	Galabov, Velichkova (1974); Galabov (1979)
	CVB1	mice	s.c. (43.3 mg/kg	
	CVB3	mice	s.c. (65 mg/kg)	
Flavonoids				
Ro 09-0179	CVB1	mice	per os (10-40 mg/kg)	Ishitsuka et al. (1982)
3-MQ	CVB4	mice	per os (20 mg/kg)	Van Hoof et al. (1984)

The next table (Table 2) presents the results from the clinical trials for anti-enteroviral chemoherapeutics carried out following the double-blind placebo-controlled schedule in which were involved compounds inhibiting enterovirus replication.

Table 2

Inhibitors of Enterovirus Replication in Clinical Trials

- **Disoxaril (WIN51711)** – poor bioavailability; crystalluria in healthy volunteers (Phase I) stopped further clinical development.
- **WIN 54954** – well tolerated in Phase I; applied orally effectively reduced the severity of coxsackievirus A21-induced natural mild respiratory infections; lack of effect against experimentally induced infections caused by rhinovirus 23 and 29 (Tutner et al. 1993); rapidly metabolized; induced reversible hepatitis (Diana et al. 1995); adverse effects (flushing, rush) precluded further development.
- **Pleconaril (WIN63843)** – rate of metabolism strongly reduced (Diana et al. 1995); displayed some efficacy in 2 of 3 neonates with enteroviral hepatitis (Aradottir et al. 2001); marked efficacy against chronic meningoencephalitis (78% improvement adverse effects been minimal) (Rotbart and Webster 2001); no efficacy in another double-blind trial in children with enteroviral meningitis, twice as many adverse effects have been registered (Abzug et al 2003); in phase III double-blind placebo-controlled trial some efficacy was shown in coxsackievirus rhinovirus A21-induced respiratory infections (common cold) when the treatment start within 24 hours of symptom onset (Hayden et al. 2003); drug-increased levels of the CYP3A4 liver microsomal enzyme stopped further trials, as decided by FDA in 2002 (Senior 2002). Schering-Plough carried in 2006-07 a placebo-controlled study of the effects of pleconaril nasal spray on common cold symptoms and asthma exacerbations following rhinovirus exposure (Clinical Trials. gov.U.S.NIH, 2007), the results of which have not yet been announced. In meanwhile, pleconaril manifested properties of a virucidal for hand treatments for prevention of rhinovirus infection (Turner and Hendley 2005).
- **BTA-798 (oxime ether analogue of pirodavir)** – company Biota Scientific Management Pty Ltd scheduled a phase II of placebo-controlled double-blind clinical trial of this compound in asthmatic adults with symptomatic rhinovirus infection, starting on August 2010. This study is ongoing, but no information about the course and results till February 8th, 2012 (according FDA) (Clinical Trials.gov Identifier: NCT01175226).

The failure of the clinical trials of the inhibitors of enterovirus replication selected through the preclinical studies is undoubtedly due mainly to the lack of selectivity, substantiated by the well expressed side effects in the human body. The results obtained are in agreement with the manifested lack of efficacy in experimental models in vivo of the great majority of the active in vitro substances, and more precisely only less than 20 out of hundreds inhibitors of enterovirus replication manifested some in vivo activity.

In conclusion, at actual time clinically effective antivirals for use in the treatment of enteroviral infection do not exist. The realization of anti-enteroviral chemotherapy is a problem open for the future.

The totality of experimental data in the antiviral field and the knowledge accumulated on the molecular biology of picorna/enteroviruses explicitly demonstrates that the main reason for the lack of antivirals for clinical use in enterovirus infections is the development of drug-resistance.

Enterovirus replication inhibitors developed by SAIM Department of Virology team

Two groups among the compounds included in the list have been developed as a result of previous studies of ours and in the Department of Virology of the Stephan Angeloff Institute of Microbiology. These are N,N'-diphenylthiourea derivatives and oxoglaucine.

PTU-23 and related compounds

The N,N'-diphenylthiourea derivative PTU-23 have been characterized as a strong and specific inhibitor of picornavirus replication. Its antiviral spectrum embraces polioviruses 1-3, CVB 1-6, CVA 7/IV, echoviruses 1, 3, 6-9, 11-14, 17, 19, 20, enterovirus 71, ECSO II-IV, EMCV, rhinoviruses 1B-632 and H-17, FMDV type C (Galabov 1979). Another derivative from the same group, PTU-20, showed a marked activity against polio 1, CVB1 and echovirus 19. PTU-24 is active against CVB1, some ECSO and HRV H-17, and possessing strongest effect vs. FMDV (Galabov 1979). Based on structure-activity relationship study, a planned synthesis has been carried out attaining to a several highly active derivatives (Galabov et al. 1980). Studies on the mode of anti-picornavirus effect of PTU-23 characterized this compound as a selective inhibitor of specific viral 37S ssRNA synthesis as a result of suppression of the synthesis of a viral protein with regulatory functions in the replication cycle (Galabov 1979; Galabov and Dmitrieva

1983; Galabov et al. 1983). Moreover, PTU-23 antagonizes the anti-enteroviral effect of guanidine hydrochloride (Galabov AS, unpublished data).

