Are Non-Structural Proteins From SARS-CoV-2 the Target of Hydroxychloroquine? An *in Silico* Study

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Abstract- COVID-19 is caused by SARS-CoV-2 which has structural and non-structural proteins (NSP) essential for infection and viral replication. There is a possible binding of SARS-CoV-2 to the beta-1 chain of hemoglobin in red blood cells and thus, decreasing the oxygen transport capacity. Since hydroxychloroquine (HCQ) can accumulate in red cells, there is a chance of interaction of this drug with the virus. To analyze possible interactions between SARS-CoV-2 NSP and hemoglobin with the HCQ using molecular docking and implications for the infected host. This research consisted of a study using bioinformatics tools. The files of the protein structures and HCQ were prepared using the AutoDock Tools software. These files were used to perform molecular docking simulations by AutoDock Vina. The binding affinity report of the generated conformers was analyzed using PyMol software, as well as the chemical bonds formed. The results showed that HCQ is capable of interacting with both SARS-CoV-2 NSP and human hemoglobin. The HCQ/NSP3 conformer, HCQ/NSP5, HCQ/NSP7-NSP8-NSP12, HCQ/NSP9, HCQ/NSP10-NSP16 showed binding affinity. In addition, the interaction between HCQ and hemoglobin resulted in polar bonds. Interaction between SARS-CoV-2 NSP and HCQ indicates that this drug possibly acts by preventing the continuity of infection.
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Introduction

In December 2019, an outbreak of pneumonia of unknown origin was reported in Wuhan City, Hubei Province, China, mainly linked to a seafood market. Studies of epidemiology and etiology later revealed that it is a new type of coronavirus, currently called SARS-CoV-2 (1). Due to the significant increase in the number of cases in China and other parts of the world, the World Health Organization (WHO) declared a global health emergency in January 2020 (2).

Coronaviruses (CoVs) of the family Coronaviridae are spherical viruses that have spike proteins protruding from the surface, resulting in the appearance of a crown, so the name coronavirus (3). There are in total four subfamilies of coronavirus, named alpha, beta, gamma, and delta, and SARS-CoV-2 is in the beta subfamily, along with MERS-CoV and SARS-CoV, possessing approximately 50% of the identity of the genetic sequence with the first and 70% with the latter (2,3). This subfamily, pathogenic to humans, has a simple-tape positive RNA genome encapsulated by a membrane.

SARS-CoV-2 has structural proteins such as spike surface, envelope, membrane, and nucleocapsid glycoproteins, which gives it a typical coronavirus structure (3-5). In addition, it has several non-structural proteins (NSP) essential for infection and viral replication; among them, is the polyprotein ORF1ab, encoded by the *orf1* gene that represents approximately 67% of the entire virus genome. The polyprotein ORF1ab is cleaved into 16 proteins-the NSP, which perform several functions for the success of viral infection, with activities of endonuclease, exoribonuclease, and polymerase, among others (6-8).

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The disease caused by SARS-CoV-2 was named COVID-19 in February 2020 by the WHO and has a broad spectrum of symptoms ranging from asymptomatic, mild upper respiratory tract disorders, and severe viral pneumonia with respiratory failure that can lead to death (9). Evidence indicated that the main form of transmission occurs by close contact with another infected person, mainly by droplets of saliva, sneezing, cough, phlegm, and also by the formation of aerosols, i.e. in orotracheal intubation procedures (10-12). Most affected patients are between 30 and 79 years old, reaching more men, and with the greater manifestation of symptoms such as fever, fatigue, dry cough, myalgia, and dyspnea (10,11). Other less common manifestations also recorded were headache, sputum production, hemoptysis, and diarrhea (10,11).

One of the theories proposed to explain the respiratory failure caused by COVID-19 is that SARS-CoV-2 binds to the porphyrin of the hemoglobin beta-1 chain, dissociating iron from porphyrin, and thus decreasing oxygen transport capacity (13). Studies also indicate that the virus can be coupled to the CD147 receptor in erythrocytes (14), making it a possible gateway for the virus to interact with hemoglobin. The dissociation of porphyrin iron generates an intense inflammatory response in lung cells due to the impairment of normal gas exchange function, which decreases oxygen saturation and results in "frosted glass" images on radiographs and tomographies (13,15).

