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RESEARCH ARTICLE

Anti-malarial Drugs are Not Created Equal for SARS-CoV-2 Treatment: A Computational Analysis Evidence

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ARTICLE HISTORY

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Abstract: ***Background*:** The evolution of the pandemic has burdened the national healthcare systems worldwide and at present, there is no preferred antiviral treatment for COVID-19. Recently, the SARS-Cov-2 protease structure was released that may be exploited in *in-silico* studies in order to conduct molecular docking analysis.

***Methods*:** In particular, we compared the binding of twoantimalarial drugs, already in use, (*i.e*. chloroquine and hydroxychloroquine), which showed some potential clinical effects on COVID-19 patients, using ritonavir, lopinavir and darunavir as positive control tree antiviral recognized compounds.

***Results*:** Our results showed that hydroxychloroquine but not chloroquine exhibited a significant binding activity to the main protease similar to that possessed by protease inhibitors tested for other viral infections.

***Conclusion*:** Our data suggest that hydroxychloroquine may exert additional direct antiviral activity compared to chloroquine. In the absence of clinical studies comparing the efficacy of these two compounds, hydroxychloroquine may offer additional effects and may be considered as the first choice.

Keywords: COVID19, protease inhibitor, SARS-CoV-2, computational analysis, Anti-malarial, hydroxychloroquine.

1. INTRODUCTION

The evolution of the pandemic has led to a lot of efforts by the national healthcare systems worldwide [1]. At the time of writing this article, the pandemic seems to have been overcome in China, but Italy and other western countries are still seriously affected . As of April 10, 2020, a total of 96877 confirmed cases have been reported in Italy, and 18279 deaths have been reported. At present, there is no preferred antiviral treatment for COVID-19 and for this reason, it is necessary to develop effective therapeutic protocols for the disease and its possible complications. In this regard, various possible drugs can be considered as treatment options such as vaccines [2], monoclonal antibodies [3], oligonucleotide-based therapies [4], peptides, *etc*. [5]. However, new drugs will require months and years to develop; in the meanwhile, it is strictly necessary to repurpose existing agents, exhibiting antiviral activity and either approved or under development development for treating different infections [6, 7].

The sulfate and phosphate salts of chloroquine are both commercially available as antimalarial drugs. Hydroxychloroquine has also been used as an antimalarial, in addition, it is now broadly used in autoimmune diseases such as lupus and rheumatoid arthritis [8]. Of note, chloroquine and hydroxychloroquine are considered to be relatively safe, so that therapy at home is not unrecommended. In particular, attention is now focused on hydroxychloroquine, with special regard to possible combinations with antibacterials used as adjuvant therapy in SARS-CoV. However, chloroquine treatment has been associated with serious adverse reactions, such as cardiovascular disorders [9]. Therefore, chloroquine and hydroxychloroquine should be administered under strict medical observation and prescription. Chloroquine is known to block virus infection and reduce virus/cell fusion by increasing endosomal pH, and in addition, it interferes with the glycosylation of cellular receptors of SARS-CoV. In this regard, previous data on Vero E6 cells demonstrated chloroquine to be effective in various stages of viral infection, including both entry and post-entry stages of the 2019-nCoV infection [10]. Furthermore, besides its antiviral activity, chloroquine possesses an immune-modulating activity, which may further enhance its antiviral effect *in vivo*.

Similarly, hydroxychloroquine is also licensed for the chemoprophylaxis and treatment of malaria as a disease-modifying antirheumatic drug [8]. Previous data suggest that prophylaxis with hydroxychloroquine may have good clinical efficacy on SARS-CoV-2 infection and ameliorate viral shedding [11].

Following administration, both chloroquine and hydroxychloroquine are adsorbed and rapidly dealkylated *via* cytochrome P450 enzymes (CYP) into the pharmacologically active metabolites, desethylchloroquine and bisdesethylchloroquine, which are responsible for the extended pharmacological actions and increased toxicity [12].

Finally, additional potential therapeutic agents that may exhibit direct effects on viral infection are the inhibitors of protease (*i.e.* lopinavir), representing a key target in viral polyprotein processing. Interestingly, early administration of antiviral drugs after symptom onset can reduce the spread of the infection to others by reducing viral shedding in the respiratory secretions of patients and targeted prophylactic treatment could further reduce the risk of infection [13].

