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Article

QCT-Based 3CL Protease Inhibitor: Tollovir

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Abstract: Beginning in December 2019, a novel coronavirus known as SARS-CoV-2 has caused an international outbreak of respiratory illness termed COVID-19. This disease may cause mild to moderate respiratory illness. However, older individuals and those with underlying medical conditions like diabetes, cancer, and cardiovascular disease are more likely to develop serious infections (progressive pneumonia, multi-organ failure, and even death)[1]. The main target for antiviral therapy for COVID-19 is the 3CL protease (3CLpro, Main protease, Mpro, Nsp5). The 3CL protease is integral to viral replication and is often known as the "Achilles heel" of the SARS-CoV-2 virus[2]. Thus, NLC Pharma utilized a unique and novel Quantum Core Technology (QCT) technology to design a mechanism-based potent inhibitor against the SARS-Cov-2 3CL protease. 3CL Pharma has developed an oral anti-viral for COVID-19 called Tollovir. Biochemical assays have revealed that 40uM of Tollovir in PBS can achieve 90% maximum inhibition of 3CL protease. Furthermore, the oral anti-viral has completed clinical trial phase 2b/3. Tollovir has proven to be safe and significantly decreases mortality rates of hospitalized patients with COVID-19 infection.

Keywords: coronavirus; 3CL protease; drug target for coronavirus; SARS-CoV

1. Introduction

Coronaviruses are responsible for multiple novel diseases in the human population, ranging from acute respiratory infections such as SARS-CoV, SARS-CoV2, and MERS-CoV to viruses that cause the common cold like HCoV-229E and HCoV-NL63 [3]. SARS and SARS-CoV-2 belong to the Nidovirus group of positive-sense single-stranded RNA viruses. SARS first emerged in 2003, and recent genome sequencing studies revealed up to 89% sequence similarity to the SARS-CoV-2 genome.

Currently, there are very few approved therapeutic agents for coronavirus infection: Boosted Protease Inhibitors, which combine a protease inhibitor with a booster molecule to improve its antiviral activity and Pharmacokinetic properties, and natural inhibitors. Examples of boosted protease inhibitors are Nirmatrelvir/ritonavir (Paxlovid) (a combination of 3cl protease inhibitor and a booster that increases nirmatrelvir's levels in the body[4]; Molnupiravir (Lagevio) (it introduces errors into the viral RNA during replication, thus inhibiting the virus's viral growth and disrupting its function indirectly); Non -boosted Protease Inhibitors are currently going through clinical trials (GC343, which presently failed to show significant efficacy in large clinical trials for COVID-19 and VX-745: Another inhibitor with promising preclinical data, currently in Phase 2 clinical trials for COVID-19) [5].

Natural Inhibitors include plant extracts, marine natural products, and naturally accruing peptides: Extracts from various plants, like licorice root, green tea, and Andrographis paniculata, have shown potential for inhibiting 3CL protease in laboratory studies. However, their efficacy in humans remains unclear and requires further investigation[6]. Compounds isolated from marine organisms like sponges and algae have also exhibited 3CL protease inhibitory activity. Like plant extracts, more research is needed to assess their potential as antiviral agents[7]. Naturally occurring peptides derived from various sources, including food or microorganisms, have shown promise inhibiting 3CL protease. However, challenges remain in optimizing their stability and delivery for therapeutic use[8].

While synthetic inhibitors like Nirmatrelvir/Ritonavir and Molnupiravir have shown clinical efficacy against COVID-19, continued research is crucial to develop even more potent and broadly

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effective drugs. Natural inhibitors hold potential, but challenges often hamper their development in isolating, purifying, and optimizing them for therapeutic use. Combination therapies that target different aspects of the viral life cycle, including the 3CL protease, could be a promising strategy for combating coronaviruses and other noroviruses. The main target for antiviral therapy for COVID-19 is the 3CL protease (3CLpro, Main protease, Mpro, Nsp5). The 3CL protease plays an integral role in viral replication.