The rapid spread of the disease around the world and the severity of some of the clinical manifestations aroused an intense search for drugs that could help control the pandemic and, among the possibilities, HCQ was suggested. HCQ is a medicine already used in the treatment of malaria, a disease caused by the protozoan *Plasmodium* sp., and also for the therapeutic control of some autoimmune diseases such as lupus, having its side effects already well known in the literature. In addition, it is known that HWH crosses the red blood cell membrane and can accumulate in this cell (16-20). Thus, this study aims to analyze, through bioinformatics tools, whether there is an interaction between HCQ and NSP of SARS-CoV-2, as well as, the relationship of the interaction of this drug with human hemoglobin and the implications for the infected organism and viral infection.

Materials and Methods

Three-dimensional structure files

The files of the three-dimensional structure of SARS-CoV-2 NSP and human hemoglobin (receptors) were obtained from the RCSB PDB-Protein Data Bank (https://www.rcsb.org), extension ".pdb" (Table 1). The ligand used for molecular docking was HCQ (ZINC1530652), whose file with extension ".sdf" was obtained from the Zinc15 database (https://zinc15.docking.org).

Table 1. Protein structures (receptors) used in molecular docking

Hemoglobin	NSP	PDB
Desoxihemoglobin A		2W6V
	NSP3 (RNA binding protein)	2W2G
	NSP5 (Replicase poliprotein 1ab)	6YB7
	NSP7 and NSP8 (cofators), NSP12 (RNA-dependent RNA polymerase)	6M71
	NSP9 (Replicase protein)	6W4B
	NSP10, NSP16	6W4H
	NSP15 (Endoribonuclease)	6VWW

Font: Authors (2020)

Ligand and receptors preparation

The HCQ.sdf file was converted to HCQ.pdb input file using the PyMol software. AutoDock Tools software was used to detect and choose HCQ torsion points, and the file was saved in PDBQT format. The NSP of SARS-CoV-2 and human hemoglobin were prepared using

AutoDock Tools software removing the ligands coupled to the proteins, removing crystallographic water, and adding hydrogens. After, the protein files were saved in PDBQT format. In these analyses, grid boxes with spacing equal to 1Å which covered the entire dimension of the protein structures were used.

Molecular docking

Molecular Docking is a molecular modeling approach that aims to predict the structure formed between two or more molecules, identify the essential interactions of amino acids and the selected ligands, predicting the modes of predominant bindings of this complex (21-23). The protein and HCQ files and the parameters of the grid box were submitted in AutoDock Vina, a molecular coupling and virtual screening software (24) for molecular docking, resulting in a report with the binding affinity of the generated conformers. The conformers and chemical bonds between receptors and ligands were analyzed using PyMol software.

Results

HCQ and human hemoglobin interaction

Under the conditions of molecular docking in this study, HWH to human hemoglobin (2W6V) using the basic side chain residue Arginine 141 (ARG 141) present in the alpha 2 chains utilizing two polar bonds, with distances of 2.3Å and 2.4Å. The affinity of the drug's binding to hemoglobin was -7.1 kcal/mol in its most energetically negative configuration, using random seed 1298683712 (Figure 1).

