REWIEV ARTICLE

Triazavirin might be the new hope to fight Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

Triazavirín by mohol byť novovu nádejou v súboji s koronavírusom 2 vyvolávajúcim ťažký akútny respiračný syndróm (SARS-CoV-2)

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Summary

Since the beginning of the outbreak, a large number of clinical trials have been registered worldwide, and thousands of drugs have been investigated to face new health emergency of highly contagious COVID-19 caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Drug repurposing, i.e., utilizing an approved drug for a different indication, offers a time- and cost-efficient alternative for making new (relevant) therapies available to physicians and patients. Considering given strategy, many approved and investigational antiviral compounds, alone or in various relevant combinations, used in the past to fight Severe Acute Respiratory Syndrome Coronavirus-1, Middle East Respiratory Syndrome Coronavirus, Human Immunodeficiency Virus type 1, or Influenza viruses are being evaluated against the SARS-CoV-2. **Triazavirin** (**TZV**), a non-toxic broad-spectrum antiviral compound, is efficient against various strains of the Influenza A virus (*Influenza Virus A*, *Orthomyxoviridae*), i.e., swine flu (H1N1, or H3N2), avian influenza (H5N1, H5N2, H9N2, or highly pathogenic H7N3 strain), Influenza B virus (*Influenza Virus B*, *Orthomyxoviridae*), Respiratory Syncytial Virus (*Orthopneumovirus*, *Pneumoviridae*), Tick-Borne Encephalitis Virus (known as Forest-Spring Encephalitis Virus; *Flavivirus*, *Flaviviridae*), West Nile Virus (*Flavivirus*, *Flavaviridae*), Rift Valley Fever Virus (*Phlebovirus*, *Bunyaviridae*), and *Herpes* viruses (*Simplexviruses*, *Herpesviridae*) as well. In regard to COVID-19, the molecule probably reduced inflammatory reactions, thus limiting the damage to vital organs and reducing the need for therapeutic support, respectively. In addition, *in silico* computational methods indicated relatively satisfactory binding affinities of the **TZV** ligand to both structural (E)- and (S)-proteins, non-structural 3-chymotrypsin-like protease (3-CL^{pro}) of SARS-CoV-2 as well as human angiotensin-I converting enzyme-2 (ACE-2). The interactions between **TZV** and given viral structures or the ACE-2 receptor for SARS-CoV-2 might effectively block both the entry of the pathogen into a host cell and its replication. Promising treatment patterns of COVID-19 positive patients might be also based on a suitable combination of a membrane fusion inhibitor (**umifenovir**, for example) with viral RNA synthesis and replication inhibitor (**TZV**).

Key words: SARS-CoV-2 • COVID-19 • drug repurposing • triazavirin • structural proteins • 3-chymotrypsin-like protease • ACE-2

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Súhrn

Od začiatku pandémie vysokoinfekčného ochorenia COVID-19 (Coronavirus Disease-19), ktoré je spôsobené koronavírusom 2 vyvolávajúcim ťažký akútny respiračný