One of the most critical viral enzymes produced by the following two coronaviruses is nsp5 ($3CL^{pro}$). 3C-like protease ($3CL^{pro}$), the main protease (M^{pro}), refers to the cysteine protease's critical role in coronavirus gene expression and replicase processing. The following enzyme is conserved across all nidoviruses and is considered the "Achilles heel of the SARS-CoV-2 virus"[9].

Among coronaviruses, the 3CL protease within the same genus exhibits greater than 80% sequence identity. The greatest sequence conservation exists in and around the enzyme's active site. Sequence analysis of the SARS-CoV $3CL^{pro}$ and SARS-CoV-2 $3CL^{pro}$ revealed only a difference of 12 amino acid residues, allowing for approximately 96% sequence identity between the 3CL proteases for the two coronaviruses[10]. The 12 amino acid residues are: T35V, A46S, S65N, L86V, R88K, S94A, H134F, K180N, L202V, A267S, T285A, I286L. It is important to note only A46S was revealed to be close to the protease's active site but does not affect the enzyme's proteolytic activity. The other 11 variant positions are distant from the enzyme's binding site and do not affect the enzymatic activity. Furthermore, the superposition of the active site with and without ligands for the SARS-CoV $3CL^{pro}$ and SARS-CoV-2 $3CL^{pro}$ revealed high similarity[11].

Regarding the 3CL protease's function, the enzyme mediates the proteolytic cleavage of pp1a and pp1ab by processing at more than ten distinct cleavage sites with a QS cleavage site. This produces 16 non-structural proteins (nsp1 to nsp16)[12]. The polyprotein pp1a contains non-structural proteins 1-11, while polyprotein pp1ab contains the translated coding regions of nsp1-16, including the nsp12 RNA-dependent RNA polymerase and helicase. The proteolytic processing of the two viral polyproteins by 3CL protease is essential for viral replication because it creates mature and functionally active replication machinery for the virus. In other words, the proteolytic processing of the two viral polyproteins by 3CL protease acts as a critical regulatory mechanism in expressing the coronavirus replicase proteins. Thus, since the SARS-CoV outbreak of 2003-2004, research has demonstrated 3CL protease as a desirable antiviral medication target against SARS and other coronaviruse[13]s.

Drugs primarily targeting viral and bacterial diseases such as SARS and Coronavirus infection are prone to developing resistance to viral and bacterial mutations[14]. For example, as Paxlovid continues to be the only known treatment for the SARS-CoV-2 virus, many recent studies have noted potential resistance towards the 3CL-protease inhibitor due to mutations[15]. For instance, a study led by a virologist revealed that after 12 rounds of Nirmatrelvir treatment, the SARS-CoV-2 virus accumulated three mutations at amino acid positions 50, 166, and 167 in 3CLpro that reduced the virus' susceptibility to Nirmatrelvir by 20-fold. Furthermore, another study spotted potential resistance-conferring mutations at positions 50 and 166 in 3CL pro that, when combined, the virus was 80 times less susceptible to Nirmatrelvir[16]. In other words, viruses have an extremely high mutation rate, and these mutations can occur in any region of the viral genome. More specifically, mutations in the protease region of the COVID-19 genome have proven to create drug resistance and reduce the protease inhibitory effect of Paxlovid. Thus, it is essential to develop potent inhibitors that target the 3CL protease's mechanism to prevent drug resistance in the future. *The QCT technology overcomes the major underlying disadvantage of the structural drug design method: drug resistance.*

1.1. Catalytic Mechanism-Based Drug Design: QCT

Catalytic mechanism-based drug design, also known as Quantum Core Technology (QCT), stems from a combination of quantum calculations and physical organic chemistry developed by Dr. Dorit Arad. The novel methodology for drug design incorporates basic concepts regarding enzymatic mechanisms, such as the Koshland-induced fit model and the Molecular Orbital theory[17]. More specifically, the *QCT applies quantum mechanical calculations to protein structure data sets to systematically*

