ASSESSMENT OF CYTOTOXICITY AND ANTIVIRAL ACTIVITY AGAINST SARS-COV-2 OF THE MIXTURE OF LACTOFERRIN, ARTEMISININ, AND AZITHROMYCIN *IN VITRO*

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Lactoferrin, artemisinin, and azithromycin exhibit a broad spectrum of antiviral, immunomodulatory, and anti-inflammatory effects. The experiments show that these drugs partially inhibit the infection caused by SARS-CoV-2 *in vitro*. This allows us to conclude that the effects on the entry of virions into cells mediated by each of these substances taken separately are insufficient for complete inhibition of the SARS-CoV-2 infection. The study was aimed to perform *in vitro* assessment of cytotoxicity and antiviral activity against the laboratory SARS-CoV-2 strain of the mixture of active ingredients: lactoferrin, artemisinin, and azithromycin. We used the Vero CCL81 (ATCC) cell line and the Dubrovka laboratory strain of SARS-CoV-2 (GenBank ID: MW161041.1), isolated in the Vero CCL81 cell culture from the nasopharyngeal swab of patient with COVID-19. Cytotoxic effects and antiviral activity against SARS-CoV-2 of the drug mixture were assessed based on the cytopathic effects using the MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay. Hydroxychloroquine was used as a reference drug. It has been shown that at high (MOI 100) and low (MOI 20) multiplicity of infection used in the Vero CCL 81 cell culture, the mixture of artemisinin, lactoferrin and azithromycin has a significant effect on the SARS-CoV-2 reproduction, and IC50 (half maximal inhibitory concentration) is estimated as the 1 : 2 dilution in both cases. The findings make it possible to conclude that the studied mixture is low toxic and shows significant antiviral effects *in vitro*.

Keywords: artemisinin, azithromycin, lactoferrin, cytotoxicity, antiviral activity, SARS-CoV-2, COVID-19, drug repurposing

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Compliance with ethical standards: the study was performed in accordance with the principles of the World Medical Association Declaration of Helsinki.

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ОЦЕНКА ЦИТОТОКСИЧНОСТИ И ПРОТИВОВИРУСНОЙ АКТИВНОСТИ СМЕСИ ЛАКТОФЕРРИНА, АРТЕМИЗИНИНА И АЗИТРОМИЦИНА В ОТНОШЕНИИ SARS-COV-2 *IN VITRO*

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Лактоферрин, артемизинин и азитромицин обладают широким спектром противовирусного, иммуномодулирующего и противовоспалительного действия. Экспериментально показанное частичное ингибирование ими инфекции, вызванной SARS-CoV-2 *in vitro*, позволяет заключить, что влияния на проникновение вирионов в клетки, опосредованное каждым из этих веществ в отдельности, недостаточно для полного ингибирования инфекции SARS-CoV-2. Целью работы было оценить *in vitro* цитотоксичность и противовирусную активность смеси активных действующих веществ лактоферрина, артемизинина и азитромицина в отношении лабораторного штамма SARS-CoV-2. Использовали перевиваемую культуру клеток Vero CCL81 (ATCC) и лабораторный штамм коронавируса SARS-CoV-2 «Дубровка» (идентификационный № GenBank: MW161041.1), выделенный на культуре клеток Vero CCL81 из назофарингеального мазка больного COVID-19. Определение цитотоксического действия смеси препаратов и изучение противовирусной активности в отношении вируса SARS-CoV-2 оценивали по эффекту цитопатического действия с использованием МТТ (метилтиазолилдифенил-тетразолия бромид). В качестве препарата сравнения использовали гидроксихлорохин. Показано, что при высокой множественности заражения (100 MOI) и низкой (20 MOI) в культуре клеток Vero CCL81 смеси артемизинина, лактоферрина и азитромицина оказывает значимый эффект на вирусную репродукцию SARS-CoV-2, ИК50 (полумаксимальная ингибирующая концентрация) оценивается в обоих случаях как разведение 1 : 2. Полученные результаты позволяют сделать вывод о низкой цитотоксичности изучаемой смеси и о наличии значимого противовирусного действия *in vitro*.

Ключевые слова: артемизинин, азитромицин, лактоферрин, цитотоксичность, противовирусная активность, SARS-CoV-2, COVID-19, перепрофилирование препарата

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Currently, the issue of the spread of COVID-19 coronavirus infection caused by SARS-CoV-2 is still relevant. Despite the fact that timely vaccination can decrease the risk of severe infection, the development of additional safeguard that can either alleviate severe course or prevent COVID-19 infection is still a priority, especially because antibodies against vaccine antigens may not recognize new variants of the virus.