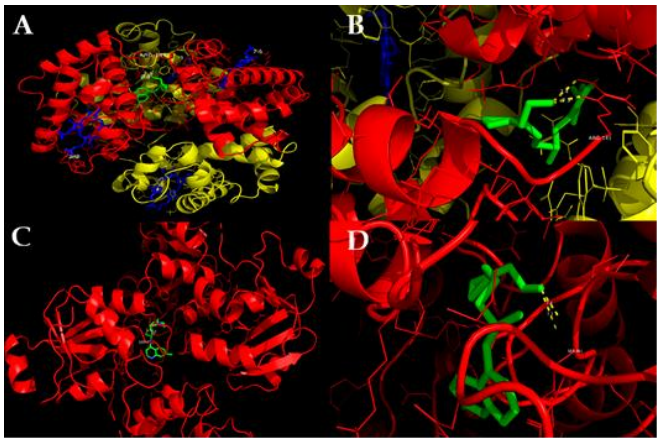

Figure 1. A: Panoramic view of the in silico interaction between HCQ (green) and alpha chains (red) and beta chains (yellow) of hemoglobin. The heme groups are in blue. B: Polar bonds (yellow dotted) between HCQ and ARG 141 of hemoglobin alpha chain 2. C: Panoramic view of the in silico interaction between NSP3 (red) and HCQ (green). D: Polar bond (yellow dotted) between SER 461 and HCQ

SARS-CoV-2 NSP and HCQ interactions

Interaction of NSP3 (2W2G) of SARS-CoV-2 with HCQ was observed utilizing two polar bonds (2.3Å and 2.4Å) with the Serina 461 hydrophilic lateral chain residue (SER 461). The binding affinity was -6.2 kcal/mol with seed 829874916 (Figure 1).

The interaction between NSP5 (6YB7) and HCQ showed a binding affinity of -6.8 kcal/mol using seed 319743316. Two polar links were formed between the drug and the residues Threonine 198 (THR198) and Aspartate 298 (ASP298) of The NSP5, with bind lengths equal to 3.5 Å and 3.4 Å, respectively (Figure 2).

The complex formed by NSP7, NSP8, and NSP12A (6M71) also interacted with HCQ through NSP12. The binding affinity between HCQ and the protein complex was equal to -6.3 kcal/mol. Three polar bonds were formed between the drug and the Threonine 141 (THR141), Lysine 47 (LYS47), and Histidine 133 (HIS133), with a length of 2.6Å, 2.3Å, and 3.6Å, respectively (Figure 2).

The interaction between NSP9 (6W4B) and HCQ showed a binding affinity of -5.5 kcal/mol with seed -2024625604. Three polar bonds were observed between HCQ and Valine 42 (VAL42), Arginine 40 (ARG40), and Serine 60 (SER60) residues from NSP9; the bind lengths were 1.9Å, 3.4Å and 3.2Å, respectively (Figure 3). NSP9 is a homodimer, so these interactions are observed in the two subunits (not shown). The conformer formed between the NSP10-NSP16 complex and the HCQ presented binding affinity equal to -6.3 kcal/mol with seed -1376471192, in which the drug performed a polar bond of 3.0 Å with Threonine 4364 (THR4364) (Figure

3). THR4364 residue belongs to NSP10.

HCQ interacted with SARS-CoV-2 NSP15 with a binding affinity equal to -6.3 kcal/mol, utilizing a polar bond of 2.9Å with Tyrosine 279 residue (TYR279) (seed -1286225520). NSP15 is a homodimer, possibly the TYR279 of the two subunits are involved in binding with distinct HCQ molecules.

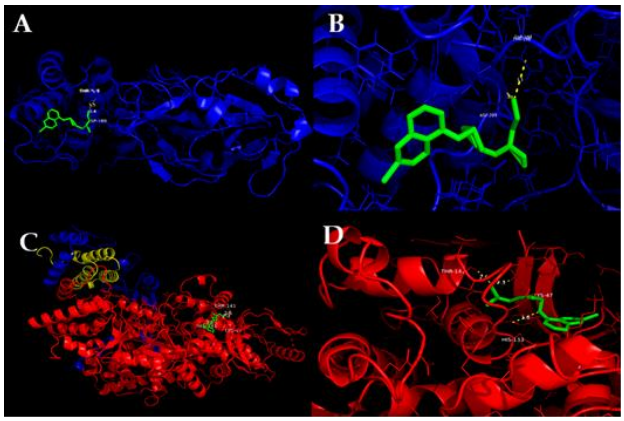

Figure 2. A: Panoramic view of the in silico interaction between NSP5 (blue) and HCQ (green). B: Polar bonds (yellow dotted) between ASP298 e THR198 and HCQ. C: Panoramic view of the in silico interaction between HCQ (green) and NSP7 (yellow)-NSP8 (blue)-NSP12 (red) complex. D: Polar bonds (yellow dotted) between THR141, LYS47, HIS133 (NSP12) and HCQ

