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Original Research

Inhibition of SARS-CoV-2 M^{pro} with Vitamin C, L-Arginine and a Vitamin C/L-Arginine Combination

Ivana Đukić[1], Nevena Kaličanin[2], Milan Sencanski[3], Snezana B. Pajovic[4], Jelena Milicevic[3], Jelena Prljic[3], Slobodan Paessler[5,6], Radivoje Prodanović[1], Sanja Glisic[3,*]

[1]University of Belgrade-Faculty of Chemistry, 11000 Belgrade, Serbia
[2]University of Belgrade-Institute of Chemistry, Technology and Metallurgy, 11000 Belgrade, Serbia
[3]Laboratory of Bioinformatics and Computational Chemistry, Institute of Nuclear Sciences Vinca, National Institute of the Republic of Serbia, University of Belgrade, 11001 Belgrade, Serbia
[4]Department of Molecular Biology and Endocrinology, VINCA Institute of Nuclear Sciences, National Institute of the Republic of Serbia, University of Belgrade, 11001 Belgrade, Serbia
[5]Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA
[6]Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX 77555, USA
*Correspondence: sanja@vinca.rs (Sanja Glisic)
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Abstract

Background: Drug resistance is a critical problem in health care that affects therapy outcomes and requires new approaches to drug design. SARS-CoV-2 M^{pro} mutations are of concern as they can potentially reduce therapeutic efficacy. Viral infections are amongst the many disorders for which nutraceuticals have been employed as an adjunct therapy. The aim of this study was to examine the potential *in vitro* activity of L-arginine and vitamin C against SARS-CoV-2 M^{pro}. **Methods**: The M^{pro} inhibition assay was developed by cloning, expression, purification, and characterization of M^{pro}. Selected compounds were then screened for protease inhibition. **Results**: L-arginine was found to be active against SARS-CoV-2 M^{pro}, while a vitamin C/L-arginine combination had a synergistic antiviral action against M^{pro}. These findings confirm the results of our previous *in silico* repurposing study that showed L-arginine and vitamin C were potential M^{pro} inhibitors. Moreover, they suggest a possible molecular mechanism to explain the beneficial effect of arginine in COVID patients. **Conclusions**: The findings of the current study are important because they help to identify COVID-19 treatments that are efficient, inexpensive, and have a favorable safety profile. The results of this study also suggest a possible adjuvant nutritional strategy for COVID-19 that could be used in conjunction with pharmacological agents.

Keywords: anti SARS-CoV-2; M^{pro}; COVID-19; arginine; vitamin C/arginine combination; M^{pro} candidate inhibitors

1. Introduction

The SARS-CoV-2 virus quickly spread around the world and was classified by the World Health Organization on March 11, 2020 as the second pandemic of the 21st century [1]. SARS-CoV-2 illness is frequently accompanied by unremitting fever, hypoxemic respiratory failure, systemic complications, encephalopathy, delirium, and thromboembolic events [2–4]. Following infection with SARS-CoV-2, patients with severe and critical illnesses suffer frequent neurological complications [4]. The blood-brain barrier (BBB) is a critical interface that regulates the entry of circulating molecules into the central nervous system (CNS). The BBB is therefore essential for the treatment of viruses that can infect the CNS, such as SARS-CoV-2 [5].

Nutraceuticals, phytochemicals from medicinal plants, and dietary supplements have been used as adjunct therapies for many diseases, including viral infections. The use of adjunct antiviral therapy may be beneficial in the treatment and prophylaxis of COVID-19 [6].

Arginine is a natural molecule that crosses the BBB through a transporter with specificity for amino acid analogs that possess cationic terminal guanidine groups, such as those contained in L-arginine [7]. Recently, it was shown that amino acids can improve immunity and shorten disease length in patients with COVID-19 [8]. In a randomized clinical trial of adults with severe COVID-19, L-arginine plus standard care significantly reduced the need for respiratory support and reduced the length of hospitalization. Large doses (1.66 g) of L-arginine were given orally twice per day for the entire hospitalization period [8]. The molecular mechanisms that underlie the significant modulating effect of arginine are still to be clarified.

