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Consecutive Alternating Administration of Antiviral Combinations: a Novel Treatment Approach against Coxsackievirus B1 Neuroinfection

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Editorial

The role of enteroviruses (EV) in human infectious pathology has been increased and substantially clarified in recent decades [1-3]. This is in part due to the large number of investigations carried out on a series of EV-induced infections manifested for the first time by epidemic spread in several regions of the globe, for example the EV71 epidemic in Southeast Asia [4,5] and EV68 in the USA [6]. Out of the 116 "classical" human EV serotypes of species A–D, there are 65 EVs that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, 5 other non-polio enteroviruses, and 3 polioviruses [7]. Also belonging to the Enterovirus genus are about 150 human rhinovirus serotypes of A, B, and C species [8].

There is no analogy in the biological world to the proven EV replication mutation rate of 10^{-3} [9,10] nor to the connected, unusual phenomenon of one virus, in one region, during one period of time (the summer season), causing more than ten different clinical pictures affecting different human tissues and organs (e.g., brain, meninges, uvea, conjunctiva, smooth muscles, myocardium, pericardium, endocardium, and pancreatic beta cells). Besides, an unusual obstacle to introducing traditional epidemiologic measures was manifested — more than 80% of infected individuals were asymptomatic (lack of a clinical picture) [1,11,12]. This unusual clinical phenomenon, for epidemiology, was explained by the existence of EV progeny consisting of billions of quasispecies [13,14]. Such quasispecies are at the basis of the rapid development of drug resistance to each established enterovirus replication inhibitor. Herrmann and Herrmann [15] postulated that the development of resistance is an obligatory indicator for considering a substance that inhibits viral replication to be a specific virus inhibitor. For the development of drug resistance substantially contributed the monotherapeutic treatment as the only approach applied in the anti-enterovirus studies: among the several hundred substances with different modes of action that are active *in vitro*, fewer than 20 have demonstrated some effect *in vivo*, and none have passed clinical trials.

The double-blind placebo-controlled clinical trials of enterovirus replication inhibitors selected through preclinical studies have so far failed. This is undoubtedly due mainly to the lack of selectivity, substantiated by well-expressed side effects in the human body. There are several examples of such trials: disoxaril (WIN51711) and WIN 54954 [16]; pleconaril (WIN63843) [17,18]; and BTA-798 (an oxime ether analogue of pirodavir) [19]. These trial results show that, at present, clinically effective antivirals for treating enteroviral infections do not exist. Thus, the realization of anti-enteroviral chemotherapy is a problem for the future.

There are convincing indications for chemotherapy application in enterovirus infections: (i) the severity of a series of EV-caused illnesses, (ii) the large number of EV species and serotypes, (iii) the social importance of certain EV infections, which is connected with their widespread occurrence, and (iv) the absence of vaccinal prophylaxis, excluding the anti-poliomyelitis vaccines. Recently, progress has been made in the development of a vaccine against enterovirus 71 [20]. Evidently, the problem of counteracting drug resistance in EV infections remains unsettled; it has been found to be much more complicated than counteracting drug resistance in AIDS, influenza, and hepatitis C.

Another special indication is the 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 controlling polio in the post-eradication era [21]. These will be of great benefit for post-exposure prophylaxis and outbreak control [22,23].

Combination chemotherapy could be considered as a perspective approach for preventing the development of anti-EV drug resistance. The carried out, by our team mainly, systemic investigation of multiple anti-EV inhibitory substances used in double combinations against a broad spectrum of enteroviruses identified a considerable number of such combinations that had synergistic combined effects. Some combinations proved to have an additive effect, and only a small number of combinations manifested an antagonism, in particular those that included ribavirin [24-26]. Ribavirin's mutation-rate-enhancing action toward EVs was determinant in these cases [27]. A double drug resistance was proven initially in the course of investigation on the synergistic combination disoxaril + enviroxime against poliovirus 1 [25]. The validity of this phenomenon needs additional studies to be confirmed.

Our experiments both *in vitro* and *in vivo* were carried out on Coxsackie B viruses. Why have we targeted representatives of Coxsackie B viruses in our anti-enteroviral investigations? The Coxsackie B viruses cause many diseases [10]: meningitis and soft paralyses (B1–6); pleurodynia (epidemic myalgia, Bornholm disease; B1–6); acute respiratory diseases (B2–5); eye diseases (uveitides; B2); heart diseases (acute myocardiopathy and acute pericardiopathy; B1–6); chronic diseases of the heart and vessels (dilative cardiomyopathy; B3–5), insulin-dependent diabetes mellitus (IDDM; B2–4 predominantly); diseases of newborns (B2–5); gastrointestinal diseases (hepatitis in newborns, pancreatitis; Coxsackie B); FMD-like disease (B2, B5); and chronic asthenia syndrome (Coxsackie B).