Oxoglaucine

In a pilot study performed by our research group, a series of aporphinoid alkaloids isolated from *Glaucium flavum Crantz* (yellow horn poppy) or obtained synthetically, had been tested *in vitro* for their antiviral activity against viruses belonging to several taxonomic groups, including picorna-, orthomyxo-, paramyxo-, and herpesviruses. One of the compounds, oxoglaucine, manifested a well-pronounced inhibitory effect against the replication of poliovirus type 1 (Mahoney) from the *Enterovirus* genus in the *Picornaviridae* family (Galabov et al. 1995).

Oxoglaucine can be isolated from the epigean parts of *Glaucium flavum Crantz* (Kuzmanov et al. 1998) and it could also be obtained synthetically from the main plant alkaloid glaucine (Philipov et al. 1998).The concentration of oxoglaucine, which produced no visible cytotoxic effect on monolayer FL cells, the maximal tolerated (non-toxic) concentration (MTC), was 18 μM, and the concentration reducing cell growth by 50% (cell growth inhibitory concentration 50, $CGIC_{50}$) was 12 μM.

The antienteroviral spectrum of oxoglaucine was tested against a panel of 16 enteroviruses – poliovirus type 1 (Mahoney), poliovirus type 1 (LSc-2ab), and a series of viruses belonging to human enterovirus B: coxsackievirus A9 (CV-A9), the six coxsackie B viruses (CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, and CV-B6), and echoviruses (EV)-2, EV-4, EV-6, EV-9, EV-13, EV-15, and EV-19. The endpoint dilution method in the multi-cycle cytopathic effect (CPE)-inhibition setup and the plaque-inhibition test was used for determining the antiviral effect. Oxoglaucine revealed a marked inhibitory effect on all of the tested viruses. Concentrations that reduced virus titer by 1, 1.67, and 2 lg as compared to that of virus control (with no oxoglaucine in the maintenance medium) were determined. The concentrations reducing virus titer by 1 lg ranged from 0.03 to 3.0 μM. Inhibitory concentrations 50 (IC_{50}) ranged from 0.5 to 5.0 μM. CV-B4 and CV-B5, followed by CV-B3, CVA9, and EV-9 were the most susceptible to the antiviral effect of oxoglaucine. On the opposite end, the highest dose of oxoglaucine was required to inhibit the replication of CV-B1. On the basis of these results and the cytotoxicity parameters of oxoglaucine, the selectivity index (SI) was calculated as the ratio of MTC and IC_{50}. As expected, oxoglaucine exerted the highest selectivity against CV-B4 ($SI \approx 400$), followed

by EV-9, EV-13, and EV-19, as well as by CV-B3 and CV-A9 (Nikolaeva et al. 2007, 2008). A marked anti-rhinovirus activity was found (Georgieva and Galabov 2008).

In general, oxoglaucine revealed a broad-spectrum antienteroviral activity accompanied by high selectivity.

Research on the mode of action of oxoglaucine is in progress. In a preliminary stage of the research, the direct virucidal effect of the compound was tested in the quantitative suspension test at three temperature regimens (4°C, room temperature, and 37°C). The results obtained indicated undoubtedly that oxoglaucine possessed a virus-specific mode of antiviral action and its effect was not due to the direct inactivation of extracellular virions. In a time-of-drug-addition set-up oxoglaucine inhibited the production of infectious virus particles when added early in infection. The effect is the strongest when oxoglaucine was added on hour 0 p.i. The antiviral effect was also quite strong when the compound was added up to the 3rd hour. Addition after the 5th hour did not result in an inhibition. It could be concluded that oxoglaucine exerts its antiviral effect in the early stages of the virus replication cycle (Nikolaeva et al. 2007, 2008).

4. Development of drug resistance: the main problem in the chemotherapyof enteroviral infections

Viral RNA-dependent RNA polymerase manifests high infidelity, e.g. each newly synthesized molecule of poliovirus RNA contains, on average, one mutation (Agol 2006).

Therefore, in natural conditions the picornaviral progeny is quite heterogenous. The unlimited number of mutants in the picorna- viral population is a result of point mutations occuring randomly during the viral replication, the so called pseudospecies (quasispecies).

Drug-resistant phenomenon attains unusually high levels in the case of picornaviruses, as random genetic mutations can occur at a very high frequency (10^{-3}-10^{-4}) (Richards and Ehrenfeld 1990). A drug-resistant picornaviral progeny will develop quite easily as a result of selection of pre-existing mutants.