syndróm (Severe Acute Respiratory Syndrome Coronavirus 2; SARS-CoV-2), bol celosvetovo registrovaný veľký počet klinických štúdií, v ktorých sú hodnotené tisícky liečiv. Stratégia reprofilizácie liečiv, t.j. utilizácia liečiv, ktorých terapeutické použitie už bolo schválené v inej indikácii, získa čas a zníži náklady na liečbu dovtedy, kým budú lekárom a pacientom k dispozícii nové (relevantné) terapeutické alternatívy. Z tohto dôvodu je proti SARS-CoV-2 hodnotených aj mnoho zlúčenín, ktoré sú schválené alebo experimentálne používané v liečbe infekcií zapríčinených koronavírusom 1 vyvolávajúcim ťažký akútny respiračný syndróm (Severe Acute Respiratory Syndrome Coronavirus 1), koronavírusom vyvolávajúcim stredovýchodný respiračný syndróm (Middle East Respiratory Syndrome Coronavirus), vírusom ľudskej imunodeficiencie typu 1 (Human Immunodeficiency Virus type 1) alebo vírusmi chrípky (*Influenza viruses*). **Triazavirín** (**TZV**) je netoxickým širokospektrálnym antivirotikom, ktoré efektívne pôsobí proti rôznym kmeňom chrípky antigénneho typu A (*Influenza Virus A*, *Orthomyxoviridae*), t.j. prasacej chrípke (H1N1 alebo H3N2), vtáčej chrípke (H5N1, H5N2, H9N2 alebo proti vysokopatogénnemu kmeňu H7N3), kmeňom chrípky antigénneho typu B (*Influenza Virus B*, *Orthomyxoviridae*), respiračnému syncyciálnemu vírusu (*Orthopneumovirus*, *Pneumoviridae*), vírusu kliešťovej encefalitídy (*Flavivirus*, *Flaviviridae*), vírusu západonílskej horúčky (*Flavivirus*, *Flavaviridae*), vírusu horúčky Rift Valley (*Phlebovirus*, *Bunyaviridae*) a aj herpesvírusom (*Simplexviruses*, *Herpesviridae*). V kontexte terapie ochorenia COVID-19 táto zlúčenina pravdepodobne redukovala zápalové reakcie, a takto limitovala poškodenie vitálnych orgánov a oddialila eventualitu terapeutickej podpory. Výpočtové metódy *in silico* tiež indikovali relatívne uspokojivé väzbové afinity tohto ligandu k štruktúrnym (E)- a (S)-proteínom, neštruktúrnej proteáze podobnej 3-chymotrypsínu (3-CL^{pro}) SARS-CoV-2 a aj receptoru ľudí, enzýmu-2 konvertujúcemu angiotenzín-I (ACE-2). Interakcie medzi **TZV** a vírusovými štruktúrami alebo ACE-2-receptorom pre SARS-CoV-2 by mohli efektívne blokovať vstup patogéna do hostiteľskej bunky a tiež jeho intracelulárnu replikáciu. Sľubné terapeutické schémy pre liečbu COVID-19-pozitívnych pacientov by mohli spočívať vo vhodnej kombinácii inhibítora fúzie membrán (napr. **umifenoviru**) s inhibítorom syntézy a replikácie vírusovej RNA (**TZV**).
Kľúčové slová: SARS-CoV-2 • COVID-19 • reprofilizácia liečiv • triazavirín • štruktúrne proteíny • proteáza podobná 3-chymotrypsínu • ACE-2

Introduction

The world is presently face to face with a pandemic, which is spreading rapidly across the globe caused by a new zoonotic coronavirus referred to as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2; previously 2019-nCoV)[1]. The virus is an oval enveloped, linear positive-sense, single-stranded RNA *β*-coronavirus (subgenus *Sarbecovirus*, genus *β-Coronavirus*) with a diameter of around 60–140 nm belonging into the subfamily *Coronavirinae* and family *Coronaviridae*, which, in turn, comprises the order *Nidovirales*[2, 3].

There has been no specific therapy for Coronavirus Disease (COVID-19) caused by the virus as its pathogenesis and proliferation pathways still remain unknown. Given the severity of the COVID-19 pandemic, identifying efficient and safe drug/vaccine treatment options as soon as possible is critical for adequate response to the outbreak.

Recent Emergency Use Authorization of Pfizer and BioNTech's mRNA vaccine may provide a pathway forward, but monitoring of long-term immunity is still required, and diverse vaccine candidates are still under development. One of the biggest drawbacks of Pfizer and BioNTech's COVID-19 vaccine is quite long-term storage, as the vaccine must be stored at –70 °C, creating challenges in reliable cold chain distribution[4]. Furthermore, as the SARS CoV-2 pathogen continued to replicate and transmit, more infectious mutant strains have been developed[5, 6], which, in addition, could possess remarkable alterations in their antigenicity[7].

The treatments for COVID-19, one of the most contagious diseases in human history, have been mainly based on the experience with the diseases caused by "similar" viruses such as Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-CoV-1), Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Human Immunodeficiency Virus type 1 (HIV-1), or both Influenza A and B viruses.