The aim of the present study was to model *in silico* the potential inhibitor activity of antiviral and antimalarial agents on virus

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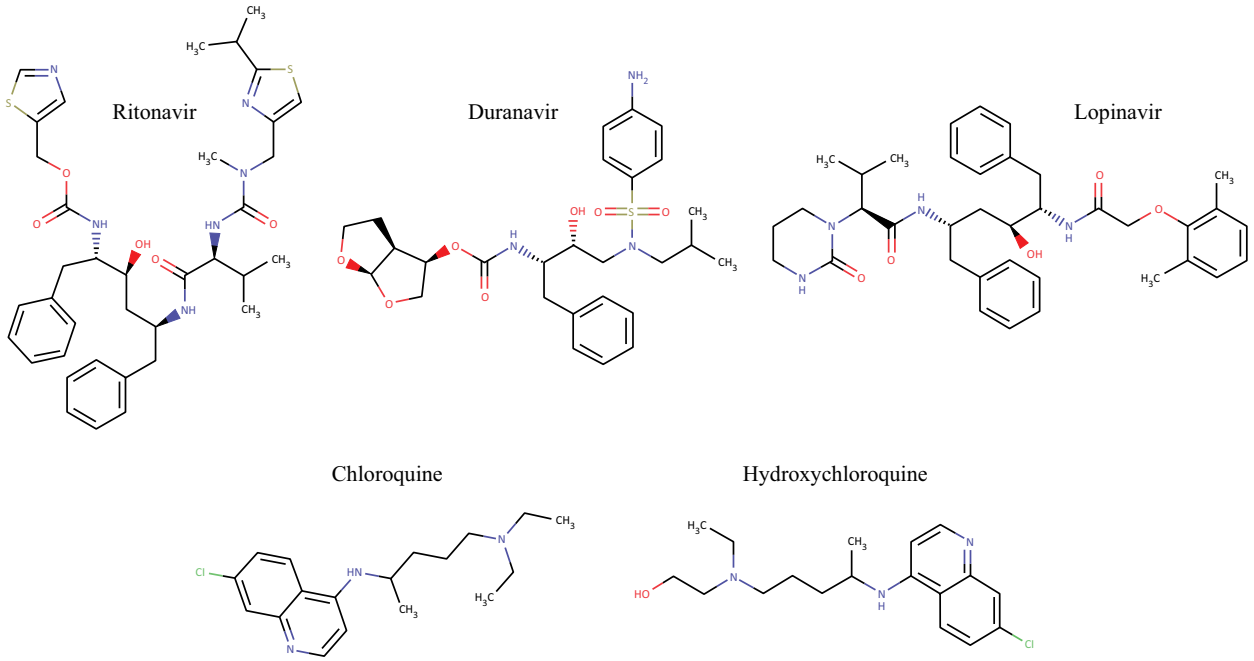

Fig. (1). 2D Representation of molecules used for docking studies: ritonavir, darunavir, lopinavir, chloroquine and hydroxychloroquine.

protease in order assess their potential clinical use and provide a criterion of choice in the absence of reliable available clinical trial data.

2. MATERIALS AND METHODS

The docking studies were performed according to the following protocol. All compounds were built and energy minimized with "Flare preparation ligand" [14]. The protease coordinates were downloaded by protein databank with the code 6Y84 that possesses a resolution of 1.39 angstrom. According to the literature, the binding site was identified in a specific region named TAEDMLN. The nearest amino acids and right charges were calculated with "Flare protein preparation". Docking calculations were performed with Flare in "Accurate but Slow" modality, which applies an extra precision type of calculation, using more rigorous sampling and scoring algorithms to increase the accuracy and reliability of predictions. Flare in addition to the dG, provides an accurate estimation of the free energy of protein-ligand binding, an additional extra-score value, called VS-score, which provides an estimate of the efficiency of the ligand in the case of assessment of activity/inactivity in virtual screening experiments.