First description of drug-resistance in enteroviruses is done by Melnick et al. (1961). They isolated poliovirus 1 mutants resistant to guanidine in monkey kidney cell culture experiments. Loddo et al. (1962) confirmed these data adding proofs for development of

drug-dependent poliovirus mutant. Later drug-resistant enterovirus mutants have been isolated to approximately all specific enteroviral replication inhibitors, independently of their mechanism of action (Loddo1980). Herrmann and Herrmann (1977) postulated the drug-resistance development as an indicator of specific antiviral activity. The only experiments on development of drug-resistant enterovirus mutants in an animal model were done by Barrera-Oro and Melnick (1961): in guanidine-treated monkeys infected with poiliovirus 1.

Disoxaril mutants of CVB1

We investigated the resistance phenomenon on the model of CVB1 with disoxaril, a typical representative of the WIN compounds – a highly effective inhibitor of a broad spectrum of entero- and rhinovirus serotytpes. We selected WIN compounds because they are considered as the most potential inhibitors of enterovirus replication . They inhibit the virion uncoating process (Fox et al. 1986). The direct crystal X-ray analysis of virus-inhibitor complexes showed that the primary target structure of this actionis the hydrophobic pocket beneath the "canyon" on the VP1 protein (Rossmann 1989). WIN compounds inserted into a cleft formed by a twisted β-sheet increase the structural rigidity of the VP1 subunit thus preventing virus uncoating.

Enterovirus resistance to disoxaril has been established initially with poliovus type 3/Sabin (Mosser et al. 1994). Later this resistance was observed in CVB3 to another WIN compound - pleconaril (Croarke and Pevear 1999).

In the first stage of our research (Nikolova and Galabov 2003), we have demonstrated the development of resistance to desoxaril during treatment of experimental CVB1 infection in newborn mice. In fact, the obtaining of drug-resistance in in vivo model of enterovirus infection was the second after the classic data of Barrera-Oro and Melnick (1961) on guanidine-resistance registered within poliovirus 1 infection in monkeys. Moreover, our findings represented the first development on a model in vivo of drug-resistance to a WIN compound. In these experiments we showed that inefficacy of disoxaril course on the enterovirus infection is due uniquely to the accumulation of a drug-resistant progeny in the target organ, the mouse brain since day 6th after virus inoculation (IC_{50} = 3.3 μM, 0.64-1.37 μM in placebo group) and reaching maximal levels (IC_{50} = 35->40) within 24-48 h (on the 7th-8th day).

Only three passages of the wild CVB1 (CVB1/SOF, disoxaril-sensitive) in FL cell

cultures in the presence of disoxaril were sufficient for the development of disoxaril-resistant Coxsackievirus B1 mutant (CVB1/RES). The *in vivo* and *in vitro* generated disoxaril-resistant CVB1 mutants demonstrated almost identical phenotype characteristics.

Table 3
Phenotypic charactristics of disoxaril mutants of Coxsackievirus B1: summarized data

Cox B1 disoxaril mutants	MIC_{50} (μM)	Plaque diam. (mm)	Plaque Shape	Stability at 50° C $ET_{\Delta=3\ logs}$ (min)	Pathogenicity for mice
Wild SOF/FL	0.84	0.9 ± 0.3	round	31	normal
RES-30μM/FL	>30.0	1.9 ± 0.1***	irregular	7	slightly increased
Wild SOF/mice	0.52-1.37	0.9 ± 0.3	round	26	normal
RES-25mg/kg/mice	>40.0	1.9 ± 0.1***	irregular	13	slightly increased

***, $p < 0.001$ for CVB1/RES vs. CVB1/SOF (dis-sensitive) mutants, Student's *t*-test.

Nine consecutive passages of CVB1/RES in FL cells in the presence of disoxaril (30μM) resulted in the selection of a disoxaril-dependent mutant (CVB1/DEP) (Nikolova and Galabov 2002).

The analysis of the phenotypic markers of the isolated disoxaril mutants showed (Nikolova et al. 2011) that the changes in the sensitivity to disoxaril (WIN) correlated with the changes of the size and the shape of virus plaques under agar (Table 4}. This marker of the CVB1/RES mutant also correlated with the significant increase of thermosensitivity. It is well known that the marker $T_{50}°_{C}$ is connected to the stability of virions which for the enteroviruses is to a large extent determined by the VP1 integrity. The ligand pocket structure of this protein in the disoxaril-resistant mutant (CVB1/RES) has a nature that prevents the binding of any ligand (palmitate or disoxaril). This compromises the virions' stability toward drug (WIN) treatment. In the case of dependent mutants the presence of such an inhibitor in the cell culture medium protects them from thermoinactivation.