The main protease of SARS-CoV-2, M^{pro} (also called 3CLpro), is an important drug target due to its crucial role in the life cycle of the virus. M^{pro} is one of the best characterized drug targets in coronaviruses that lack homologous human protease, thus making it one of the most attractive SARS-CoV-2 drug targets [9].

We previously proposed a simple theoretical criterion for rapid virtual screening of molecular libraries for candidate inhibitors of M^{pro} [10]. After initial *in silico* screen-

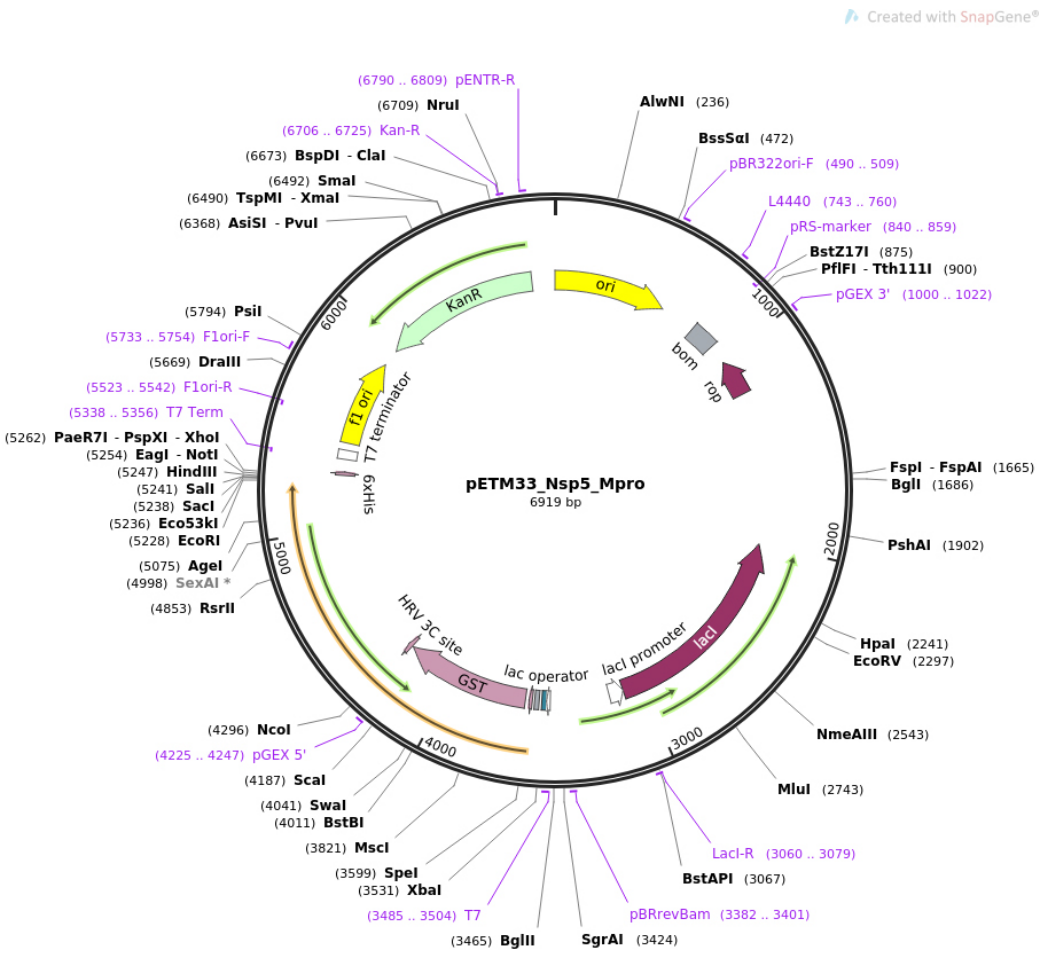

Fig. 1. Plasmid map of cloned GST-M^{pro} (adapted from [17]).

ing of Drugspace using EIIP/AQVN filter [11], followed by further filtering of drugs by virtual ligand-based screening and molecular adhesion, we identified arginine as a candidate M^{pro} inhibitor [10]. In another computer-based study, arginine was identified amongst the 20 amino acids as the best inhibitor of the main protease in SARS-CoV-2 [12]. In our earlier *in silico* work, we also proposed vitamin C as a candidate M^{pro} inhibitor. It was shown in another study that vitamin C inhibits SARS-CoV-2 3CLpro *in vitro* [13]. Ascorbic acid, or vitamin C, is an important water-soluble nutrient. It is produced by plants and by virtually all animals, with the exception of primates, most bats, guinea pigs, and a few rodents which, as a consequence, require vitamin C in their diet [14].