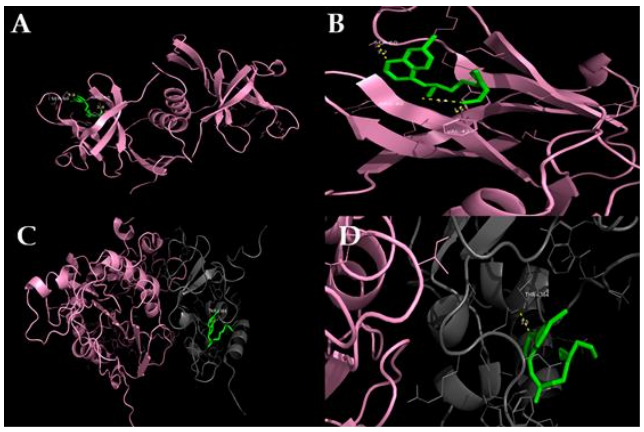

Figure 3. A: Panoramic view of the in silico interaction between HCQ (green) and NSP9 (pink). B: Polar bonds (yellow dotted) between VAL42, ARG40, SER60, and HCQ. C: Panoramic view of the in silico interaction between HCQ (green) and NSP10 (gray)-NSP16 (pink) complex. D: Polar bonds (yellow dotted) between THR 4364 (NSP10) and HCQ

Discussion

According to the theory that SARS-CoV-2 binds to the hemoglobin of erythrocytes and can reduce its affinity for O2 and the amount of hemoglobin itself, this may be one of the possible causes of the severity of the disease by decreasing the levels of O2 available in tissues, which may lead to hypoxemia (13); this would contribute to morbidity and mortality in patients affected by COVID-19.

Considering the ability of HCQ to accumulate inside the red blood cells (25), molecular docking was performed between this drug and human hemoglobin, which resulted in a significant binding affinity value of -7.1 kcal/mol with the alpha chain of hemoglobin. This indicates that HCQ binding could alter the conformation of this protein and affect its interaction with SARS-CoV-2. Thus, HCQ would prevent the decrease of the host's oxygen saturation, which would supposedly be a consequence of the capture of the iron atom of the porphyrin of the hemoglobin heme group by the virus after entering the red blood cells through the CD147 receptor (14).

The binding between HCQ and hemoglobin takes place near the 2.3-bisphosphoglicerate (BPG) binding site, a chemical compound that is found in red blood cells

in amounts equivalent to hemoglobin and has as main function to reduce hemoglobin affinity for oxygen facilitating the release of O2 to tissues. It is suggested that this binding could interfere with BPG binding, and thus in the O2 affinity. The interference in the regulation of hemoglobin affinity by O2 would make it difficult for oxygenation of tissues and may cause a clinical picture in the patient of hypoxemia, which could contribute to the worsening of the prognosis of COVID-19.

NSP3 is a large non-structural protein with varied functions in viral replication and main action in the entry of SARS-CoV-2 into cells of the human organism (26). It is discussed that this protease is one of the main differences between the current coronavirus and the others that were once responsible for outbreaks and epidemics, such as SARS-CoV (2002) and MERS-CoV (2012). Another important aspect of the expression of this protein, which occurs in macrophages activated by Interferon-γ (IFN-γ), is a prolonged inflammatory condition, which also involves the Angiotensin Converter Enzyme 2 (ACE2), also activated by IFN-γ (27).

Molecular docking analysis performed between HCQ and NSP3 indicated that this drug can interact with this protein through a serine residue. The SER461 is located in the ADP-ribose phosphatase domain of NSP3, which is a homodimer, near the interface between the subunits that compose it. Because it is a homodimer, possibly two HCQ molecules are necessary for a functional activity on NSP3.