Presently, there are over one thousand clinical studies focusing on the treatment for COVID-19 all over the world. The trials aim at various membrane fusion inhibitors targeting viral entry – **umifenovir** (**UFV**), a dual acting direct antiviral/host targeting compound, inhibitors of angiotensin-I converting enzyme-2 (ACE-2; peptide inhibitors, **nitrofurantoin**), RNA polymerase inhibitors (**remdesivir**, **favipiravir**, **ribavirin**), protease inhibitors (**lopinavir**/**ritonavir** combination, **camostat**, **danoprevir**, **darunavir/cobicistat** combination, **ebselen**), Janus-associated kinase inhibitors (**baricitinib**, **ruxolitinib**), endosomal acidification inhibitors (**chloroquine**, **hydroxychloroquine**), drugs/entities targeting inflammatory responses and cytokine storms (**corticosteroids**, **thalidomide**), immunomodulators (**interferon alpha-1b**, **interferon alpha-2a**), drugs inducing host innate immune responses to produce interferons (**nitazoxanide**), immunosuppressive anti-interleukin-6 receptor monoclonal antibodies (mAb) and other mAb (**tolicizumab**, **sarilumab**, **siltuximab**), compounds/entities neutralizing interferon gamma (**emapalumab**), modulators of sphingosine-1-phosphate receptors (**fingolimod**), biologically active compounds from traditional Chinese medicine (**hesperidin**, **quercetin**), or mesenchymal stem cells, for example[8–10].

The fact is that the world has been in varying states of lockdown since March 2020, causing profound economic and social consequences. In addition, despite implementation of masks and other safety protocols, a number of lives have been lost due to COVID-19. Many

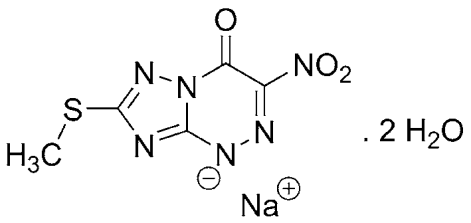

*Fig. 1 Chemical structure of **triazavirin** (**1**)*

clinical trials[8–10] have been conducted for the treatment of this disease, however, no drug has been verified to be really effective. To find "very specific drugs" for COVID-19, multi-center clinical trials should be performed with enough enrolled patients to analyse carefully the clinical outcomes, which will bring new knowledge and help to define the best way how to treat such zoonotic disease as well as to reduce its symptoms and complications.

Regarding mentioned, there is no surprise that some compounds belonging into small drugs classes, such as **triazavirin** (**TZV**; **1**) (Fig. 1), might be considered new hopes for the treatment of COVID-19. The molecule (**1**) showed therapeutic potential in a very recent pilot randomized controlled trial. Authors of the trial[11] suggested that antiviral effect of **TZV** (**1**) was connected with the ability to reduce inflammatory reactions, thus reducing both the damage to vital organs and need for therapeutic support.

Tsvetov et al. (2020) noted[12] that routine use of **TZV** (**1**) for the prevention of infection caused by SARS-CoV-2, or treatment of COVID-19 can not be recommended until results of relevant clinical trials will be accessible and critically reviewed. The authors concluded that implementation of **TZV** (**1**) into therapeutic protocols designed for such zoonotic disease might be possible in the framework of clinical trials in accordance with current regulations and ethical standards. In fact, such legitimate statement was based on the information available until May 1, 2020 and was published[12] before the results of pilot randomized control trial as well as trial protocol were accessible for (not only) scientific community[11, 13]. There was concluded that **TZV** (**1**) might benefit COVID-19 patients by controlling symptoms and reducing frequent usage of concomitant therapies for vital organ supports[11, 13].

The most recent information is that **TZV** (**1**) underwent clinical investigation for the treatment of COVID-19 and was in the Phase-3 Clinical Study in China[14], Chinese Clinical Trial Register Number ChiCTR2000030001.

Origin and previous therapeutic experience with triazavirin

TZV (**1**), also known as **riamilovir** (CAS Registry Number: 123606-06-4), belongs into the nitro substituted [1,2,4]-triazolo[5,1-*c*][1,2,4]triazines class. Development of the compound (**1**) was a result of joint efforts of the Postovsky Institute of Organic Synthesis, Ural Federal University (Ural Branch, Russian Academy of Sciences), Research Institute of Influenza (Ministry of Health of the Russian Federation), Virology Center of the Research Institute of Microbiology (Ministry of Defence of the Russian Federation), State Science Research and Test Institute of Military Medicine (Ministry of Defence of the Russian Federation), Ural Center for Biopharma Technologies in Novouralsk (Russian Federation) and Medsintez Pharmaceutical Plant in Yekaterinburg with the support of the Ministry of Education and Science of the Russian Federation. The molecule (**1**) passed the entire cycle of required clinical trials as an antiflu drug and was entered into the Drug Register of the Russian Federation[15] on August 28, 2014.