Some of the important effects of vitamin C following viral infection are reduced pro-inflammatory response, improved epithelial barrier function, enhanced alveolar fluid clearance, antiviral activity, and immune system stimulation. Vitamin C is also a crucial factor in the production of type I interferons during the antiviral immune response, and acts as an inactivating agent for RNA and DNA viruses [15].

Due to its antioxidant, anti-inflammatory, and immunomodulatory properties, vitamin C is a possible therapeutic option for the prevention and treatment of COVID-19 infection, as well as a possible adjuvant therapy for COVID-19 critical care [16].

The results of the present study showed that arginine has inhibitory activity against M^{pro} *in vitro*. Furthermore, a vitamin C/arginine combination was found to have synergistic inhibitory activity *in vitro*.

2. Materials and Methods

2.1 Equipment

Microorganisms were grown using the thermostat-controlled "Environmental Shaker-Incubator ES-20" and the shaker "Thermo-shaker TS-100 Biosan" (Ratsupites iela 7 k-2, Riga, Latvia). The "Consort E122" system was used for protein electrophoresis and the HPLC AKTA (Emeryville, Cytivia, CA, USA) system for enzyme purification. A "Thermo Scientific Appliscan" (ThermoFischer Scientific, Waltham, MA, USA) device was used to measure enzyme activity by fluorescence.

2.2 Chemicals

The antibiotic Kanamycin was purchased from Invitrogen, catalog number: 11815024, Carlsbad, CA, USA.

Various components for media preparation (agar, peptone and tryptone) were purchased from Torlak, Belgrade, Serbia. Other substances were ordered from Centrohem, Belgrade, Serbia.

2.3 M^{pro} Gene

The gene for M^{pro} was ordered from Addgene and cloned into the pETM33 vector with N-terminal GST and His-tag (Fig. 1, Ref. [17]).

The M^{pro} gene was cloned using NcoI and EcoRI restriction enzymes. Chimera: His-GST-HRV_3C-MP has 1656 bp with a Mr of 62.3 kDa. Recommended expression conditions when using *E. coli* BL21 DE3 gold cells are growth at 37 °C, induction with IPTG at a final concentration of 1 mM, and expression for 16 h at 18 °C. *E. coli* STAR strain was used for intracellular expression, while the DH5α strain was used for storage and propagation of plasmids.

Amino Acid Sequence of M^{pro}

DGSGFRKMAFPSGKVEGCMVQVTCGTTTLNGLWLDDVVYCPRHVICTSEDMLNPNYEDLLIRKSNHNFLVQAGNVQLRVIGHSMQNCVLKLKVDTANPKTPKYKFVRIQPGQTFSVLACYNGSPSGVGSVGFNIDYDCVSFCYMHHMELPTGVHAGTDLEGNFYGPFVDRQTAQAAGTDTTITVNVLAWLYAAVINGDRWFLNRFTTTLNDFNLVAMKYNYEPLTQDHVDILGPLSAQTGIAVLDMCASLKELLQNGMNGRTILGSALLEDEFTPFDVVRQCSGVTFQ.

2.4 Isolation and Purification of M^{pro} Protease

2.4.1 Cell Lysis

Collected cells were resuspended in 5 mL of lysis buffer composed of 50 mM Na-phosphate buffer, 300 mM NaCl and 10 mM imidazole (pH 7.5). Samples were sonicated on ice. Aliquots were taken before induction (0 h) and after expression (24 h). Sonication was performed using an ultrasound probe, 10 times for 10 seconds each, with a 20 second pause in between. After lysis, the mix was centrifuged for 5 min at 13,000 rpm and the supernatant then passed through a sterile 0.22 μm filter.