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ascertain the catalytic transition state of specific enzyme families to design and synthesize particular enzyme inhibitors. In other words, the novel technology considers both the conditions required for the enzymatic reaction and the core of the enzyme's catalytic site, known as the reaction center, to create potent and selective viral inhibitors[18]. One of the significant challenges in drug design is creating inhibitors that can both target and fit perfectly into the enzyme's active site. Thus, NLC Pharma utilizes the "Structural Mechanistic Enzymology" (SME) approach[19] to classify enzymes based on their enzymatic mechanism and protein structure to create inhibitors that react and form a covalent bond with the enzyme's reaction center, allowing the molecule to bind to the enzyme[20] tightly. Furthermore, Quantum Core Technology designs molecules that directly interact with the molecule and the enzyme's critical catalytic residue in the active site (the reaction center), activating the enzyme (triggering). In the enzyme's resting state, the catalytic residues cannot interact and activate the enzyme due to a lack of spatial alignment. When the substrate interacts with the enzyme, it forms the enzyme-substrate complex (ES complex), which consists of numerous non-covalent interactions between the active site residues and the substrate, thus increasing the ES binding energy[21]. The formation of the ES complex activates the catalytic machinery of the enzyme, inducing a change in the spatial conformation of the catalytic residues and allowing the catalytic residues to react among themselves chemically. As a result, the catalytic residue reacts with the substrate in a step called "triggering" and initiates the enzymatic reaction[22]. In application, QCT identifies the triggering reaction of each enzyme using *ab initio* quantum mechanical calculations[23]. The QCT drug development process can be broadly broken down into three stages: analysis, creation of core structures, and optimization. During the analysis stage, the main task is classifying enzyme families according to their quantum mechanism. This is followed by designing core structures based on previous cores of similar enzymes, mechanical analysis, known inhibitors, modeling, and screening of focused libraries[24]. The optimization stage can be performed in three forms: modeling and computation chemistry, virtual libraries, or combinatorial chemistry[25]. The goal of the optimization stage is to gain the potency and specificity of the molecule. Utilizing the Quantum Core Technology as a means for mechanism-based drug designs provides the pharmaceutical industry with a unique and efficient method to obtain novel core molecules that can serve as the basis for developing new drugs, raising the overall productivity in the sector.

2. Materials and Methods

2.1. SARS-Cov-2 3CL Protease:

Recombinant SARS-CoV 3CL Protease 50 UG E-718-050 from Doron Scientific, Israel.

2.2. Cloning and Production of SARS-Cov-2 3CL Protease with P132H Mutation:

A G-block of 3CL P132H was ordered from IDT. The fragment was inserted into the pET14b plasmid as an XbaI-BamHI fragment. pET14b-XopC was then utilized as a template vector. The plasmids were then purified from bacteria using the MiniPrep kit, NEB. The G-Block (total lug DNA) was re-suspended in 20ul TE, 50ng/µl pET14b-XopC, and then digested using restriction enzymes XbaI and BamHI (HF) in CutSmart buffer (10X) for 1hr at 37°C. We then ran samples in preparative agarose gel (1-2%) and excised the bands according to the specific size (G-block: ~1000bp, digested pET14b: ~4500bp). Extracted DNA from gel slices (Gel extraction kit, NEB). Ligation- A 'self' reaction was prepared with 13ul nuclease-free water instead of inserting DNA. The reaction was incubated overnight at RT. To create competent E. coli bacteria (XL-1 strain, prepared in-house, Benhar lab), a starter was made with a colony from the transformation plate that was inoculated into 4ml LB-amp (100ug/ml) and incubated ON at 37°C, 250RPM shaking. We then purified the plasmids from the bacteria (MiniPrep kit, NEB) using 3ml of culture. 1ml of the culture was stored at 4C. pET14b-3CL mut P132H: 80ng/µl. The plasmid was then sent for sequencing. In addition, a glycerol stock was prepared using 1ml of the culture mixed with 250ul of 80% glycerol and stored at -80°C. The plasmid was transformed into E. coli BL-21 strain (Rosetta, prepared in-house, Benhar lab): We then made a starter with a colony from the transformation plate, which we inoculated into 10ml LB-amp