This analogue of a purine nucleoside (guanosine) has been established as a first line of the defense against acute respiratory viral infections showing good efficacy, tolerability and favorable toxicological properties in terms of acute toxicity[16–19]. Eventual long-term therapy based on **TZV** (**1**) should be considered carefully because compensatory-adaptive mechanisms of a body might be affected[20]. Furthermore, inhibition of enzymes' activity in endoplasmic reticulum of hepatocytes could influence negatively a detoxication function of the liver[21].

The molecule (**1**) was efficient against various strains of Influenza A virus (genus *Influenza Virus A*, family *Orthomyxoviridae*), i.e., swine flu (H1N1, or H3N2), avian influenza (H5N1, H5N2, H9N2, or highly pathogenic H7N3 strain) as well as Influenza B virus (*Influenza Virus B*, *Orthomyxoviridae*). Target of given compound was a specific viral protein, hemagglutinin. Important role of interactions between **TZV** (**1**) and SH groups of amino acids of specific biological targets, resulting in the formation of covalent disulfide bonds, was confirmed. Briefly, the drug (**1**) inhibited enzymatic activity of protein sulfide isomerase, an enzyme responsible for the formation and isomerisation of disulfide bonds, thus disturbing the formation of a tertiary structure of hemagglutinin and replication cycle of the Influenza viruses[22, 23].

Pharmacokinetic studies showed that the maximal concentration of **TZV** (**1**) in plasma was achieved in quite a short time (1–1.5 hrs), and maintained in adequate concentrations. Biphasic behaviour of the elimination curve was observed. The treatment of patients with this drug administered *per os* proved its ability to reduce significantly the duration of main clinical symptoms of influenza, i.e., intoxication, fever, respiratory symptoms, thus decreasing a number of incidents of influenza-related complications, and avoiding use of symptomatic drugs[15].

In fact, the antiviral spectrum of **TZV** (**1**) is much more complex. This compound is effective against RNA as well as DNA viruses, i.e., Respiratory Syncytial Virus (*Orthopneumovirus*, *Pneumoviridae*), Tick-Borne Encephalitis Virus (known as Forest-Spring Encephalitis Virus; *Flavivirus*, *Flaviviridae*), West Nile Virus (*Flavivirus*, *Flavaviridae*), Rift Valley Fever Virus (*Phlebovirus*, *Buny-*

aviridae), and *Herpes* viruses (*Simplexviruses*, *Herpesviridae*) as well[24–28].

Structural arrangement and stability of triazavirin

X-Ray diffraction analysis showed that the molecule (**1**) crystallized as a dihydrate, i.e., 2-methylthio-6--nitro-1,2,4-triazolo[5,1-*c*] [1,2,4]triazin-7-one sodium salt dihydrate. The sodium ions were bonded to a centrosymmetric binuclear complex, in which carbonyl groups of the heterocycle acted as bridges. Additional binding was observed by means of oxygen atoms of a nitro group and nitrogen of an azole heterocycle. Water molecules were coordinated on sodium ions and formed hydrogen bonds with nitrogens of azine fragments of neighboring heterocycle molecules. In general, the presence of multiple hydrogen bonds was a distinctive feature of **TZV**'s (**1**) crystal packing. The stability of given molecule up to 130 °C showed the applicability as a dosage form[29].

Triazavirin *versus* SARS-CoV-2: The fight has begun

Hypothesis to treat COVID-19 with **TZV** (**1**) as the monotherapy or if being included in relevant combination(s) of drugs was based on a drug repurposing strategy. The approach is also termed drug repositioning, drug reprofiling, or re-tasking. The drugs that have been approved for the treatment of some disease are safe for human use, and only their effectiveness against the disease of interest needs to be established. The fundamental principle involved in drug repurposing is that a common molecular pathway is responsible for many diseases and a host of detailed information is available on the formulation, dose, toxicity, pharmacology and clinical trial data of the approved/shelved or discontinued drugs. Drug repurposing may leave some vital information on druggable targets that could be capitalized in target--based drug discovery[9, 30].