The best docking poses were refined by the ligand-protein complex energy minimization using Flare. Finally, to improve the stability of each complex, short (1ns of production) molecular dynamic runs were performed at constant temperature, followed by a quick minimization of all the atoms involved in the binding site (T = 300 K; P = 1.01325 bar; ts = 2.0; cutoff = 10.0 Å). Molecular dynamics were performed using NAMD software and input files were prepared by tLeap, subprogram of Amber16 and Ambertools17 with 'ff12SB', 'ff99SBildn' and 'gaff' forcefields [15, 16]. Ligand charges were obtained using Gaussian 09. tLeap was also used to neutralize the complex charge. For water box creation, a pre-equilibrated water box (TIP3PBOX) implemented in tLeap was used. In an initial phase, protein and ligand were fixed to obtain a good merge. After this initial step, all components were released slowly (backbone and ligand in a first phase and later α-carbon) in a periodic boundary condition. This protocol permitted us to obtain a complete confirmation of the H-bonds involved and analyze the ligand-receptor complex in the binding site more accurately. All conformers that show lower energy scoring functions values and a good RMSD profile were selected and analyzed to identify the most probable conformers interacting with the binding site.

3. RESULTS

In order to evaluate affinities of potential drug candidates to counteract COVID-19, docking studies of selected compounds were accomplished using the main protease of the SARS-Cov-2 virus, published on the protein data bank in March 2020 (PDB ID 6Y84; resolution: 1.39 Å).

There are several aminoacidic residues that might play a relevant role in the enzyme function, as shown by Chen *et al.* [17]. The catalytic domain is located between residue 41 and residue 145, specifically from amino acids 45 to 51 (TAEDMLN). Starting from this information, a lot of antiviral drug combinations have been analyzed to find the suitable one able to slow down the replication of COVID-19. Computational docking is widely used to study protein-ligand interactions, in general, for drug discovery and development. Docking studies are actually used to predict the bound conformation and binding free energy of small molecules to the target. The three compounds that are involved in these studies, as a positive control, respectively darunavir, lopinavir and ritonavir, were prepared for docking using Flare by Cresset (Fig. **1**) [14, 18].

The grid was created large enough to contain the above-mentioned amino acids and all others involved in the putative docking site. During this process, the protein was considered capable of modifying only amino acids in the grid and the ligand . The docking protocol gave information for the evaluation of the binding energy prediction and virtual screening binding energy. The docking results highlighted the importance of the formation of different and stable hydrogen bonds that involved one or more amino acids in the binding (Table **1**).

In particular, in our study, ritonavir seems to be the best compound as it links the protease binding site with a good dG and VSscore (-9.791). This is confirmed by the H-bond established between the nitrogen of the unsubstituted thiazole moiety and the backbone amino group of Thr190. A number of bonds involve three carbamide portions that bind the amino groups of Gln189, His41 and the carboxyl group [C=(O32) -- NH] of Glu166. Moreover, there is a formation of 2 H-Arene bonds with Asn142 and

Table 1. Results of the docking studies and compounds characteristics.

Compounds	MW	LogP	TPSA	dG	VSscore	no. H-bond
Ritonavir	628.8	4.7	120	-9.791	-11.206	9
Lopinavir	720.9	6.3	145.8	-8.717	-9.913	6
Darunavir	547.7	2.8	140.4	-7.576	-10.199	7
Chloroquine	320.9	4.8	7.2	-7.927	-8.499	2
Hydroxychloroquine	336.9	4.2	9.2	-7.865	-9.134	3