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(100ug/ml) and incubated ON at 37°C, 250RPM shaking. We added Ampicillin to the LB medium (100ug/ml), and the medium was inoculated with 5mL of the overnight culture of E. coli BL-21 with pET14b-3CL mut P132H. The culture was grown at 37°C 250rpm, shaking until OD_{600} = 0.6. At this point, IPTG was added to a final concentration of 1mM (by directly adding the powder to the culture). The culture was incubated at 18°C overnight for induction of protein expression. In addition, we centrifuged bacterial cells (Sorvall, 500mL cups) at 4000rpm for 20min. The sup was discarded, and the pellet was stored at -20°C ON. Protein was separated in the FF HisTrap HP column (1mL, GE). Protein size: 35.6Kda. Protein Ext. Co: 0.966. All eluted fractions (including FT and washes) were quantified by nano-drop, and protein concentration was calculated (Ext. co: 0.966). SDS-PAGE analyzed all relevant fractions to verify the purity. Fractions with sufficiently high concentration (>1.2mg/mL) were mixed and loaded on the Zeba™ Spin Desalting column (Thermo Fisher, Cat# 89892) for buffer exchange. Storage buffer without maltose, DTT, and glycerol was prepared x2 (meaning 80 Trism 220mM NaCL, 4.4mM KCl) and used to wash and elute the protein from the Zeba column (according to the manufacturer's instructions). Following buffer exchange, the protein was quantified by a BCA protein assay kit (Thermo Fisher) according to the manufacturer's instructions. Storage buffer x2 was used as blank.

2.3. Bioactivity Assay for 3CL Protease

The following assay was utilized to determine the raw materials required to produce Tollovid—the assay tested for the inhibitory activity of various plant extracts against recombinant 3cl protease. Inhibitory activity (IC50) of plant extracts was performed using 488 (CBR2, by Cambridge UK) and 670 (Covidyte™ TF670) substrates. NLC provided all plant-based extracts and diagnostics. Thus, specific inhibitory activity cannot be attributed to particular aspects or impurities of the extract but rather to the entire plant. Any reference to Shikonin (active ingredient) in the following report originates from the names of the materials designated by NLC.

"Shikonin" refers to 5,8-dihydroxy-2-(1-hydroxy-4-methylpent-3-en-1-yl)naphthalene-1,4-dione and more particularly to the (R)-enantiomer thereof, which is found in the roots of Lithospermum erythrorhizon (purple gromwell), a plant used in Chinese herbal medicine (in which it is referred to as "Zicao") for treating a variety of inflammatory and infectious diseases. The (S)-enantiomer is known as "alkannin," a plant that has been used in folk medicine to treat abscesses and inflammations, and racemic mixtures are also known as "Shikalkin."

Shikonin has been reported to exhibit activities related to the treatment of cancer, inflammation, and wound healing, as well as antiviral activity against HIV type I, adenovirus 3 (AdV3), and hepatitis C virus (HCV) and distinct anti-inflammation mechanisms, such as inhibition of leukotriene B4 synthesis, suppression of mast cell degranulation, inhibition of neutrophil respiratory burst, alteration of phosphatidylinositol-mediated signaling, and blockade of chemokine binding to CCR-1 [Andujar et al., Planta Med 2013, 79:1685;] and reduction of interleukin-6, interleukin-8 and chemokine C—C motif ligand (CCL)20 production[26]

The controls for the following bioactivity assay were samples with no assay and samples with DMSO. A stock solution of 20mM was added to DMSO to prepare the sample based on the percentage of Shikonin provided by NLC. The solution then underwent serial dilutions in DMSO, all while light-protected. To prepare a buffer, enzyme solution of 150 ng final concentration, substrate solution of 1 M final concentration, and reagent buffer with DTT (RB+) were added. Extract dilutions were incubated with enzyme for 30 minutes in the dark. Percentage interference was calculated by 1 – [FU measurement of free fluorophore in RB in RB at the specific extract concentration divided by FU of no extract]. Normalized slope calculations were determined by slope x (1 + % interference).

2.4. Preparation of NLC-V

NLC-V is a liposomal formulation of Shikonin, 95% extracted from Lithospermum erythrorhizon gromwell root and Arnebia euchromatin extract. Patients were administered NLC-V through an oral capsule containing 10mg of Shikonin.