In the disoxaril-resistant mutants the hydrophobic pocket cavity is empty and this structure is highly restrictive to take any ligand(s); it comes water accessible and thus – thermolabile (thermounstable). In disoxaril-dependent mutant the hydrophobic pocket is still occupied – it has one (or two) molecule(s) disoxaril and this ensures of that mutant pentamer "cross links" with increased thermostability of the wild disoxaril-sensitive strain. This guarantees a successful virus replication. The new population of disoxaril-dependent mutant needs new molecules of disoxaril for assembling of virions and to continue the virus replication.

Differences between CVB1/RES and CVB1/DEP were also observed in the marker pathogenicity for mice. In contrast to increase pathogenicity registered for CVB1/RES (Nikolova and Galabov 2003), CVB1/DEP pathogenicity was strongly dependent on the presence of disoxaril. On one hand, an almost full similarity was established between the pathogenicity found in CVB1/DEP in the presence of disoxaril and in CVB1/SOF in the absence of disoxaril. On the other hand, a considerably stronger decrease of the pathogenicity of CVB1/DEP in the absence of disoxaril, as compared to the protective effect of the compound on the wild CVB1/SOF (dis-sensitive), was registered (Nikolova et al. 2011). This data correlate well with the results from the cell cultures experiments.

Results of our studies on disoxaril mutants of CVB1 showed that the determination of a panel of phenotypic markers of the viral drug-resistant mutants is of significant importance and should be recommended as an obligatory requirement in the experimental chemotherapy of viral infections. This study has to be introduced as an obligatory step in the program of development of an antiviral. This is especially valid for development of antivirals against enteroviruses.

The main goal of the next stage of our investigations was to clarify the molecular basis of the antiviral effect of disoxaril targeting the CVB1 VP1 protein by comparing phenotypic and genotypic characteristics of the wild strain (disoxaril-sensitive), disoxaril-resistant, and disoxaril-dependent mutants.

The analysis of the results from the timing-of-addition study following the one-step virus growth cycle experimental setup with the disoxaril-dependent mutant of CVB1 shows that even the late addition of the compound, *i.e.* during the exponential phase (5 - 6 hours after the virus inoculation) guarantees full production of the infectious virions at the end of the one-step cycle. These data lead to the conclusion that structural changes

of the capsid protein VP1 in the disoxaril-dependent mutant do not affect the process of uncoating and the following synthesis of viral RNA and proteins. Such changes in VP1 of the disoxaril-dependent mutant appear to affect the stage of mature virions' assembly only and not the stage of uncoating and synthesis of viral RNA and proteins (Nikolova et al. 2011). A similar drug-dependent virion assembly process was established in the HRV16 mutant dependent to another WIN compound, WIN 52035 (Wang et al. 1998).

Table 4
Phenotypic characteristics of disoxaril mutants of CVB1 strains

CVB1 disoxaril mutants	MIC_{50} [μmol/L]	Plaque diameter [mm]	Plaque shape	Stability at 50 °C ET_{50}[a] [min]	Pathogenicity for mice
CVB1/SOF	0.84	0.9 ± 0.3	round	31	normal
CVB1/RES	>30.0	1.9 ± 0.1*	irregular	7	slightly increased
CVB1/DEP	---	1.9 ± 0.1	irregular	23	normal

[a]ET_{50}, effective time 50%: the time (in minutes) necessary for a 50% reduction of the infectious virus titer
*$p < 0.001$ for CVB1/RES vs. CVB1/SOF mutants, Student's *t*-test.

The results with CVB1/SOF (dis-sensitive) show that the inhibitory effect of the compound strongly depends on the moment of its addition during the latent period, data being in compliance with the literature references pointing out that disoxaril blocks virus uncoating (Zeichhardt et al. 1987; Diana et al. 1989; Fox et al. 1991).

VP1 genes sequencing and BLAST analysis of CVB1 disoxaril mutants

DNA fragments containing the complete VP1 sequence of wild CVB1/SOF and both

disoxaril mutants were generated by RT-PCR (Nikolova et al. 2011). A constant specific feature was observed in all DNA fragments of the CVB1/RES and CVB1/DEP mutants when compared to the sequence of CVB1/SOF was the deletion of a nucleotide TTG / insertion of a nucleotides TTT at the same location in relation to the sequence of the CVB1/SOF (ntt 2749 - 2751 from M16560/ins TTTnt.). These changes did not lead to any change of the reading frame.

According to the BLAST data, the comparison of the particular fragments demonstrated a high degree of similarity of CVB1/SOF, CVB1/RES and CVB1/DEP sequences, except for the area starting from approximately 200th to 240th amino acid residue which corresponds to the ligand-binding site (canyon region) of VP1.

Substantial difference in the primary sequence between VP1 of CVB1/SOF, CVB1/RES and CVB1/DEP is observed in the 196th to 258th amino acid residues. It is remarkable that SOF and DEP mutants have higher degree of similarity than SOF and RES.