2.4.2 Purification

M^{pro} (25 mL) was purified by HPLC with a 5 mL Ni-NTA FF Sepharose column. The lysis buffer described above was used for column equilibration, and the same buffer with a gradient from 10 mM to 300 mM imidazole was then used for protein elution. Absorbance at 280 nm was monitored and fractions were examined by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) electrophoresis. Samples (17 mL) were dialyzed against 50 mM Tris-HCl buffer with 150 mM NaCl, pH 7.5. The purified enzyme was stored in 50% glycerol at –20 °C.

2.4.3 Measurement of Activity

Changes in fluorescence were monitored every 135 s for 45 min at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The total volume of reaction mixture was 200 μL and the reaction buffer was 20 mM Tris with 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) (pH 7.3). Five μL of enzyme and 1 μL of fluorescent substrate dissolved in dimetilsulfoksid (DMSO) were added so that the final concentration was 5 μM. L-arginine and vitamin C were tested as possible inhibitors. These were dissolved in 20 mM Tris (pH 7.3) and the pH adjusted to 7.3 if necessary. Final inhibitor concentrations used in the experiments were 2 mM, 10 mM, 20 mM, 50 mM, 100 mM, 150 mM, and 200 mM. Buffer was used as a blank, and buffer plus substrate was used as a control.

3. Results

3.1 M^{pro} Inhibition with Vitamin C, Arginine, and Vitamin C/Arginine Combination

Figs. 2,3 show the inhibition of M^{pro} with vitamin C and arginine, respectively. The addition of 75 mM arginine to 20 mM (3.52 mg/mL) vitamin C increased the level of inhibition from 44% to 61%, while the addition of 75 mM arginine to 50 mM (8.81 mg/mL) vitamin C increased inhibition from 67 to 82% (Table 1). Therefore, vitamin C and the amino acid arginine have an additive inhibitory effect on the *in vitro* proteolytic activity of the M^{pro} protease from the SARS-CoV-2 virus (Table 1).

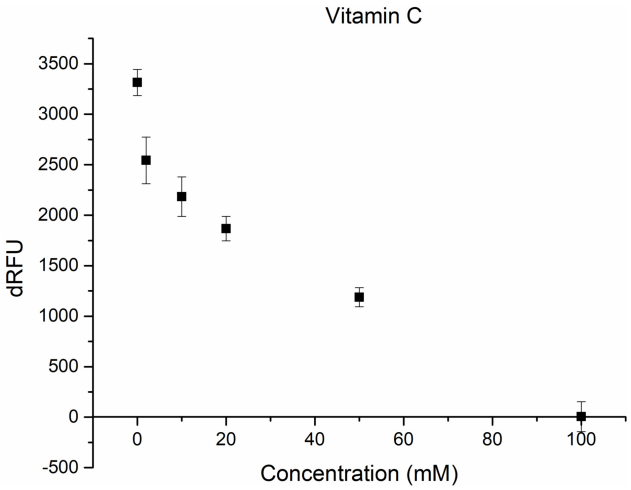

Fig. 2. Inhibition of M^{pro} by vitamin C (IC 20 mM).

3.2 Inhibition of M^{pro} by Vitamin C (IC 20 mM)

It could be seen from the obtained results that around 44% inhibition was achieved using 20 mM vitamin C concentration (Fig. 2), while in the case of arginin 39% of inhibition was achieved at 75 mM concentration (Fig. 3).

When both compounds were added at the same con-

Table 1. M^{pro} inhibition with vitamin C, arginine, and vitamin C/arginine.