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3. Results

3.1. In-Vitro Assay Results:

40uM of XA ZB-201217 (in DMSO) displayed 100% inhibitory activity against 3CL pro (B), and 40uM of XA ZB-201217 (in DMSO) showed 94% inhibitory activity against modified 3CL (Figure 1).

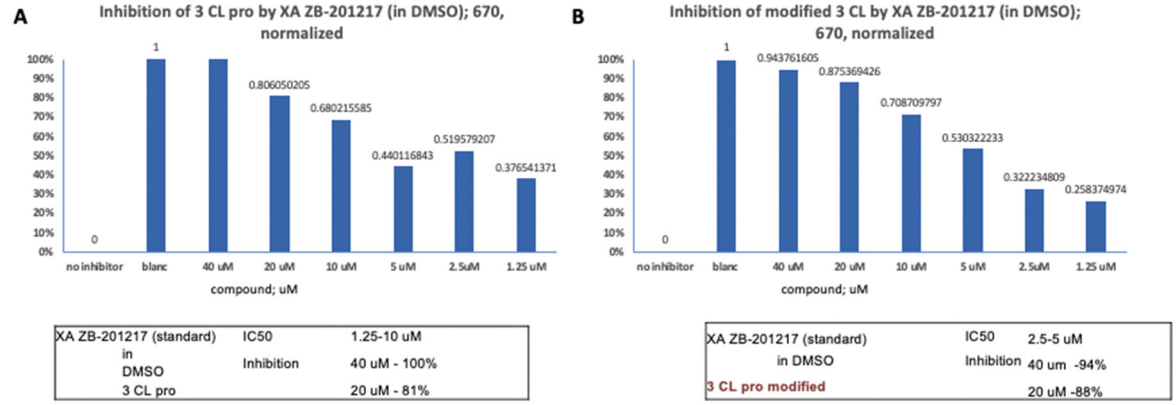
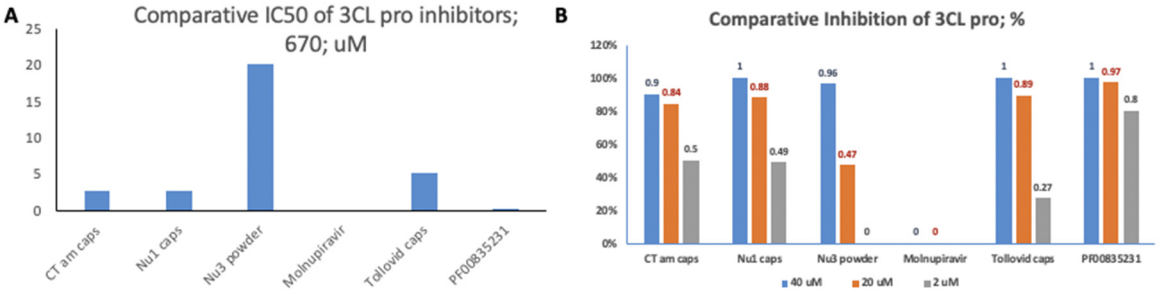


Figure 1. Inhibitory Activity of XA ZB-201217 (in DMSO). (A) 40uM of XA ZB-201217 (in DMSO) presented 100% inhibitory activity of 3CL pro. (B). 40uM of XA ZB-201217 (in DMSO) presented 94% inhibitory activity of modified 3CL.

In the assessment of 3CL pro inhibitors, Nu3 powder exhibited the highest IC50 value compared to the other inhibitors tested. Moreover, 40uM of Tollovid capsules and 40uM of Nu1 capsules demonstrated complete inhibitory activity against 3CL pro. The IC50 values of all 3CL pro inhibitors were also examined (Figure 2).