Conventional drug development usually includes five main stages: 1. discovery and development, 2. pre-clinical research, 3. clinical research, 4. review by relevant authority(-ies), e.g., U.S. Food and Drug Administration or European Medicines Agency, and v) post-market safety monitoring by the authority(-ies) and development. However, there are only four steps in drug repurposing: 1. compound identification, 2. compound acquisition, 3. clinical research, and 4. post-market safety monitoring by the authority(-ies) and development. In life-threatening cases, where there is no alternative medicine or vaccine, such a strategy is particularly attractive[30, 31].

Mechanism of action of **TZV** (**1**) against SARS-CoV-2 might be more complex, involving not only the reduction of inflammatory reactions[11, 13]. Molecular modelling, docking investigations and molecular dynamic simulations indicated relatively satisfactory binding affinities of given drug to both structural envelope (E) and spike (S) proteins as well as non-structural 3-chymotrypsin-like protease (3-CL^{pro}) of SARS-CoV-2. The compound (**1**) might interfere with proper functions of these viral structures, thus preventing both binding and replication of the pathogen within a host cell[32].

The (E)-protein of SARS-CoV-1 is an integral membrane protein about 76–109 residues long and has been known to be most dynamic at the membranous functional interface[33]. Primary structural elements of the protein make it particularly interesting that delve into its possible role in viral propagation. Very interestingly, primary sequence of the protein remains mostly conserved. The SARS-CoV-2 (E)-protein shows approximately 97% sequence similarity with that of SARS-CoV-1. The finding alternatively directs into understanding the uniqueness of the sequence that is conserved to mediate crucial functional roles of this smallest structural protein, as viroporin-like activity[34].

The molecule (**1**) also interacted *in silico* with a human ACE-2 structure, a type I membrane zinc-containing metalloenzyme, which was primarily considered a therapeutic target for cardiovascular diseases. ACE-2 hydrolyses angiotensin-I to produce angiotensin (1–9). The enzyme is a homologue of angiotensin-I converting enzyme (ACE), a central enzyme of the renin-angiotensin system (RAS), which functions as a fundamental regulator of RAS by proteolytic cleavage of angiotensin-I to the potent vasoconstrictor, angiotensin-II, a critical signalling molecule[35].

The ACE-2 protein is expressed in various organs of the human body, including the lungs, cardiovascular system, gut, kidneys or central nervous system – the compartments, which are regarded as prime targets of SARS-CoV-2. Specific interactions between the ligand and viral ACE-2 receptor migt block entry of the virus into a host cell[36].

At first sight it could be assumed that **TZV** (**1**) interacted with particular viral surface proteins *via* substitution reactions of its nitro group with *N*- and *S*-nucleophilic moieties of amino acids, such as L-lysine, L-arginine, or L-cysteine[37]. The interactions might not be a property of **TZV** (**1**) itself but of its metabolites, i.e., **AMTZV** (**2**), as being suggested (Fig. 2). These products of biotransformation interacted covalently with protein amino acid residues. Thus, it was proposed that **TZV** (**1**) did not directly react with L-cysteine[38, 39].

Besides, alkylation or glycosylation of **TZV** (**1**) at nitrogen atom is metabolically also possible, since given compound is a bioisostere of purine bases, for

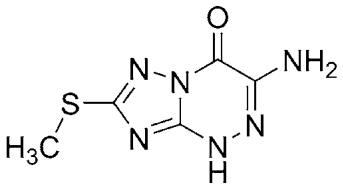

*Fig. 2 Chemical structure of **AMTZV** (**2**), a metabolite of **triazavirin** (**1**)*

which such biotransformation pathways have been known. The drug can undergo *N*-methylation and addition of glucuronic acid residue in the human organism as well[37].

Recent studies[40, 41] concluded that 3-CL^{pro}, which is also termed main protease (M^{pro}), or non-structural protein 5 (nsp5), is highly conserved between both SARS-CoV-2 and SARS-CoV-1. The 3-CL^{pro} cysteine protease directly mediates the polyprotein processing and maturation of viral non-structural proteins during replication. Moreover, no human protease counterpart to 3-CL^{pro} has been known yet. Thus, the drugs targeting 3-CL^{pro} could be highly virus-selective and safe[40, 41].

Following the findings from previous *in vitro* experiments connected with other broad-spectrum antivirals, the interactions between **TZV** (**1**) and viral non-structural protein 12 (nsp12), which is well-known under the name of RNA-dependent RNA polymerase (RdRp), should be also inspected in detail. The RdRp enzyme mediates the transcription and replication of the RNA genome during viral infection[42–44].