To date, COVID-19 prevention and treatment consist primarily of the use and development of vaccines for the formation of neutralizing antibodies binding spike protein, immune sera and monoclonal antibodies, antiviral drugs [1], and drugs directed against hyperactivation of the immune response [2] along with the symptomatic supportive treatment and respiratory support of the infected individuals. During the fight against the COVID-19 pandemic, special attention was paid to drug repurposing, since the known safety and pharmacokinetic profiles allowed for timely introduction of the drugs, in contrast to the new medications that required full scale testing and registration. Currently, the most recent (16th) update of the Temporary Guidelines on the "Prevention, Diagnosis, and Treatment of Novel Coronavirus Infection (COVID-19)" includes favipiravir, molnupiravir, nirmatrelvir + ritonavir, remdesivir, and umifenovir as the direct-acting antiviral agents. Biotechnology medications are also recommended: interferon alpha, synthetic small interfering ribonucleic acid (double-stranded) [3]. However, the clinical trials of these drugs used for treatment of COVID-19 are limited and often controversial, there is no indisputable evidence and experience of using the drugs. The presence of multiple mutations in the S protein suggests its capability of acquiring new ligand specificity properties [4].

The mechanism underlying the SARS-CoV-2 cell entry, that is associated with the angiotensin converting enzyme 2 (ACE2), is a complex multifactorial process that involves many accessory molecules: proteinases, co-receptors and activators of their expression. Availability of co-receptors allows SARS-CoV-2 to infect cells with low ACE2 expression on the membranes.

Thus, as a glycoprotein, S protein can interact with receptors not only via its protein component, but also by binding to the lectin receptors via its carbohydrate component (N-glycans of S1 subunit containing oligomannose and complex carbohydrates that protect the virus against antibodies) [5, 6]. Binding of the lectin-like S1 sites to the target cell glycocalyx via O-acetylated sialic acids [7] and heparan sulfate [8] may facilitate cell infection. It has been shown that heparan sulfate promotes cell entry in viruses of many types [9], including SARS-CoV-2 [10]. The majority of polysaccharide chains found in the heparan sulfate proteoglycans are strongly negatively charged. This makes it possible to recruit SARS-CoV-2 viral particles on the cell surface due to interaction with S protein, thereby increasing its local concentration for further binding to ACE2. There are reasons to believe that the positively charged binding groove located in the S protein RBD domain might be the putative binding site for negatively charged polysaccharide chains of the heparan sulfate proteoglycans [8, 11], and the binding specificity depends largely on the complementary spatial arrangement of the main protein groups and of sulfate and carboxyl groups on the polysaccharide [12–14].

As for strong inhibitors of the SARS-CoV-2 cell entry, the drug repurposing screening has made it possible to identify a number of compounds targeting the heparan sulfate proteoglycans and dependent on them endocytosis pathways. One such compound is lactoferrin (LF), the naturally occurring non-toxic glycoprotein that is available as dietary supplement [15].

Assessment of the LF antiviral activity in the model of the human colon adenocarcinoma cell line Caco-2 and monkey kidney epithelial cell line Vero 6 infected with coronavirus has shown that LF partially inhibits infection and SARS-CoV-2 replication [16]. A number of studies focused on assessing the effects of LF binding with to the receptor show that binding affects various signaling systems and pathways, including NF-κB and various interferon regulatory factors. This results in modulation of antiviral immune response [17]. The effects of LF on regulation of TLR, especially TLR3 and TLR7, involved in recognition of RNA viruses [18, 19] and inhibition of cathepsin L [20] have been also shown. These result in blocking the SARS-CoV-2 entry into the human embryonic kidney 293/hACE2 cells [21]. The experimental study [16] shows that LF can inhibit the TGFB1 immunosuppressive cytokine expression, suppress the expression of thymic stromal lymphopoietin, high levels of which have been found on the bronchial mucosa of patients with asthma and chronic obstructive pulmonary disease, and reduce the expression of pro-inflammatory cytokines IL1B and IL6. These immunomodulatory effects of LF may counteract the cytokine storm activation.