Inhibitor	Concentration (mM)	Inhibition (%)	Relative error
Water	0	0	0
Vitamin C	20	43.66	± 3.66
Vitamin C	50	67.29	± 2.87
Arginine	75	39.48	± 16.25
Vitamin C + Arginine	20 + 75	60.97	± 12.52
Vitamin C + Arginine	50 + 75	82.23	± 14.82

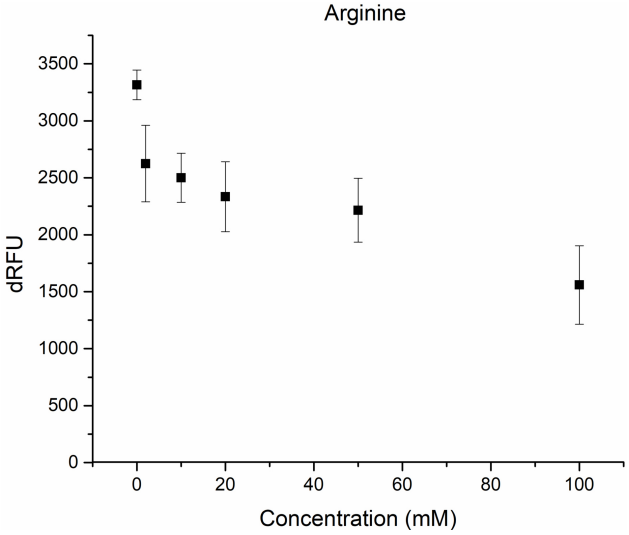

Fig. 3. Inhibition of M^{pro} by arginine (IC about 150 mM).

centration's inhibition was increased to 61% showing an additive inhibitory effect on the *in vitro* proteolytic activity of the M^{pro} protease from the SARS-CoV-2 virus (Table 1). Additive inhibitory effect was achieved also using 50 mM vitamin C in combination with 75 mM argining giving 82% of M^{pro} protease decrease in activity.

4. Discussion

Drug resistance is a critical problem in health care that affects therapy outcomes and requires new drug design approaches. SARS-CoV-2 mutations are therefore of great concern as they could lead to drug resistance. The SARS-CoV-2 main protease (M^{pro}) is one of the most attractive drug targets. However, more than 19,000 mutations encompassing 282 amino acid positions have already been reported in M^{pro}. These "hotspots" could change the M^{pro} structure and activity and potentially reduce any therapeutic effects against this protease [18]. Safe and inexpensive treatments for SARS-CoV-2 prevention and as adjunct therapy to pharmacological agents are therefore urgently required during the current pandemic.

Nutraceuticals are defined as any food or food component that provides medical or health benefits, including the prevention and treatment of disease [19]. These may have potential therapeutic efficacy in the fight against the SARS-CoV-2/COVID-19 pandemic. In a randomized clinical trial of adults with severe COVID-19, L-arginine given with standard care was found to significantly reduce the need for respiratory support and the length of hospitalization (NCT04637906, registration date November 20, 2020; [8]). Recently, the L-Arginine and Vitamin C improves Long-COVID (LINCOLN) survey found that L-arginine when taken with vitamin C improved long-COVID, thus demonstrating for the first time the beneficial effect of this combination. The survey was completed by 1390 patients who were divided into two groups: those who received L-arginine plus vitamin C, and those who received a multivitamin combination. According to the survey, supplementation with L-arginine plus vitamin C had beneficial effects for long-COVID patients, with less symptoms and significantly lower effort perception [20].

The important roles of amino acids, including arginine, in immune responses were recently reviewed [21]. L-arginine is converted in the body to nitric oxide, which has been suggested as a therapeutic option for COVID-19. Nitric oxide was shown to be an effective antiviral against SARS-CoV *in vitro*, as well as *in vivo* by inhalation of very low concentrations in a small clinical trial [22]. Although some published studies have linked arginine supplementation with increased nitric oxide production, other reports claim that acute L-arginine supplementation does not increase nitric oxide production in healthy subjects. The molecular mechanisms that underlie this important modulating effect of arginine therefore remain to be clarified [23].

The focus of the current study was to investigate the action of a non-toxic, natural amino acid against the M^{pro} of SARS-CoV-2. In our previous *in silico* study we proposed that arginine inhibits SARS-CoV-2 M^{pro}. This was confirmed experimentally in the present study, and was not unexpected since amino acid building blocks are often found in drugs and in drug candidates whose molecular targets bind naturally to amino acids or peptide structures. Many antiviral enzymatic inhibitors incorporate amino acid motifs or are themselves amino acids, which is also common with protease inhibitors [24].