The activity of the ADP-ribose phosphatase domain of NSP3 correlates with virulence and the ability of the virus to evade the host's innate immune response (28). Recently it has been suggested that the activity of ADP-ribose phosphatase of NSP3 on other proteins may be related to cytokine storm syndrome observed in severe cases of COVID-19 (27). Thus, the binding of HCQ could block this catalytic activity of NSP3, preventing cytokine storm syndrome and, consequently, improving the clinical prognosis of COVID-19.

NSP5, also known as 3-chymotrypsin-like cysteine protease (3CLpro), is one of the most important viral proteins because it is essential for the life cycle and replication of the virus (30). NSP5 plays a central role in the processing of viral proteins, and cleavage of a viral polyprotein in 11 different sites, besides being essential for the replication of SARS-CoV-2, so its inhibition may prevent the progression of infection in the human organism (29). This protease has been studied as a possible target for drugs in the treatment of viral diseases for almost 20 years, since the SARS-CoV epidemic in 2002 and, given the genomic similarity between the two viruses, it is estimated that it may also be one of the targets in the current pandemic. Since no human protease with characteristics similar to 3CLpro is known, it is unlikely that a drug that inhibits it is toxic to the organism (30).

The result of molecular docking with the anti-malarial drug revealed that this drug can interact with NSP5 through two polar bonds with THR198 and ASP298 residues. NSP5 is responsible for processing viral polyproteins that participate in the coronavirus replication process. A common feature of NSP5 of different coronaviruses is its ability to cleave the human DCP1A enzyme. DCP1A is an mRNA pickling enzyme, its activity makes mRNA unstable, so it has an anti-viral activity when acting in coronavirus RNA. NSP5 also cleaves an essential modulator of the transcription factor NF-kB and participates in a mechanism of inhibiting the production of IFN-γ and its signaling. By inhibiting the DCP1A enzyme and the IFN-γ production, NSP5 assists in the process of immune evasion of coronavirus (31). Possibly, the blockade of NSP5 could help in the reduction of the cytokine storm, one of the major problems in the fight against COVID-19 and the severity of the disease, which could be a potential therapeutic target.

Like NSP5, NSP7 and NSP8 are non-structural proteins of SARS-CoV-2. NSP7 and NSP8 form a single hollow hexadecameric structure, consisting of eight copies of NSP8 firmly joined by eight copies of NSP7, similar to a cylinder, and act as cofactors of NSP12 (32). Studies suggest that the hexadecameric structure is a general component of the Coronaviridae family and may be essential to bind and track the RNA polymerase enzyme (RdRp) of SARS-CoV-2, which confers efficiency in coronavirus genome replication (32). RdRp, or NSP12, is a central key factor in the viral replication and transcription process (33), and has an N-terminal extension with a kinase-analogous fold and binds to two NSP8 cofactors, a common structure of viral polymerases (34,35). There is experimental evidence for SARS-CoV that the NSP7 and NSP8 complex activates and confer processability to the RNA synthesis activity of NSP12-RNA-polymerase (35,36). The RNA polymerase complex is formed by the NSP7-NSP8 heterodimer complex, together with the NSP12 and an NSP8 monomer complexed to NSP12 (33).

Molecular docking analysis between HCQ and the complex formed by NSP7, NSP8, and NSP12 indicated that the drug was able to interact through three polar bonds. Although a considerable affinity interaction between HCQ and the complex has been identified, the

functionality of this interaction has not yet been elucidated. Recent studies suggest that Chloroquine is a zinc ionophore, increasing intracellular levels of this ion, which at high concentrations is harmful to viral replication because it inhibits RNA-dependent RNA polymerase (37,38). It is suggested that HCQ should act similarly to Chloroquine about zinc (39).

Another non-structural protein of SARS-CoV-2 analyzed in this study was the replicase protein NSP9, a homodimer that can participate in viral replication by acting as a single-stranded RNA-binding protein of the virus (40). There is possible evidence in the literature of some points of mutations in NSP9 that block viral replication (41,42). Some studies have also pointed out a possible interaction of NSP9 with NSP8 in the SARS-CoV, in which the NSP9 dimer may have a possible protective action for the nascent single-stranded RNA that emerges from the channel of the NSP7-NSP8 complex, and which do not yet have a stable secondary structure (31,43).