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3CL pro inhibitors	IC50 (uM)
CT am capsule	2.5
Nu1 capsule	2.5
Nu3 powder	20
Molnupiravir	0
Tollovid capsule	5
PF00835231	0.1
Shikonin	10
Tollovir	2.5

Figure 2. IC50 Values of 3CL Pro Inhibitors. (A). Nu3 powder had the highest IC50 value compared to other 3CL pro inhibitors tested. (B). 40uM of Tollovid capsules and 40uM of Nu1 capsules both presented 100% inhibitory activity against 3CL pro. (C). IC50 values of all 3CL pro inhibitors.

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At a concentration of 40µM, Tollovid in DMSO showed 100% maximum inhibition of 3CL pro. Similarly, at the same concentration, Tollovid in PBS demonstrated 100% maximum inhibition of 3CL pro. However, Tollovir in PBS, at a concentration of 40µM, presented 90% maximum inhibition of 3CL pro (Figure 3).

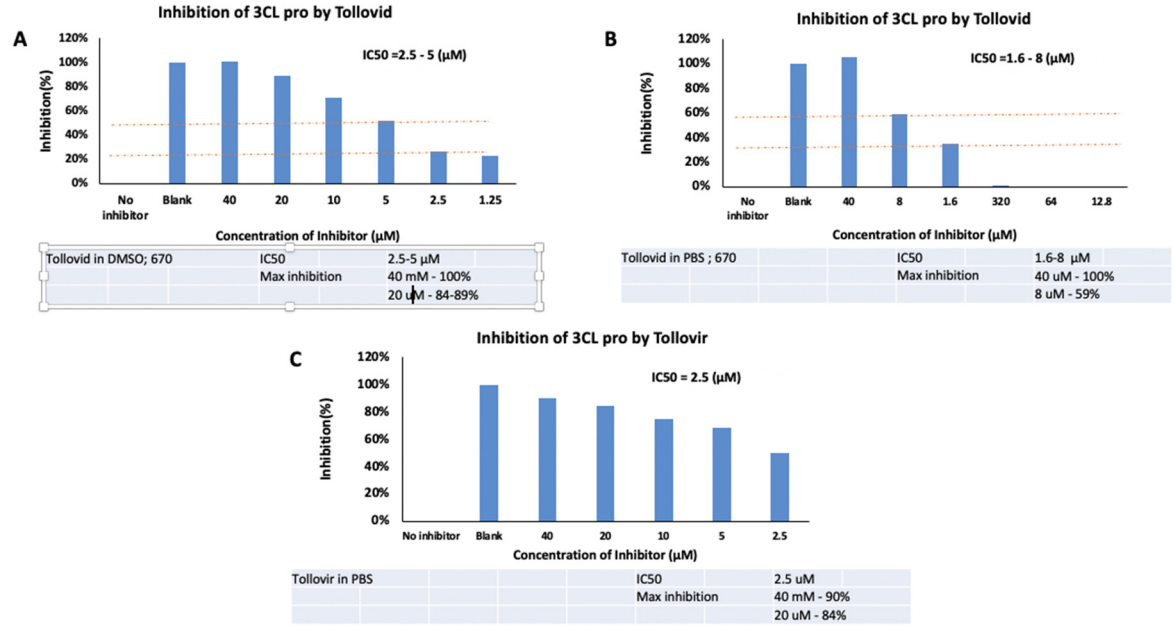

Figure 3. Inhibition of 3CL pro by Tollovid and Tollovir. (A). 40uM of Tollovid in DMSO presented maximum inhibition of 3CL pro of 100%. (B). 40uM of Tollovid in PBS presented maximum inhibition of 3CL pro of 100%. (C). 40uM of Tollovir in PBS presented maximum inhibition of 3CL pro of 90%.