Azithromycin that affects a variety of processes is one more promising repurposed drug. First of all, azithromycin affecting the decline in the expression of matrix metalloproteinases related to CD147 attracted attention of the researchers, who hypothesised that azithromycin was capable of inhibiting CD147 and eventually blocking viral entry into host cells [22]. It has been shown that CD147 induces the PI3K/AKT signaling pathway activation, thus promoting NF-κB induction and production of pro-inflammatory cytokines [23, 24]. The PI3K/AKT signaling pathway increases the TMPRSS2 serine protease expression, thus enchancing viral entry.

Immunomodulatory properties of azithromycin [25] may play a vital part in treatment of hyperinflammation caused by cytokine storm associated with COVID-19. *In vitro* studies of azithromycin have shown the decreased secretion of pro-inflammatory cytokines and chemokines [26, 27]. Furthermore, azithromycin reduces accumulation of inflammatory cell infiltrates in the bronchoalveolar lavage fluid [28]. In fibroblasts, azithromycin inhibits proliferation and collagen production by reducing the concentration of transforming growth factor (TGFβ) and demonstration of pulmonary antifibrotic activity [29, 30]. Azithromycin exhibits mucoregulatory effects: it reduces mucus hypersecretion and improves mucociliary clearance [31].

Research has shown that azithromycin can modify ACE2 glycosylation, thus preventing SARS-CoV-2 entry into cells. Molecular mimicry of azithromycin and cellular GM1 ganglioside (ganglioside-lipid that acts as a cofactor of the respiratory virus attachment to host cells) is the other proposed mechanism underlying antiviral effects. Azithromycin can bind the ganglioside-binding domain of S protein, thus blocking the S protein-GM1 interaction on the host cell plasma membrane [32].

Indirect blocking of furin system promoting viral entry after the S1-ACE2 binding is one more mechanism underlying the effects of azithromycin. The furin system is activated in the acidic conditions of the trans-Golgi network. In the active form furin cleavages S1 subunit from spike protein. It is assumed that azithromycin reduces furin activity by increasing the organellar pH [33]. Furthermore, azithromycin can alkalinize vesicles containing SARS-CoV-2 virions, thus preventing the pH-dependent membrane fusion.

Artemisinin, the anti-malarial drug that exhibits immunomodulatory properties is the third candidate remedy against SARS-CoV-2. Artemisinin, together with chloroquine and quinine, has a long history of clinical use, it shows a broad-

spectrum antiviral potential. It has been shown that chloroquine, the anti-malarial drug possessing immunomodulatory activity, that is well-known for decades, and hydroxychloroquine, the chloroquine derivative, can effectively inhibit SARS-CoV-2 *in vitro* [34, 35].

In addition to its role in treatment of malaria, artemisinin was studied for its potential effects on the immune responses under physiological and pathological conditions [36–38]. Many bacteria and viruses, including SARS-CoV-2, activate the NF-κB signaling pathway in human cells. Activation of the NF-κB signal transduction results in subsequent activation of the p50/p65 transcription factors. Artemisinin and artesunate can act as the NF-κB signaling pathway inhibitors by blocking the function of p50/p65. Research shows that artemisinin can interact with the cell surface via inhibition of the SARS-CoV-2 S protein binding to the cell surface receptors. This potentially prevents both endocytic entry of the virus and activation of the NF-κB signal transduction. Thus, artemisinin can prevent cytokine storm by inhibiting the IκB kinase [39–41]. However, the molecular docking studies show that artemisinins can also bind to coronavirus proteins, such as E protein, helicase protein, N protein, protein 3CL PRO, S protein, non-structural protein 3 (nsp3), nsp10, nsp14, nsp15, cathepsin L, and GRP78 [42, 43]. Therefore, biological activities of artemisinin may be partially based on inhibition of functions of these viral proteins.

Partial inhibition of the SARS-CoV-2 infection by lactoferrin, artemisinin, and azithromycin *in vitro* suggests that potential blocking of the entry of virions into cells mediated by each of these substances is insufficient for complete inhibition of the SARS-CoV-2 infection. However, the combination use of these drugs may be more promising in terms of clinical use. That is why the search and development of new drugs effective against novel coronavirus infection are going on, and the relevance of such studies is beyond doubt. The cell culture study of the drug antiviral activity is the first step of the search.

The study was aimed to assess cytotoxicity and antiviral activity of the mixture of active ingredients, lactoferrin, artemisinin, and azithromycin, against SARS-CoV-2 and to compare the mixture with hydroxychloroquine, since many *in vitro* studies that involved assessment of antiviral activity were limited by non-utilization of reference drugs.