The use of suitable combinations of antiviral agents, which interfere efficiently with the fusion of SARS-CoV-2 and host cell membranes and inhibit RNA synthesis as well as replication of the pathogen, respectively, might also provide benefits in the COVID-19 treatment. For example, **UFV** (**3**), a compound well-known under the name of **arbidol** (Fig. 3), is considered a very efficient membrane fusion inhibitor[45, 46]. Invention of this molecule is attributed to a joint consortium of Russian scientists from the Chemical-Pharmaceutical Scientific Research Institute, the Scientific Research Institute of Medical Radiology in Obninsk and the Leningrad-Pasteur Scientific Research Institute for Epidemiology and Microbiology (the former Soviet Union), some forty years ago.

The broad-spectrum antiviral drug (**3**) inhibited a large panel of viral pathogens, enveloped, or not, displayed a dual binding activity to lipid membranes and to viral or cellular proteins, i.e., (S)-glycoprotein. This trimeric glycoprotein responsible for crown-like appearance mediates attachment of SARS-CoV-2 to the host receptor. The small indole-derivative molecule (**3**) blocks viral endocytosis and replication in membranous intracellular compartments. In regard of the use in clinical practice, **UFV** (**3**) is licensed in the Russian Federation and the People's Republic of China for the treatment of influenza and other viral infections[47–49].

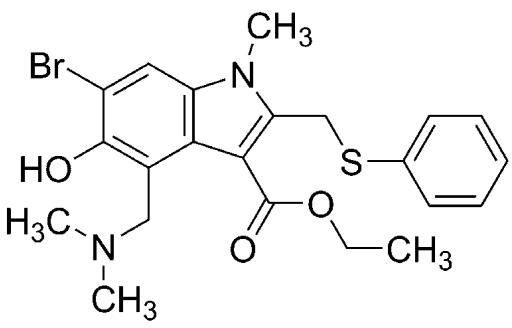

*Fig. 3 Chemical structure of **umifenovir** (**3**)*

However, there was no clear evidence to support extensively the **UFV** (**3**) monotherapy in order to achieve significant improving patient-important outcomes in patients with COVID-19, as published in a systematic review and meta-analysis of Huang et al. (2020)[50].

Conclusions

The emerging and re-emerging viral diseases are continuous threats to the wellbeing of human life. The current COVID-19 pandemics has created an unprecedented medical, economic and social crisis all over the world. Considering the rapid spread of this highly infectious disease, the drug repurposing strategy is necessary, but it requires caution. Anti-malarial therapeutics, i.e., **chloroquine** and **hydroxychloroquine**, were reported to be effective against SARS-CoV-2, but could not convince in clinical trials yet. Furthermore, when initial unfounded speculations about alleged dangers of antihypertensive therapies based on ACE inhibitors were frequently published in the media, they caused notable uncertainty among patients. Such impetuous communications can have serious consequences and should not be proclaimed carelessly.

TZV (**1**) is an efficient broad-spectrum antiviral drug with favorable toxicological properties. The compound might influence not only inflammatory reactions of the human body suffering from COVID-19 but could also interact with structural (E)- and (S)-proteins as well as non-structural 3-CL^{pro} of SARS-CoV-2, a causative agent of COVID-19 and, in addition, with ACE-2 protein of host cells. Indeed, highly conserved (E)-protein can be a potential target for SARS-CoV-2 therapeutic interventions, i.e., small drug molecule candidates as well as vaccines, to reduce of pathogenicity.

Promising treatment patterns of COVID-19 positive patients would be also based on a combination (etiotropic) drug therapy. Relevant antiviral compounds might prevent the penetration of the SARS-CoV-2 pathogen into the host cell and block its replication. The processes can be carried out *via* direct antiviral action of the drugs belonging into a group of viral RNA synthesis and replication inhibitors (**TZV** (**1**)). In addition, membrane fusion inhibitors, e.g., **UFV** (**3**), might interfere with the fusion of pathogen's and host cell's membranes due to interactions with a viral surface (S)-glycoprotein and blockade of its trimerization. The molecule (**3**) also acts as an effective blocker of the virus entry and possibly a blocker of intracellular vesicle transport. Anyway, the lessons learned from current emerging pandemic can help to prepare in advance for possible future pandemic situations.

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Conflicts of interest: none.

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