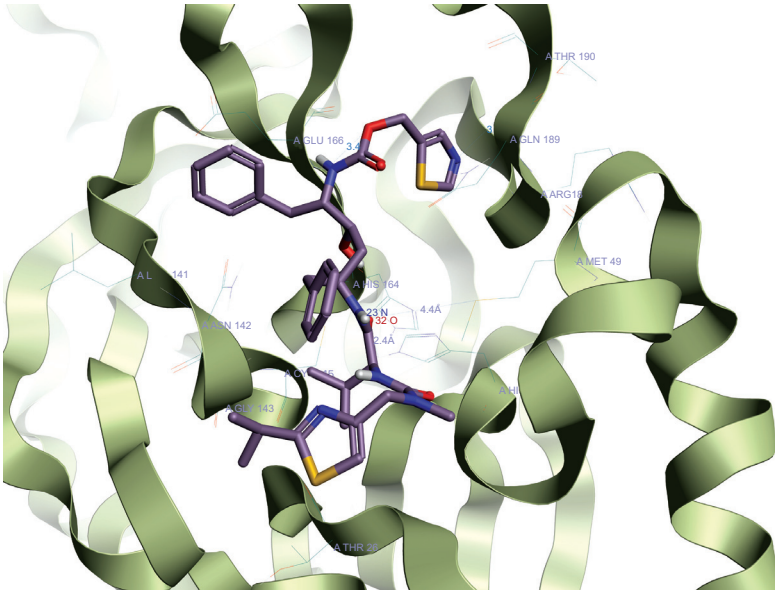

Fig. (2). Docking position of ritonavir in the main protease of SARS-COVID-19 virus binding site. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Glu166. In addition, Met49 is likely to bind with an NH-S bond with N23. All these bonds remain stable after the 1ns of dynamic simulation (Fig. **2**).

Lopinavir maintains a high dG (-8.717) but lacks in the VSscore value, probably because it involves a portion of the binding site rather different and water exposed by the unsubstituted aromatic portion, in comparison with those occupied by ritonavir. In fact, here, it looses arene interactions but makes many stable hydrogen bonds between the dimethylphenoxy portion and Gly143. Interesting ones are the H-bonds between N12 and Asn142 and between N27 and Gln189. All of these bonds maintain throughout the dynamic simulation (Fig. **3**).

On the other hand, darunavir exhibited different features. In fact, it demonstrates unsatisfactory binding energy prediction value (-7.576) despite a high value of VSscore (-10.199). Moreover, when any bond is lost during the dynamic equilibration, it demonstrates that it is not stable enough in the binding site. The maintained bonds are, for example, between the hydroxyl group and Gln189, like lopinavir and ritonavir, demonstrating that this amino acid is important for binding. Another H-bond involves the sulphonyl group and Glu166, like ritonavir. In addition, it is present a pi-pi stacking between the aromatic portion of darunavir and His41 (Fig. **4**).

Most of the above mentioned interactions are consistent with the data reported in the literature. The relevance of the interaction with Cys145, His41 and Asn142 amino acids or the involvement of Glu166 and His164 were also confirmed by Muramatsu T. *et al.* [19].

Docking studies on two of the evaluated compounds showed that the interaction of ligands with His41 and Cys145 amino acid residues is critical for good positioning of the ligand in the binding site, as these amino acids give the ligand the right disposition in the binding site and the highest binding energy. Moreover, the phenyl portion is important, because it is able to form pi-pi stacking with some tyrosine residues [20, 21].

Considering the interactions of the compounds that classically have a protease as the primary target of attack on the virus, chloroquine and hydroxychloroquine compounds have an interesting positioning inside the binding pocket. In fact, due to the formation of H- bonds, they have the ability to interact with some essential amino acids that we have already analyzed for the previous compounds. Of course, the number of bonds to H- that these two molecules, and in particular chloroquine, form, are considerably lower than the other compounds. Despite this, the bonds that are formed are stable and probably define, in a stronger way, the essential amino acids for an irreversible action on viral proteases.

Due to their chlorine atoms, chloroquine and hydroxychloroquine are capable of forming strong halogen bonds, with the same amino acid, Glu166. In addition, the N12 of both also interacts with the His164 backbone. Both have a useful steric size given by the diethyl and ethylamino ethanol portions, respectively. Naturally, the additional feature of hydroxyquinoline is certainly the hydroxylic portion, capable of binding the sulfur of Met165 very strongly through a hydrogen bond. The molecules also undergo an improvement in maintaining their arrangement by the Ar-Ar interface between the quinoline rings and Phe140 (Fig. **5**). In conclusion,