Through molecular docking, it was possible to observe that HCQ was able to interact with NSP9 through three polar bonds with the residues VAL42, ARG40, and SER60. Like NSP3, NSP9, because it is a homodimer, two HCQ molecules would probably be required for a desired functionality in NSP9. Although there are still no antiviral drugs focused on NSP9, it could be a potential target for treatment since a deficiency in NSP9 activity implies a deficiency in viral replication and consequently infection progress. Due to the ability to interact with NSP9, despite the many controversies, HCQ could be a possible candidate for treatment studies.

According to some studies, SARS-CoV-2 NSP16 is an enzyme whose structural stability depends on the interaction with NSP10; in addition, NSP10 functions as a stimulating factor for performing its methyltransferase activity (44,45). The NSP16-NSP10 complex is important for the stability of viral RNA, for the translation of proteins, and for the evasion of the host immune system (44,46).

In a study to evaluate mutations in the SARS-CoV-2 genome, it was observed that some viral proteins such as NSP10 did not accumulate mutations (47). Thus, the interface between NSP16-NSP10 may be an important specific therapeutic target to eliminate the new coronavirus (44), because it is a conserved region. A virtual screening study showed that the drugs capable of interacting with the NSP16-NSP10 methyltransferase complex were the antiviral Telbivudine, the broad-spectrum antibiotic Oxytetracycline, methyl gallate, which has anti-inflammatory potential, 2-deoxy-glucose that interferes with glucose and dafnetin metabolism, a type of coumarin (48). In this study, we observed that HCQ interacted with NSP10 through THR4364 residue by a polar bond. The connection is close to the interaction interface region of the NSP16-NSP10 complex; a conformational alteration may be transmitted throughout the complex, interfering with its stability. There is also the possibility of HCQ binding to the NSP10 before the formation of the NSP16-NSP10 complex, which would compromise the essential methyltransferase activity, compromising the continuation of the SARS-CoV-2 infection cycle.

One of the peculiarities of SARS-CoV-2 is the immune suppression capacity contributing to the process of viral pathogenesis (49). NSP15 of SARS-CoV-2 has the potential to antagonize the production of IFN-γ (49), similar to the monomeric NSP15 of the other coronavirus family consisting of an N-terminal domain, a median domain, and a catalytic domain (50). The literature indicates that some of the drugs that bind to NSP15 were farneceno (a terpenoid compound) and farnesol (an isoprenoid lipid) (51). In this study, it was observed that HCQ interacted with NSP15 through TYR279 residue. Because it is a homodimer, probably two HCQ molecules would be necessary to obtain a desirable functionality. Once binding to NSP15, it is suggested that the immune suppression capacity by SARS-CoV-2 becomes deficient.

For molecular docking values lower than -6.0 kcal/mol were considered significant for binding affinity (25). Therefore, non-structural proteins NSP3, NSP5, NSP15, and complexes NSP7-NSP8-NSP12 and NSP10-NSP16 showed significant values of affinity of interaction with HCQ, as well as hemoglobin.

According to the theory that SARS-CoV-2 can invade red blood cells through the CD147 receptor and that HCQ can accumulate within this cell, this drug has the potential for therapeutic studies against COVID-19, since *in silico*, results suggest that this drug is capable of interacting with several viral NSP and thus, HCQ could interfere with mechanisms essential to viral action. However, the ability of HCQ to interact with hemoglobin promotes the hypothesis of worsening the symptoms of COVID-19. The findings in this study may support the controversial functionality of HCQ in patients with distinct clinical symptoms of COVID-19. Maybe, the drug has therapeutic functionality in immunocompetent individuals with clinical symptoms of lesser severity; administration of the HCQ to individuals with more severe conditions, such as those with the severe acute respiratory syndrome, could aggravate the condition due to interaction with hemoglobin. Therefore, when acting

on NSP, the therapeutic action of HCQ could not be effective since mechanisms such as cytokine storm could have already been activated by the course of infection.

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