3.2. Clinical Trial Results:

Tollovir is a highly potent dual mechanism 3CL protease inhibitor that can be utilized as a potential antiviral therapy for COVID-19. The essential compounds in the antiviral are NLC-EXT-1 and NLC-EXT-2. NLC-EXT-1 inhibits the 3CL protease and, as a result, prevents viral replication. On the other hand, NLC-EXT-2 inhibits cytokine storm, normalizing the immune function. The following study evaluates the safety and efficacy of NLC-V in patients diagnosed with COVID-19. NLC-V was administered to patients according to their weight. Patients under the weight of 70kg received two capsules four times a day, a total of 80mg NLC-V daily. On the other hand, patients above 100 kilograms received four capsules four times a day for a total of 160mg NLC-V daily. Patients weighing between 70 and 100kg received three capsules four times a day for 120mg NLC-V daily. The patients completed the treatment for ten days. The phase 2b/3 clinical trial was conducted at the Shaare Zadek Medical Center with hospitalized patients who confirmed SARS-CoV-2 infection between the ages of 40 – 90. The primary endpoint is the time to clinical improvement, defined as the time from randomization to either an improvement of two points on a seven-category original scale or discharge from the hospital, whichever comes first. The consenting subjects were randomly assigned to receive a standard of care with a placebo or a standard of care with Tollovir. The hospitalized patients received four capsules every day. The results of the phase 2b/3 clinical trial can be seen in Table 1 below.

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Table 1. Tollovir + SOC Vs. Placebo + SOC.

	Tollovir™(TLVR2) N = 11	Placebo N = 9	Difference (Tollovir vs. Placebo)
Time (days) to clinical improvement as measured by NEWS 2			
n	**11**	**9**	
Mean	**8.3**	**11**	**-2.7**
Median	**8**	**15**	**-7**
Time (days) in the hospital			
n	**11**	**9**	
Mean	**10.6**	**17.8**	**-7.2**
Median	**8**	**8**	**0**
Time (days) to discharge from the hospital			
n	**10**	**6**	
Mean	**9.7**	**8.3**	**1.4**
Median	**8**	**8**	**0**
No. of Patients Deterioration to Mechanical Ventilation, n(%)	**1 (0.09)**	**3 (0.33)**	**-24%**
No. of Deaths related to COVID-19 while on Treatment, n(%)	**0 (0.00)**	**2 (0.22)**	**-22%**
Total Deaths, n(%)	**1* (0.09)**	**3 (0.33)**	**-24%**
Need for supplemental oxygen, n(%)	**8(0.73)**	**8 (0.89)**	**-16%**
Time on supplemental oxygen			
n	**8**	**8**	
Mean	**3.8**	**5.8**	**-2**
Median	**3**	**7**	**-4**
C-Reactive Protein on Day 10			
n	**7**	**3**	
Mean	**3.44**	**14.79**	**-11.35**
Median	**1.16**	**9.98**	**-8.82**
Interleukin-6 (IL-6) at Day 10			
n	**4**	**3**	
Mean	**24.6**	**36.23**	**-11.63**
Median	**10.95**	**13.8**	**-2.85**
D-Dimer on Day 10			
n	**5**	**2**	
Mean	**2,55.8**	**29,554.00**	**-29,298.20**
Median	**779**	**29,554.00**	**-28,775.20**

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*** A patient had a stroke before receiving study treatment. The patient received the study treatment for ten days via a feeding tube and achieved COVID-19 clinical improvement as measured by NEWS2; nine days after stopping the study treatment (on day 19), the patient expired.**

4. Discussion

The Phase 2b/3 clinical trial revealed that 100% of hospitalized patients who received standard of care and Tollovir significantly improved health according to the NEWS2 scale. In comparison, only 66% of hospitalized patients who received standard of care and placebo showed improvement in their health. Furthermore, it is essential to note that the mortality rate was 0% for hospitalized patients who received Tollovir but a 22% mortality rate for hospitalized patients who received the placebo. In addition, the number of hospitalized patients who required oxygen support decreased after receiving Tollovir, and the time spent on mechanical ventilation also reduced significantly compared to hospitalized patients who received the placebo. The clinical trial results revealed a decrease in IL-6, CRP, and D-dimer levels for hospitalized patients treated with Tollovir compared to patients treated with the placebo. More importantly, neutralizing antibody levels increased for patients who received Tollovir compared to patients who received a placebo. Lastly, unlike Paxlovid (21), the renal and haptic biomarkers revealed no safety signals. Thus, based on the research and clinical results, Tollovir is safe and significantly decreases mortality rates for hospitalized COVID-19 patients. However, additional research is required to understand further the anti-viral and anti-cytokine therapeutic benefits of the botanical drug Tollovir.