We examined the inhibition of M^{pro} by arginine *in vitro*, as well as the effect of a vitamin C/arginine combination. Arginine was found to inhibit SARS-CoV-2 M^{pro} *in vitro*, while co-administration of arginine and vitamin C exerted synergistic M^{pro} inhibition due to complementary antiviral action. In our previous work we proposed

that vitamin C binds to the catalytic site and L-arginine to the allosteric site [10]. Vitamin C has antiviral, antioxidant, anti-inflammatory, and immunomodulatory effects, thereby making it a potential medical treatment for COVID-19. Indeed, several randomized controlled trials have evaluated intravenous vitamin C monotherapy in patients with COVID-19 [25,26]. The current level of evidence from these trials suggests that intervention with intravenous vitamin C may improve oxygenation parameters, lower inflammatory markers, shorten hospital stays, and lower the mortality rate, especially in the most severely ill patients. Oral vitamin C supplementation at high doses may also improve the rate of recovery in less severe cases. No adverse outcomes have been reported in published trials [27]. To validate our M^{pro} assay, we first examined the inhibitory effect of vitamin C on M^{pro} *in vitro* [12]. The previously reported inhibitory activity of vitamin C against M^{pro} was confirmed in our study. A recent study found that SARS-CoV-2 positivity and infection severity were linked to lower levels of protective Bifidobacterium genera and to lower bacterial diversity [28]. Furthermore, the addition of ascorbic acid was shown to significantly promote the growth of B. bifidum [29] and could therefore confer a protective effect to SARS-CoV-2-positive patients. Based on these findings, vitamin C could also have additional protective effects for COVID patients.

The synergistic antiviral action of arginine/vitamin C against SARS-CoV-2 M^{pro} shown in the present study may be a consequence of blocking multiple sites on one target. This strategy was previously shown to improve therapeutic efficacy [30]. Furthermore, drug combination therapy was suggested as a promising strategy to extend the lifespan of antimicrobials that must be carefully selected to minimize the evolution of resistance [31].

The inflammation triggered by oxidative stress is the cause of many chronic diseases. Oxidative stress is characterized by increased production of free oxygen radicals and represents one of the basic pathological processes of atherosclerosis. It is also closely related to endothelial dysfunction and promotes a vascular inflammatory response [32]. The correlation observed between COVID-19 and atherosclerosis suggests that effort should be directed towards cardioprotection [33]. A previous study reported that supplemental L-arginine and vitamin C could be antiatherogenic, as observed by the modulation of endothelial dysfunction biomarkers [34]. Based on these findings, the vitamin C/arginine combination could also have a cardioprotective effect in COVID patients, in addition to the direct antiviral effect suggested by our study.

The findings of our study are significant in at least two respects. First, we demonstrated that arginine exerts inhibitory action against SARS-CoV-2 M^{pro}, and that coadministration with vitamin C exerts a synergistic antiviral action against M^{pro}. These observations shed light on the potential molecular mechanism underlying the significant modulating effect of arginine against SARS-CoV-2. Second, we confirmed the findings of our previous *in silico* drug repurposing study that identified L-arginine and vitamin C as candidate M^{pro} inhibitors from amongst 1490 approved drugs in Drugbank. In that study, we used VS protocol with sequential filters based on both long-range and short-range interactions to select candidate SARS-CoV-2 M^{pro} inhibitors.

The results of the current study are important in the search for effective, safe and affordable therapeutics against COVID-19. Our findings could also help to develop an effective nutritional strategy to fight infectious diseases.

5. Conclusions

Drug resistance is an important issue in health care that affects therapeutic results and necessitates novel drug design approaches. Nutraceuticals are an interesting treatment option for COVID-19. This study examined the potential antiviral activity of L-arginine and vitamin C *in vitro*. The experimental results showed that arginine inhibits SARS-CoV-2 M^{pro}, and that a vitamin C/arginine combination has synergistic antiviral action against M^{pro}. These findings confirm the results of our previous *in silico* drug repurposing study.