The multiple alignments (parallel comparison) of the three sequences (CVB1/SOF, CVB1/RES, CVB1/DEP) all "gaps" are condensed in the ligand-binding site of the CVB1/RES and the CVB1/DEP mutants. Cys is located on position 225 in the CVB1/SOF, while the resistant and the dependent mutants feature a deletion at this position. The R251 amino acid is deleted in both CVB1/SOF and CVB1/RES, but it was reconstructed in the CVB1/DEP mutant. The critically significant Met on position 213 is substituted by His in the resistant mutant, and Phe on position 237 is replaced by Leu in both the wild and the resistant mutants. All analyses performed so far unambiguously show that the primary structure of VP1 of the resistant mutant in the segment 1965-258 is entirely altered in comparison to all other sequences. The return to original sequences is very typical for CVB1/DEP mutants.

In summary, our analysis of the primary structure of CVB1 VP1 mutants of the internal segment 196 -258 showed that it is highly changed in RES and DEP in comparison to referent REF/SOF structures (Nikolova et al. 2011). The sequence changes obtained give a satisfactory explanation for the mutant resistance. However, the CVB1/DEP mutation and mutant behavior cannot be explained based on 1D-sequencing only. The experimental evidence that CVB1/DEP needs disoxaril to provoke virus coating requires further studies to determine the 3D structure of the target VP1 protein. Such approach could contribute to the characterization of the CVB1/RES mutant and would clarify the nature of disoxaril-resistance and dependence.

Resistance to oxoglaucine

Since the development of resistant virus mutants is considered as an indicator of specific antiviral activity, oxoglaucine resistant mutants have been selected and characterized. Resistant virus strains were derived for poliovirus type 1 and the six coxsackie B viruses. A correlation was observed between the sensitivity of the ancestral virus and the necessary number of passages for selection of resistant progeny. The more sensitive the virus, the faster development of resistance. The relatively slowest development of resistance was observed in the case of CVB1. If no dose pressure was exerted a reversion to sensitivity, although not complete, was observed. The phenotypic characteristics of the oxoglaucine-resistant CVB1 were determined. The resistant mutant possessed a lower virus titer in comparison to the ancestral strain. A gradual increase of the rate of the relative resistance was established in the course of the consecutive passages in the presence of high doses of oxoglaucine (Nikolaeva, 2011).

5. Combination effects of anti-enteroviral substances

So far, despite the substantial progress made towards the development of efficient anti-enteroviral chemotherapy, no drug has been approved for clinical use. The development of a safe and effective anti-enteroviral medicine is still a challenge. The inevitable and usually fast selection of resistant and even dependent enterovirus mutants is a significant obstacle hindering the progress. The resistance occurring after monotherapy with a certain drug makes it reasonable to focus interest on combined administration of antiviral compounds. This approach has proven its efficacy against HIV and HCV infections and is considered as a perspective to be applied in a situation of pandemic flu as well. Using such combined therapy, a greater effect could be achieved at lower concentrations than those required if drugs were used alone and thus to prevent the so-called "pressure of the dose" effect, which favors the rapid development of virus-drug resistance. By use of synergistic combinations the same antiviral effect might be achieved with lower concentrations than those required if drugs were used alone. Thus, a higher selectivity ratio could be achieved.

And what is especially important and highly waited: the perspective by combining drugs to restrain the resistance phenomenon.

Research on the combined effects of antivirals may reveal the existence of some

antagonistic combinations. Their existence should not be underestimated in order to avoid possible side effects and complications.

In general, study on the antiviral effects of combinations of antivirals is important for two main reasons (i) a need for chemotherapy that is still missing, and (ii) contribution to the knowledge on the mode of action of virus inhibitors. Combination use of viral replication inhibitors could be considered as one of the most perspective components of the strategy of counteraction to enterovirus infections by etiotropic agents. Development and use of synergistic antiviral combinations is waiting to contribute to a more successful chemotherapy of enterovirus-induced diseases.

A large-scale study of combinations of anti-enteroviral agents with different modes of action was carried in the Enterovirus laboratory of Department of Virology, SAIM. The research on the character of the combined effects of antienteroviral compounds begins by careful selection of the partners. Usually compounds with a known mechanism of antiviral action are chosen. This enables the precise application of the 3D model for characterization of drug interactions, developed by Prichard and Shipman in 1990. Thus, the synergistic effect *in vitro*, as well as *in vivo*, of the dual combination between enviroxime and disoxaril against the replication of coxsackievirus B1 is revealed (Nikolaeva and Galabov 2000, 2004). To assess the possible interactions between several picornavirus replication inhibitors in dual combinations the combined effects of enviroxime, disoxaril, arildone, S-7, guanidine, PTU-23, and HBB on poliovirus type 1 and coxsackievirus B1 were tested (Nikolaeva and Galabov 1995, 1999a,b, 2000, 2004). It was established that each of the dual combinations, in which enviroxime or HBB was one of the partners, showed synergistic or additive effects. Synergy against CBV-1 replication was stronger as compared to that noted for the same drug combinations against PV-1 replication. Combining disoxaril with enviroxime, HBB or PTU-23 resulted in synergism, while combining it with guanidine, S-7 or arildone led to antagonism. Arildone showed additive or synergistic effects when combined with enviroxime, HBB and PTU-23, and antagonistic ones when combined with disoxaril, S-7 or guanidine. All dual combinations of PTU-23 were synergistic with the exception of the pair of PTU-23 + guanidine, that was antagonistic (Galabov 1978; Nikolaeva and Galabov 1999b). Guanidine had additive to synergistic interactions with HBB or enviroxime but antagonistic ones with disoxaril, arildone and PTU-23. Guanidine or PTU-23 when combined with S-7 showed an unusual effect - synergistic one with an antagonistic zone. The combinations of S-7 with enviroxime or HBB were synergistic but