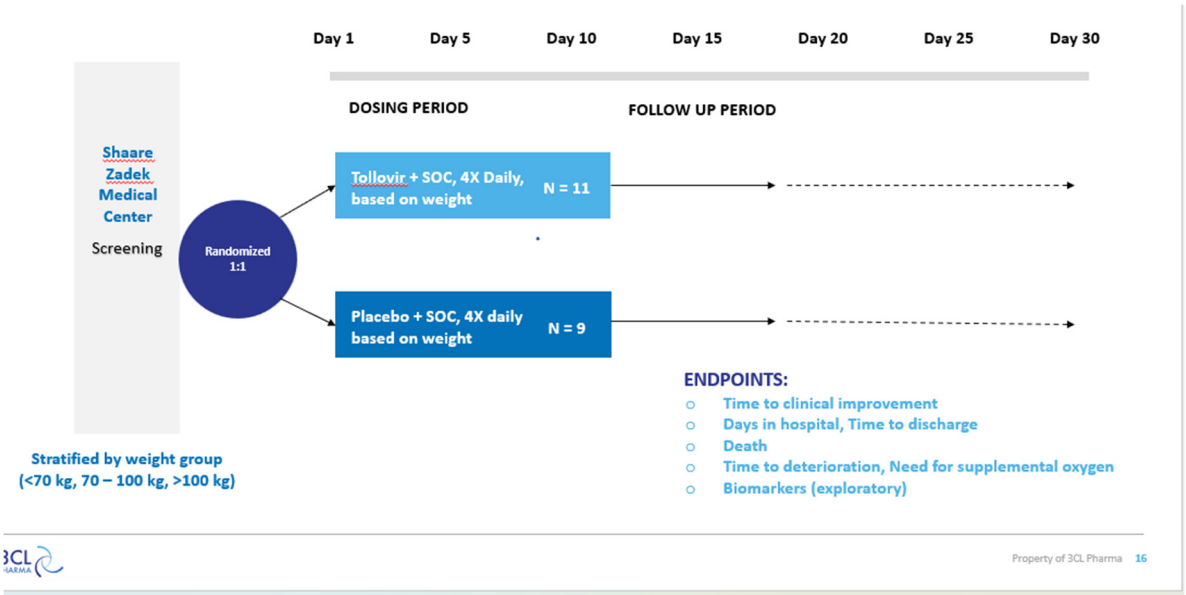

Figure 6. Tollovir Phase 2a Clinical Trial Design, Part 2.

5. Conclusions

Acute COVID-19 and Long-Haul Syndrome continue to threaten millions of lives nationally and globally. While much research focuses on developing an effective antiviral drug to target the viral 3CL protease, the study is focused mainly on creating therapeutic agents for COVID-19 using structure-based drug design. The major flaw with the following method is that it is prone to drug resistance and is not a long-term solution. Thus, 3CL Pharma has used QCT, a mechanism-based drug design, to create an oral antiviral to treat COVID-19. Tollovir has proven to be safe and decrease mortality rates in hospitalized patients.

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6. Patents

Patent number 11857517: Described herein are compounds of Formula I, wherein R1-R6 are as described herein, for use in the treatment of a coronavirus infection; a method of inhibiting a coronavirus 3CL protease by contacting the 3CL protease with a compound of Formula I; as well as methods pharmaceutical composition comprising a compound of Formula I and at least one phospholipid, wherein a weight ratio of the phospholipid(s) to the compound in the composition is in a range of from 10:1 to 1:10. Further described herein is a method of treating a coronavirus infection in a subject in need thereof, by administering to the subject at least one compound that exhibits at least two of inhibition of activity of a 3CL protease of the coronavirus; inhibition of inflammation in the subject; and inhibition of autophagy in the subject.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Funding: This research received no external funding.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Institutional Review Board Statement: https://my.health.gov.il/CliniTrials/Pages/MOH_2021-01-20_009687.aspx.

Conflicts of Interest: Dorit Arad is employed by 3CL Pharma LTD, an affiliate of Todos Medical USA.

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