The results of the current study suggest a potential dietary approach to COVID-19, in addition to pharmaceutical treatments. This is important because it is vital to develop COVID-19 therapies that are effective, affordable, and have a favorable safety profile. Furthermore, our findings might help to develop an effective nutritional approach for the prevention and treatment of infectious diseases, thereby reducing the burden on communities and healthcare systems.

Availability of Data and Materials

Not applicable.

Author Contributions

Conceptualization—RP, SP and SG; performed the experiments—RP, NK, and IĐ; validation—NK, and RP; analyzed the data—NK, IĐ, MS, and RP; investigation—NK, IĐ, and MS; resources—RP; writing, original draft preparation—RP, SG, MS, SBP, JM and JP; writing, review and editing—SG, SBP, MS, SP and RP; visualization—IĐ, NK, RP; supervision—RP; project administration—RP, SG, JM, and MS. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Guarner J. Three Emerging Coronaviruses in Two Decades: The Story of SARS, MERS, and Now COVID-19. American Journal of Clinical Pathology. 2020; 153: 420–421.

[2] Guan W, Ni Z, Hu Y, Liang W, Ou C, He J, *et al*. Clinical Characteristics of Coronavirus Disease 2019 in China. The New England Journal of Medicine. 2020; 382: 1708–1720.

[3] Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, *et al*. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395: 497–506.

[4] Najjar S, Najjar A, Chong DJ, Pramanik BK, Kirsch C, Kuzniecky RI, *et al*. Central nervous system complications associated with SARS-CoV-2 infection: integrative concepts of pathophysiology and case reports. Journal of Neuroinflammation. 2020; 17: 231.

[5] Erickson MA, Rhea EM, Knopp RC, Banks WA. Interactions of SARS-CoV-2 with the Blood-Brain Barrier. International Journal of Molecular Sciences. 2021; 22: 2681.

[6] Subedi L, Tchen S, Gaire BP, Hu B, Hu K. Adjunctive Nutraceutical Therapies for COVID-19. International Journal of Molecular Sciences. 2021; 22: 1963.

[7] Mahar Doan KM, Lakhman SS, Boje KM. Blood-brain barrier transport studies of organic guanidino cations using an in situ brain perfusion technique. Brain Research. 2000; 876: 141–147.

[8] Fiorentino G, Coppola A, Izzo R, Annunziata A, Bernardo M, Lombardi A, *et al*. Effects of adding L-arginine orally to standard therapy in patients with COVID-19: A randomized, double-blind, placebo-controlled, parallel-group trial. Results of the first interim analysis. EClinicalMedicine. 2021; 40: 101125.

[9] Kneller DW, Phillips G, O'Neill HM, Jedrzejczak R, Stols L, Langan P, *et al*. Structural plasticity of SARS-CoV-2 3CL M^{pro} active site cavity revealed by room temperature X-ray crystallography. Nature Communications. 2020; 11: 3202.

[10] Sencanski M, Perovic V, Pajovic SB, Adzic M, Paessler S, Glisic S. Drug Repurposing for Candidate SARS-CoV-2 Main Protease Inhibitors by a Novel *In Silico* Method. Molecules. 2020; 25: 3830.

[11] Veljkovic N, Glisic S, Perovic V, Veljkovic V. The role of long-range intermolecular interactions in discovery of new drugs. Expert Opinion on Drug Discovery. 2011; 6: 1263–1270.

[12] Singh P, Kumar D, Pal S, Kumari K, Bahadur I. L-amino-acids as immunity booster against COVID-19: DFT, molecular docking and MD simulations. Journal of Molecular Structure. 2022; 1250: 131924.

[13] Malla TN, Pandey S, Aldama L, Feliz D, Noda M, Poudyal I, *et al*. Vitamin C Binds to SARS Coronavirus-2 Main Protease Essential for Viral Replication. bioRxiv. 2021. (preprint)

[14] Drouin G, Godin J, Pagé B. The genetics of vitamin C loss in vertebrates. Current Genomics. 2011; 12: 371–378.