METHODS

Viruses and cells

The experiments involved the Vero CCL81 (ATCC) kidney epithelial cells from the African green monkey that were obtained from the collection of the Mechnikov Research Institute of Vaccines and Sera and the Dubrovka laboratory strain of SARS-CoV-2 (GenBank ID: MW161041.1), isolated in the Vero CCL81 cell culture from the nasopharyngeal swab of patient with COVID-19. The virus was cultivated at 37 °C in the DMEM growth medium containing glutamine and glucose (4.5 g/L), 5% fetal bovine serum (FBS), L-glutamine (300 μg/mL), gentamicin (40 μg/mL) in the 5% CO_2 atmosphere conditions (growth medium, GM). The strain derived after 20 serial passages, it caused strong cytopathic effects (CPE) of the virus. The samples of viral material were stored at a temperature of °80 °C as aliquots. Aliquots of one stock were used in all the experiments.

Preparing the drug mixture

Ten milliliters of DMSO were added to 45 mg of azithromycin to obtain the solution with a concentration of 6 μmol/mL. Then 0.5 mL of phosphate buffer were added to 10 mg of lactoferrin to obtain the solution with a concentration of 20 mg/mL. Five milliliters of DMSO were added to 21 mg of artemisinin to obtain the solution with a concentration of 15 μmol/mL. To prepare the working solution, we mixed 5 μL of azithromycin solution, 125 μL of lactoferrin solution, and 50 μL of artemisinin solution, the growth medium was used to adjust the volume to 5 mL.

The concentrated solution of the reference drug (hydroxychloroquine) was prepared using the dosage form (the pill) that was diluted in the sterile distilled water individually for each experiment on the very day of use in equimolar amounts corresponding to the amount of pure substance in the drug. All the compounds, including hydroxychloroquine, were weighted to 0.1 mg using the analytical balance.

Cell culture assay for assessment of drug cytotoxicity

The cells were seeded in the 96-well Corning plates with the average seeding density of 20,000 cells per well and grown in the DMEM growth medium containing glutamine and glucose (4.5 g/L), 5% fetal bovine serum (FBS), L-glutamine (300 μg/mL), gentamicin (40 μg/mL) in the 5% CO_2 atmosphere conditions (GM) for three days until a monolayer was completely formed. Then the medium was removed, and 100 μL of the specified test drug concentrations (eight concentrations of each drug) in appropriate medium with no serum (working medium, WM) were added to the plate. Then 100 μL of WM were added to each well of the plate. Four iterations of each experimental step were performed (n = 4). Cells containing 200 μL of WM were used as negative controls. To define the thresholds of cytotoxicity concentration (TCD_{50}), the plates were incubated for 72 hrs at 37 °C in the 5% CO_2 atmosphere. When assessing antiviral activity, cells were incubated with drugs for five days, that is why the same incubation time (five days) was used for cytotoxicity assessment in the other series of experiments in order to rule out the toxic effects of the tested samples. Cytotoxic effects of the drugs were visually estimated based on the condition of cellular monolayer and quantified using the MTT assay. For that 160 μL of DMEM growth medium containing no phenol red and 40 μL of the 5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide dye (MTT) solution were added to each well and incubated for 2 hrs at 37 °C in the 5% CO_2 atmosphere. The culture broth was removed, and 100 μL of DMSO were added to the wells, then the plates were incubated for 20 min at room temperature with continuous shaking. The optical density (OD) was measured at 530 nm taking into account the background values obtained at 620 nm using a spectrophotometer for plate reading. The maximum drug concentration that did not change the OD by more than 10–15% compared to control cells was considered as maximum tolerable concentration (MTC). The substance concentration that reduced OD by 50% compared to control cells was considered as TCD_{50}.

Assessment of drug antiviral activity against SARS-CoV-2 based on the cytopathic effect (CPE) using MTT assay

To assess antiviral activity of the samples, the Vero CCL81 cell culture was plated in the 96-well flat bottom cell culture plates (20,000 cells/well) and grown in aprropriate GM. On day three, after the monolayer was completely formed, the WM was removed from the plate wells. Then 100 μL of the tested drugs, undiluted or diluted with the WM to the specified concentrations (seven concentrations), were added to the wells. Some wells were used as virus control or cell control. Four iterations of each experimental step were performed (n = 4). In parallel, to rule out