those with disoxaril or arildone were antagonistic.

The combinations of oxoglaucine at 95% confidence interval were additive or synergistic ones with the exception of those with ribaviin. The latter were mildly antagonistic. The highest volume of synergy was observed when oxoglaucine was combined with DNB. Greater synergy was usually observed against the replication of poliovirus 1 in comparison to coxsackievirus B1 (Nikolaeva et al. 2011).

Independently of the selection in in vitro experiments of a great number of favorable, synergistic, anti-enteroviral combinations, the mentioned above tendency of some discrepancy between the activity registered in vitro and in vivo was established (Nikolaeva and Galabov 1999).

Due to the multiple virus replication cycles in the presence of the different substances in combination, even the application of synergistic combinations with proven activity *in vitro* (Nikolaeva-Glomb and Galabov, 2004) does not guarantee that the occurrence of single or multiple resistance could be avoided.

Avoiding drug-resistance by novel approach of combining anti-enteroviral substance: consecutive administration of combination of three inhibitors of enterovirus replication with different mode of action

We investigated a novel approach for combined administration of anti-enteroviral compounds with proven effect, which consists of a consecutive, not simultaneous application of the substances included in the combination in experimental enterovirus infection in vivo (in newborn mice). The first experimental model used was the neurotropic CVB1 infection (Vassileva-Pencheva and Galabov 2010).

Four compounds with different mode of action were included in the first experimental series: disoxaril (VP1 hydrophobic pocket blocker, that has been established as a favourable partner in a series of synergistic combinations *in vitro* and *in vivo*), guanidine hydrochloride (targeting attacking 2C protein), PTU-23 (viral RNA synthesis inhibitor) and oxoglaucine (attacking some early step of the virus growth cycle). The four compounds are wide-range picorna/enterovirus inhibitors. Three out of these four compounds were selected on the data for their efficacy *in vivo*. Guanidine HCl was an exception: it proved an activity only in combination with HBB (Eggers 1976). The effect of oxoglaucine applied alone against CVB1 infection in mice is relatively modest (Galabov and Spassov, unpublished data).

Table 5

Combination effects of enterovirus inhibitors

First partner	The other partner	Virus	Efect	Reference
HBB	Guanidine hydrochloride	ECHO 5	Synergistic	Tamm, Eggers (1962)
		CVA9	Synergistic	Tamm, Eggers (1962)
		CVA9	Additive	Eggers (1976)
		ECHO 9	Additive	Eggers (1976)
		CVA16	Additive	Herrmann et al. (1982)
		PV1	Additive	Nikolaeva, Galabov (1999)
	Disoxaril	PV1	Synergistic	Nikolaeva, Galabov (1999)
		ECHO13	Synergistic	Nikolaeva (2000)
		CVB1	Synergistic	Nikolaeva,Galabov (2004)
	Enviroxime	PV1	Synergistic	Nikolaeva, Galabov (1999)
		ECHO13	Synergistic	Nikolaeva., Galabov (2000)
		CVB1	Synergistic	Nikolaeva, Galabov (2002)
	S–7	CVB1	Synergistic	Nikolaeva, Galabov (2004)
Ribavirin	Enviroxime	Риновирус	Antagonistic	Al-Nakib, Tyrell (1987)
		PV1	Antagonistic	Nikolaeva, Galabov (1995)
	Sandoz 89,365	Риновирус	Synergistic	Al-Nakib, Tyrell (1987)
	Disoxaril	PV1	Antagonistic	Nikolaeva, Galabov (1995)
	Oxoglaucine	PV1 CVB1 CVB3	Antagonistic	Nikolaeva-Glomb (2011)
Disoxaril	Enviroxime	PV1	Synergistic	Nikolaeva, Galabov (1995)
		ECHO13		Nikolaeva, Galabov (2002)
		CVB1		Nikolaeva, Galabov (2004)
	Arildone	PV1	Antagonistic	Nikolaeva, Galabov (1999)
	Guanidine	PV1	Antagonistic	Nikolaeva, Galabov (1999)
	PTU–23	PV1	Synergistic	Nikolaeva, Galabov (1999)
		ECHO13		Nikolaeva, Galabov (2002)
	MDL-860 (DNB)	PV1	Synergistic	Nikolaeva et al. (2011)
		CVB3	Additive	
Enviroxime	Arildone	PV1	Synergistic	Nikolaeva, Galabov (1999)
	S–7	ECHO13	Synergistic	Nikolaeva, Galabov (2002)
	Guanidine	CVB1	Synergistic	Nikolaeva, Galabov (2004)
	PTU–23	CVB1	Synergistic	Nikolaeva, Galabov (2004)
Arildone	HBB	CVB1	Synergistic	Nikolaeva, Galabov (2004)
	PTU–23	ECHO13	Synergistic	Nikolaeva, Galabov (2002)
	S–7	PV1	Additive	Nikolaeva, Galabov (1999)
PTU–23	S–7	PV1	Synergistic	Nikolaeva, Galabov (1999)
	Guanidine	PV1	Antagonistic	Galabov (1978); Nikolaeva, Galabov (1999)
Guanidine hydrochloride	S–7	PV1	Synergistic	Nikolaeva, Galabov (1999)
Oxoglaucine	Disoxaril	PV1 CVB1	Additive	Nikolaeva, Galabov (2007)
	DNB	PV1 CVB3	Synergistic	Nikolaeva-Glomb et al. (2011)
	Guanidine hydrochloride	PV1 CVB1 CVB3	Additive	Nikolaeva-Glomb et al. (2011)
	HBB	PV1 CVB1 CVB3	Additive	Nikolaeva-Glomb et al. (2011)