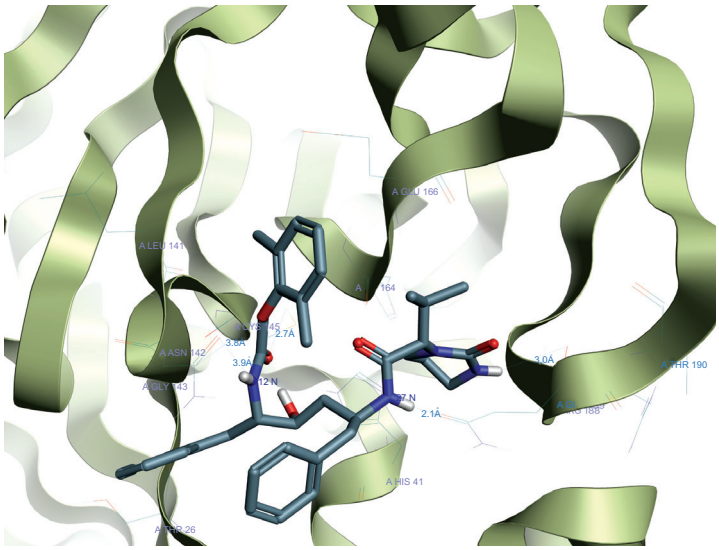

Fig. (3). Docking position of lopinavir in the main protease of SARS-COVID-19 virus binding site. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

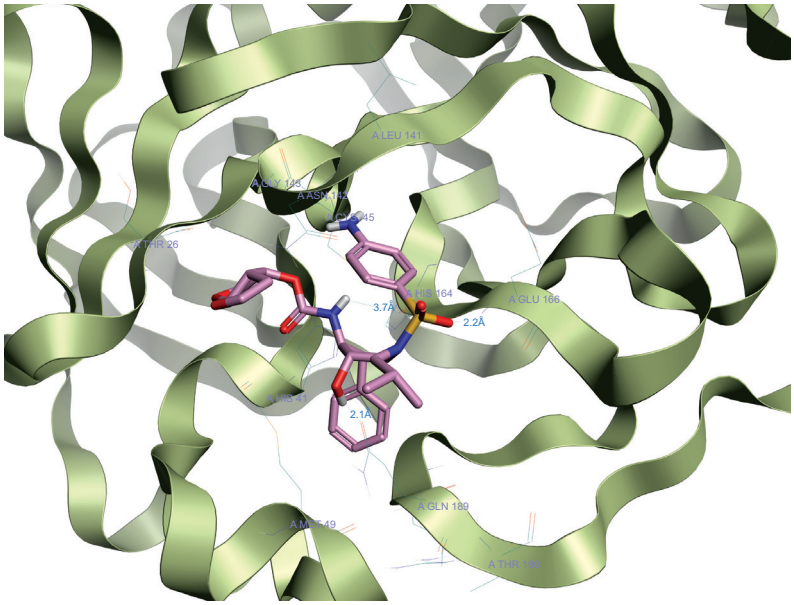

Fig. (4). Docking position of Darunavir in the main protease of SARS-COVID-19 virus binding site. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

we can surely add that the Met165 and His 164, with their particular arrangement inside the receptor pocket, forming almost a 180 degrees angle between their free portions, are among the most interesting amino acids for inhibition of the action viral.

4. DISCUSSION

Unfortunately, no regulatory agency-approved and specific treatments for any human CoV infection are so far available; therefore, the off-label antiviral strategies such as protease inhibitors and antimalarial agents are used in various stages of the disease [22]. In particular, *in vitro* studies have shown that SARS-CoV could be inhibited by LPV and since the EC50 of LPV is acceptable, it may represent a possible option for COVID-19 [23]. Consistently, two retrospective, matched cohort studies of SARS patients showed that LPV/r might have a significant impact on the clinical outcome in the early stage of the disease [24]. As far as the off-label use of antimalarial agents is concerned, the *in vitro* antiviral activity is known since the late 1960s [25-27]. There is also evidence that ritonavir and lopinavir might exert a certain inhibitory activity on the SARS-Cov2 protease [28]. This is, in a way, consistent with numerous anecdotic reports of their beneficial activity, but, to date, data available are extremely not reliable as metanalysis studies have shown that such efficacy is not consistent [29].

Thus, for severe and critical patients with COVID-19, lopinavir/ritonavir might play a role in improving outcomes, but the available evidence is still limited. A substantial number of ongoing studies should provide valuable evidence to researchers and decision-makers.