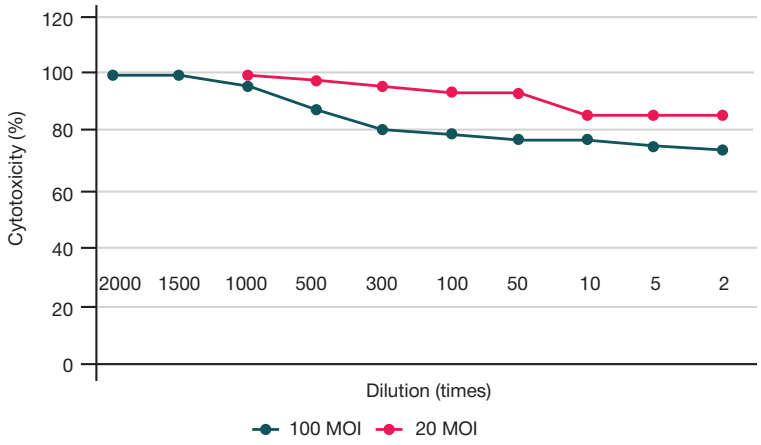

Fig. 1. Cytotoxicity of various dilutions of the mixture of active ingredients in the Vero CCL81 cell culture during the 3- and 5-day incubation

the cytotoxic effects of the drugs in the experiments focused on assessing antiviral activity, the same drug concentrations in the same conditions were added to the non-infected wells. After the 2-hrs incubation, the virus at a dose of infection (MOI) of 20 or 100 (per 100 µL) was added to all the wells, except for the cell control wells. Then the cells were incubated for five days at 37 °C in the 5% CO_2 atmosphere until the CPE was clearly visible in the virus control cells. The CPEs observed in the cells were quantified using the MTT assay as previously described. IC_{50} was calculated using the Excel application in accordance with the following formula:

$$\text{Inhibition} = \frac{100 - (OD_{\text{cell control}} - OD_{\text{experiment}})}{(OD_{\text{cell control}} - OD_{\text{virus control}})} \times 100\ (\%)$$

Inhibition of viral reproduction of 30% or more was considered significant for exhibiting antiviral activity. The drug concentration that reduced OD by 50% was considered as IC_{50}.

The dosage form of hydroxychloroquine was used as a reference drug. Hydroxychloroquine concentration of 10 µg/mL that corresponded to IC_{50} was selected for the study [34, 44].

RESULTS

Assessing cytotoxic effects of the samples in the Vero CCL81 cell culture

In the first series of experiments we studied cytotoxicity of various dilutions of the tested drugs. We used the Vero CCL81 cell line that was later used to define antiviral activity. Visual assessment performed using the inverted microscope after the 72-hrs incubation showed that there were no cytotoxic/morphological changes or cell monolayer breakdown. After adding some concentrations of substances, partial breakdown of monolayer was observed in experimental wells, the cells had a more rounded shape, and cell morphology was different from that of cell control. Complete breakdown of cell monolayer was observed in some wells. The research conducted by the more precise quantitative method involving MTT staining confirmed the data obtained by visual assessment of the cell condition. Based on the cell culture assessment of cytotoxic effects exhibited by the mixture with the use of the MTT assay, the dose–response curves were plotted (Fig. 1) that were used to define the MTC and TCD_{50} values: 1 : 500 and less than 1 : 2 for three-day incubation, 1 : 50 and less than 1 : 2 for five-day incubation, respectively. The method involving the use of MTT is also used to define antiviral activity, that is why the same dilutions of samples, with the same volume and time of incubation as in the method of assessing antiviral activity involving no cell infection, were added to the cells for control to rule out the cytotoxic effects of the mixture during the five-day incubation.

Antiviral activity of the mixture of active ingredients against SARS-CoV-2 in the Vero CCL81 cell culture

Antiviral activity against SARS-CoV-2 was assessed in the Vero CCL81 by the method of viral CPE inhibition revealed using the MTT staining. Two multiplicity of infection values, MOI 100 and MOI 20, were used to infect the cells. Inhibition was observed at no more than 15-times dilution for both variants of infection. The data obtained are provided in Fig. 2. Adding mixture to the cells significantly suppressed (viral reproduction inhibition exceeded 30%) replication of the SARS-CoV-2 coronavirus. At the same time, hydroxychloroquine with a concentration of 10 µg/mL that was used as a reference drug showed SARS-CoV-2 reproduction inhibition of 65% (data not shown).