The scheme of administration consisted of combined treatment course with 2, 3 or 4 compounds, administered (s.c.) consecutively, starting on the day of virus inoculation (20 LD_{50}/mouse) (Table 6).

Experiments carried out demonstrated the highest effectivity of the triple combination disoxaril-oxoglaucine-guanidine.HCI when the first partner is disoxaril, followed by guanidine.HCl and oxoglaucine (DGO combination). The protective effect of this combination was lengthening of the mean survival time with almost 4 days (8.8 days compared to 5 days for the placebo group). This scheme of treatment of the DGO combination clearly demonstrated a strong inhibitory effect on the virus content in the mouse brain within the whole period of virus content recording (99.9% on the day 4th, 96.8% - on day 5th, 94.8% – on day 6th, 98.7% - on day 7th). Effects of the monotherapies by the DGO partners were not found after the day 4th.

Table 6

Scheme of daily administration of the used compounds in different combinations or applied alone

	Compounds in combinations or monotherapies		Days											
			Compounds used in combination											
			1	2	3	4	5	6	7	8	9	10	11	12
Consecutive combination	DC	Dis/Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo	Dis	Oxo
		Dis/Guan	Dis	Gua	Dis	Gua	Dis	Gua	Dis	Gua	Dis	Gua	Dis	Gua
		Dis/PTU	Dis	PTU	Dis	PTU	Dis	PTU	Dis	PTU	Dis	PTU	Dis	PTU
	TC	D/O/G	Dis	Oxo	Gua	Dis	Oxo	Gua	Dis	Oxo	Gua	Dis	Oxo	Gua
		D/G/O	Dis	Gua	Oxo	Dis	Gua	Oxo	Dis	Gua	Oxo	Dis	Gua	Oxo
		Depending on the starting compound (oxo or guan) the other partners in the rest triple combinations – O/D/G; O/G/D; G/O/D; G/D/O, were swapped following the scheme, shown above.												
	QC	D/O/G/PTU	Dis	Oxo	Gua	PTU	Dis	Oxo	Gua	PTU	Dis	Oxo	Gua	PTU
Simultaneous triple combination			Combination of Dis, guan and Oxo is simultaneously applied every day											
Monotherapies of the partner substances			Each partner is applied every day											
Placebo			PBS every day											

Dis (D), disoxaril; Oxo (O), oxoglaucine; Gua (G), guanidine.HCl; PTU (P), PTU-23.

The sensitivity to disoxaril quantitated by the IC_{50} value (measured by the the plaque-inhibition test in cell culture) of the virus brain isolates in DGO treated animals (Table 7) was equal to that of the brain isolates from the control (placebo group) within the period to 6th day post infection (0.55-0.64 μM in DGO treated group, 0.38-0.57 μM in placebo group). Then, since day 7, a sharp increase of the sensitivity to disoxaril was recorded in the DGO treated animals, IC_{50} value 0.08-0.12 μM. This very well reproducible result need special consideration.

In the group subjected to the monotherapy with disoxaril, the drug-resistance phenomenon started from day 5th.