[15] Hoang BX, Shaw G, Fang W, Han B. Possible application of high-dose vitamin C in the prevention and therapy of coronavirus infection. Journal of Global Antimicrobial Resistance. 2020; 23: 256–262.

[16] Holford P, Carr AC, Jovic TH, Ali SR, Whitaker IS, Marik PE, *et al*. Vitamin C-An Adjunctive Therapy for Respiratory Infection, Sepsis and COVID-19. Nutrients. 2020; 12: 3760.

[17] Ivarsson, Y. pETM33_Nsp5_Mpro Addgene plasmid # 156475. 2021. Available at: https://www.addgene.org/156475/ (Accessed: 1 September 2021).

[18] Krishnamoorthy N, Fakhro K. Identification of mutation resistance coldspots for targeting the SARS-CoV2 main protease. IUBMB Life. 2021; 73: 670–675.

[19] Defelice SL. The nutraceutical revolution: its impact on food industry R&D. Trends in Food Science & Technology. 1995; 6: 59–61.

[20] Izzo R, Trimarco V, Mone P, Aloè T, Capra Marzani M, Diana A, *et al*. Combining L-Arginine with vitamin C improves long-COVID symptoms: The LINCOLN Survey. Pharmacological Research. 2022; 183: 106360.

[21] Li P, Wu G. Important roles of amino acids in immune responses. The British Journal of Nutrition. 2022; 127: 398–402.

[22] Chen L, Liu P, Gao H, Sun B, Chao D, Wang F, *et al*. Inhalation of nitric oxide in the treatment of severe acute respiratory syndrome: a rescue trial in Beijing. Clinical Infectious Diseases. 2004; 39: 1531–1535.

[23] Alvares TS, Conte-Junior CA, Silva JT, Paschoalin VM. Acute L-Arginine supplementation does not increase nitric oxide production in healthy subjects. Nutrition & Metabolism. 2020; 9: 54.

[24] Skwarecki AS, Nowak MG, Milewska MJ. Amino Acid and Peptide-Based Antiviral Agents. ChemMedChem. 2021; 16: 3106–3135.

[25] Abobaker A, Alzwi A, Alraied AHA. Overview of the possible role of vitamin C in management of COVID-19. Pharmacological Reports. 2020; 72: 1517–1528.

[26] Feyaerts AF, Luyten W. Vitamin C as prophylaxis and adjunctive medical treatment for COVID-19? Nutrition. 2020; 79–80: 110948.

[27] Holford P, Carr AC, Zawari M, Vizcaychipi MP. Vitamin C Intervention for Critical COVID-19: A Pragmatic Review of the Current Level of Evidence. Life. 2021; 11: 1166.

[28] Hazan S, Stollman N, Bozkurt HS, Dave S, Papoutsis AJ, Daniels J, *et al*. Lost microbes of COVID-19: *Bifidobacterium*, *Faecalibacterium* depletion and decreased microbiome diversity associated with SARS-CoV-2 infection severity. BMJ Open Gastroenterology. 2022; 9: e000871.

[29] Shu G, Yang Q, He C. Effect of ascorbic acid and cysteine hydrochloride on growth of Bifidobacterium bifidum. Advance Journal of Food Science and Technology. 2013; 5: 678–681.

[30] Kontermann RE. Dual targeting strategies with bispecific antibodies. Taylor & Francis. 2012; 4: 182–197.

[31] Hill JA, Cowen LE. Using combination therapy to thwart drug resistance. Future Microbiology. 2015; 10: 1719–1726.

[32] Jezovnik M, Poredos P. Oxidative stress and atherosclerosis. European Society of Cardiology. 2007; 6: 306–311.

[33] Grzegorowska O, Lorkowski J. Possible Correlations between Atherosclerosis, Acute Coronary Syndromes and COVID-19. Journal of Clinical Medicine. 2020; 9: 3746.

[34] Bogdański P, Suliburska J, Szulińska M, Sikora M, Walkowiak J, Jakubowski H. L-Arginine and vitamin C attenuate pro-atherogenic effects of high-fat diet on biomarkers of endothelial dysfunction in rats. Biomedicine & Pharmacotherapy. 2015; 76: 100–106.