DISCUSSION

Several studies have shown that other co-receptors and cellular molecules in addition to ACE2 are required for SARS-CoV-2 infection [45]. Currently, the complete list of them is unknown to date. Initial step of viral entry is often triggered by the low-affinity binding to the attachment sites that promotes accumulation of virions on the cell surface. The subsequent binding to the high-affinity receptor triggers the viral entry [46, 47]. The study of molecular mechanisms underlying the SARS-CoV-2 cell infection has revealed a number of drugs that make it possible to inhibit infection.

The tested mixture of artemisinin, azithromycin, and lactoferrin is low toxic, it significantly inhibits the infection and SARS-CoV-2 replication *in vitro*. It is assumed that its mechanisms of action are mediated by active ingredients. Binding to the SARS-CoV-2 entry receptors and inhibition of viral protein functions may underlie prevention of cell infection with SARS-CoV-2, since these affect the related signaling systems and pathways, including NF-κB, PI3K/AKT, various interferon regulatory factors, and pro-inflammatory cytokine production [23, 24]. Furthermore, the decrease in furin activity provided by azithromycin significantly reduces cell infection. Moreover, azithromycin can alkalinize vesicles containing SARS-CoV-2 virions and prevent the pH-dependent membrane fusion.

CONCLUSIONS

The findings show that the mixture of active ingredients containing azithromycin, lactoferrin, and artemisinin shows low

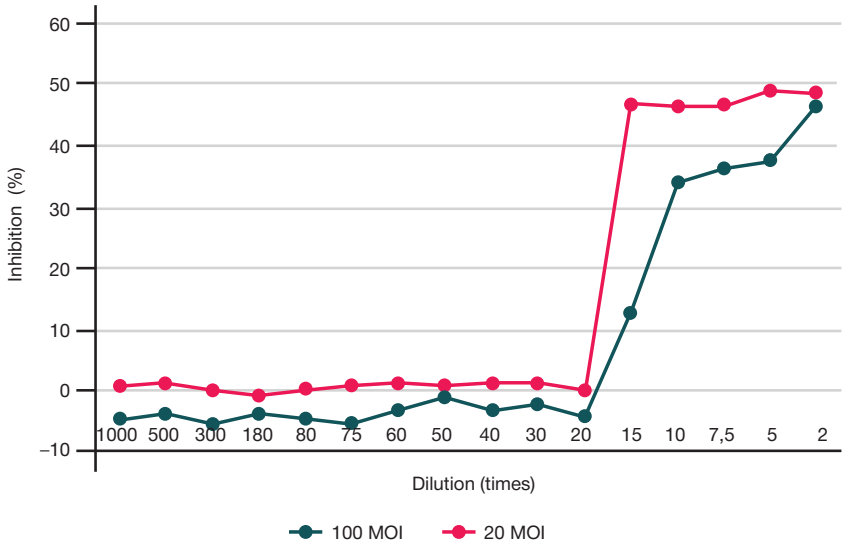

Fig. 2. Antiviral activity of the mixture of active ingredients in the culture of Vero CCL81 cells infected with MOI 20 and MOI 100 of the SARS-CoV-2 coronavirus

toxicity during the three-day and five-day incubation in the Vero CCL 81 cell culture. All the dilutions reduce cell viability by no more than 10–30%, the estimated TCD50 values are lower than the lowest dilution that is available for assessment (1 : 2). At high (MOI 100) and low (MOI 20) multiplicity of infection used in the Vero CCL 81 cell culture, the mixture has a significant effect on the SARS-CoV-2 reproduction, and IC50 is estimated as the 1 : 2 dilution in both cases. Thus, IC50 of the mixture is achieved by using the following concentrations of active ingredients: 3 μmol/L of azithromycin, 5 mg/L of lactoferrin, and 7.5 μmol/L of artemisinin.

Such molecular mechanisms underlying cell infection with the SARS-CoV-2 virions are still poorly understood, however, the combination mixtures have some benefits due to synergistic effects of the ingredients. The results obtained for the mixture of artemisinin, azithromycin, and lactoferrin *in vitro* show that the mixture can be used as a potential effective and useful adjuvant therapeutic supplement for treatment and prevention of COVID-19. Theoretical background and antiviral activity shown by the mixture of artemisinin, azithromycin, and lactoferrin during the study encourage us to plan further preclinical and clinical studies focused on assessing its safety and antiviral activity against SARS-CoV-2 *in vivo*, as well as on studying the dosage regimens of the drug and its combinations with other antivirals.

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