Table 7

Sensitivity to disoxaril in the plaque-inhibition test of virus brain isolates from DGOtreated and treated with disoxaril alone newborn mice, infected with CVB1

Test group/ Brain sample	**Disoxaril IC_{50} values (μM) of viral brain samples taken on day (after viral inoculation)**						
	4	**5**	**6**	**7**	**8**	**9**	**10**
Plaque purified							
Placebo	0.38	0.4	0.5	0.57	-[b]	-[b]	-[b]
Disoxaril	-[a]	1.71	3.03	11.1*	-[b]	-[b]	-[b]
DGO	0.55	0.52	0.64	0.09¶	0.12	0.08	0.13
Native							
Placebo	0.45	0.42	0.55	0.59	-[a]	-[a]	-[a]
Disoxaril	-[b]	2.53	5.95	14.7	-[a]	-[a]	-[a]
DGO consecutively	0.54	1.5	0.19	0.08	0.1	0.09	0.14
DGO simultaneously	1.7	1.64	5.82	16.3	-[a]	-[a]	-[a]

*P<0.05 compared to the placebo group. ¶ P<0.05 compared to the group treated with disoxaril alone. The statistical analysis was performed with one-way ANOVA with Bonferroni's Post test. [a] – due to 100% inhibitory effect of disoxaril on day 4 there is no infectious virus in the brain isolate. [b] – after day 7 there is no left alive animals, 100% mortality rate.

Extremely significant was the finding that the treatment course with the simultaneously applied daily DGA partners resulted with a well expressed development of resistance (Table 7).

The same tendency was observed with the third compound in the combination, oxoglaucine (Table 8).

As regarding brain isolates from guanidine.HCl treated groups of animals, they were not subject of MIC_{50} determination in view of the known ineffectivity of this compound in *in vivo* experiments.

Previous research of our laboratory, designed to trace longitudinally the sensitivity of infected with coxsackievirus B1 mice to chemotherapy with one of the WIN compounds – disoxaril, proved the rapid occurrence of resistance to the inhibitor just 5 days after the beginning of treatment. After day 6 the brain isolates from the group treated with disoxaril every day contain a completely resistant viral population. Moreover, when the phenotype characteristics of this population were examined, a slight increase of pathogenicity was established (Nikolova and Galabov 2003).

Severe forms of enteroviral infections pass through a viremic stage, which results in longer incubation period. In these cases a preservation of the activity of specific anti-enteroviral therapy for at least 12 to 14 days is required. This type of chemotherapy, applied in the incubation period of the enterovirus infections (chemoprophylaxis) could be effective in children, especially when epidemic outbreaks occur.

Data presented in the current paper for the first time show that the preservation of the activity of used drug partners for the time necessary (for survival of the infected animals) is possible when a triple combination of anti-enteroviral inhibitors is applied not simultaneously, in which case multiple resistance could emerge, but consecutively. We develop a model of such combination, the treatment with which, unlike monotherapies, totally prevents the occurrence of resistance. This is in full contrast to the established multiple drug-resistance regimens when the compounds are administered simultaneously every day.

Table 8

Sensitivity to oxoglaucine in the plaque-inhibition test of virus brain isolates from DGO treated and treated with oxoglaucine alone newborn mice, infected with CVB1

Test group/ Brain sample	**Oxoglaucine IC $_{50}$ values (μM) of viral brain samples taken on day (after viral inoculation)**						
	4	**5**	**6**	**7**	**8**	**9**	**10**
Plaque purified							
Placebo	0.2	0.26	0.39	0.43	-[b]	-[b]	-[b]
Oxoglaucine	3.79	3.19	3.2	-[b]	-[b]	-[b]	-[b]
DGO	0.37	0.34	0.12	0.06	0.09	0.1	0.12
Native							
Placebo	0.34	0.22	0.38	0.8	-[a]	-[a]	-[a]
Oxoglaucine	3.52	3.73	3.77	-[a]	-[a]	-[a]	-[a]
DGO consecutively	0.5	0.6	0.91	0.73	0.34	0.23	0.31
DGO simultaneously	3.12	3.53	3.45	3.4	-[a]	-[a]	-[a]

[a] – due to 100% inhibitory effect of disoxaril on day 4 there is no infectious virus in the brain isolate. [b] – after day 7 there is no left alive animals, 100% mortality rate.

The results from our study demonstrate that the way of ordering the partners plays a key role for the effectiveness of the combination. The therapy must start with an inhibitor of an early step of the viral cycle, e.g. disoxaril, which has pronounced effect in the first day of infection before the development of resistance. It must be followed by a RNA replication inhibitor, such as guanidine HCl, and then one that targets an early step again, oxoglaucine. The treatment course with the consecutively applied combination DGO confirmed its efficacy in two other models of CVB infections in laboratory animals with different infection targets (Vassileva-Pencheva and Galabov, unpublished data).

The developed by us novel approach of antiviral combinations is formulated and applied for the first time in the studies of antiviral agents.

We assume that other successful models of consecutively administered triple combinations could be researched and developed that could be the basis for new therapeutic strategies.

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