In experiments in newborn mice infected with a neurotropic strain of Coxsackievirus B1 (CVB1), Connecticut 5, treated with the VP1 ligand disoxaril (a WIN compound), drug resistance developed

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4–6 days after virus inoculation [28]. The disoxaril-resistant mutant was characterized by a panel of phenotypic markers: (i) sharply increased 50% inhibitory concentration (IC_{50}) – from 0.84 μM to >30 μM, (ii) change in the plaque shape – from round to irregular, (iii) increase of the plaque size – from 0.9 mm to 1.9 mm, (iv) increased thermosensitivity at 50°C (ET_{50}) – from 31 min to 7 min, and (v) slightly increased pathogenicity for mice. The molecular genetic basis of the drug resistance consisted of specific replacements in amino acid consequences coded in the VP1 locus.

We decided to exam the *in vivo* combination effects of EV replication inhibitors with different mechanisms of action. Preliminary, the activity of double, triple, and quadruple combinations was investigated, either administered consecutively and alternatingly (i.e., not simultaneously) (CAA) or applied simultaneously and daily, in newborn mice infected with CVB1 20 MLD_{50}. For the CAA course, we also tested the influence of the substance application order. Monotherapeutic courses of the compounds comprised in the triple combinations were used as controls, in addition to placebo groups. The best antiviral effect was produced by the triple combination via CAA with compounds applied in a specific sequence-an inhibitor targeting the capsid protein VP1 had to be administered first [29].

Initially, the effect of the CAA course with the triple combination disoxaril + guanidine-HCl + oxoglaucine (DGO) was tested on mice inoculated with CVB1. This combination (DGO via CAA) reached a protection effect of approximately 50%. It had the same efficacy against infections with neurotropic (Nancy) and cardiotropic (Woodruff) strains of CVB3 [30].

Subsequently, we replaced disoxaril with pleconaril (i.e. the combination became PGO), a VP1 blocker possessing its own *in vivo* activity, though it also has some toxicity. The PGO combination with CAA also manifested a marked protective effect (31.3% - 68%, depending on the pleconaril dose) against experimental neuroinfection with CVB1 20 MLD_{50} [31].

The WIN compounds in the DGO and PGO combinations target the VP1 protein in the enteroviral capsid, removing the pocket factor (a lipid moiety molecule) in the VP1 hydrophobic pocket [32]. The second component using the VP1 ligand disoxaril (a WIN compound) in the combinations, guanidine-HCl, is a ligand of the 2C protein, which suppresses daughter RNA (+) chain initiation during virus replication [33]. Finally, the third component is oxoglaucine, an aporphinoid alkaloid isolated from the epigeous parts of the yellow horn poppy (*Glaucium flavum* Cranz) [34,35]. Oxoglaucine's mechanism of action [36], has an enviroxime-like effect, (i.e. it acts as an inhibitor of PI4KB and therefor inhibits the formation of the replicative complex of enteroviruses). The *in vivo* antiviral effect of oxoglaucine, in analogy to enviroxime, is distinguished by modest values (S. Spasov and A. S. Galabov, unpublished data); however, the second component-guanidine-HCl-does not generally have an individual *in vivo* effect [37]. This fact compelled us to replace guanidine-HCl with another inhibitor of viral RNA synthesis, the compound 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (MDL-860) [38]. This Merrill-Dow Pharmaceuticals product (synthesized initially by L. Markley) is notable for its waste anti-enterovirus scope and for its *in vivo* effects on cardiotropic CVB3 infection in adult mice [39]. The compound's mechanism of anti-enterovirus action has not been clarified, but it is thought to interfere at an early stage, post-uncoating, in enterovirus replication [40,41]; a function of the virus replicative complex, viral RNA polymerase, has been suggested [39].

As a next research step, as mentioned above, we replaced guanidine-HCl with enteroviral RNA synthesis inhibitor MDL-860 to test the effect of a new triple combination-pleconaril + MDL-860 + oxoglaucine (PMO)-applied via CAA in newborn mice infected subcutaneously with 20 MLD_{50} of CVB1.

The PMO combination via CAA showed high activity at the 75 mg/kg MDL-860 dose: a protective effect of 50% and a pronounced suppression of brain virus titers (a decrease of 4-5 logs at day 7 post infection when compare CAA group's brain samples with that of the 25 mg/kg pleconaril monotherapy group). Moreover, along with the prevention of drug resistance, a phenomenon of increased drug sensitivity was established. MDL-860 sensitivity in PMO group on day 7 increased 8.2 times *vs.* placebo (29 times *vs.* monotherapy) and oxoglaucine sensitivity – 4.9 times *vs.* placebo (by 6.8 times *vs.* monotherapy) on Day 13. Daily, simultaneous administration of PMO showed no protective effect and a rapid development of drug resistance.

These results add new support for using CAA treatment courses to achieve clinically effective chemotherapy of EV infections.

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