Most of these studies suggested that both chloroquine and hydroxychloroquine treatment resulted in a significant reduction of replication of different viruses, including the SARS coronavirus [30]. Such effects were further confirmed in some cases, also in *in vivo* model for various viruses, including enterovirus EV-A71 [31],

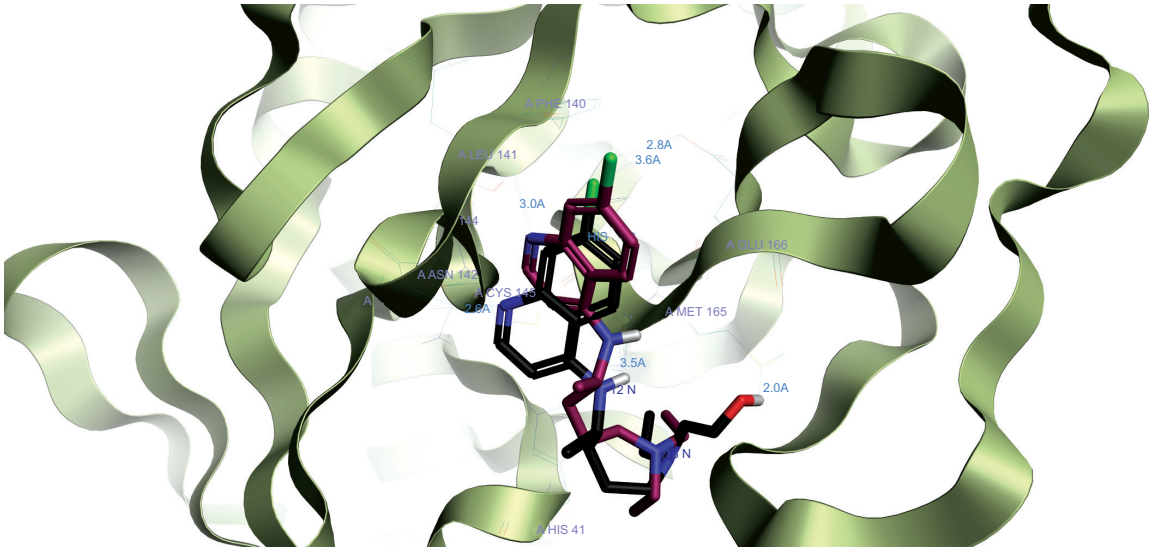

Fig. (5). Docking disposition of Chloroquine and Hydroxychloroquine in the main protease of SARS-COVID-19 virus binding site. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

human coronavirus OC43 [32] and Zika virus [33]. However, in some cases, chloroquine was not effective such as in the case of influenza infection [34]. Furthermore, a randomized, double-blind, placebo-controlled clinical trial in Vietnam demonstrated that chloroquine had no effect on the dengue-infected patients [35]. It should be taken into account that chloroquine significantly modulates the immune functions and exhibits anti-inflammatory properties [36-38] therefore, in some cases it may exacerbate acute fever and delay the cellular immune response, leading to an incomplete viral clearance [39]. Take together, these observations show that no acute virus infection is successfully treated by chloroquine in humans. As far as chronic viral diseases are concerned, the use of chloroquine in the treatment of HIV-infected patients has been considered inconclusive [40], and therefore it is not included in the protocols for HIV patients. Consistently, the effect of chloroquine in chronic hepatitis C is modest, and the introduction of new antiviral agents for HCV has excluded chloroquine in current protocols.

Our *in silico* results may in part explain the different effects of chloroquine and hydroxychloroquine, at least in *in vitro* studies. In fact, hydroxychloroquine exhibited a higher affinity to Sars-Cov-2 protease, thus providing an additional effect to those provided by chloroquine. However, no clinical data are available regarding the different efficacy of these two compounds in viral infection and in particular, in Sars-Cov-2 infection. In this respect, the European Medicine Agency recommends high awareness of pharmacovigilance signals (23 April 2020 EMA/202483/2020), with special regard to QT alterations. In fact, according to a review of ongoing RCTs, lesser attention is focused on chloroquine, and efforts are being made in the attempt to define the clinical efficacy of hydroxychloroquine. Now, while RCT data on hydroxychloroquine are still, in part, conflicting, they rather suggest borderline efficacy. On the other hand, the major concern emerges from the safety reports, indicating a statistically significant increased incidence of lethal cardiovascular events in SARS-CoV-2 patients (total n = 956.374) who are on combined therapy with azithromycin (about one third of the total; HR: 2.19). In the actual emergency phase, on the basis of the clinical data available, short term therapeutic use of hydroxychloroquine in SARS-CoV-2 infection may be recommended in less severe patients who may be followed at home, as well as in hospitalized patients, provided that patients with *per se* higher cardiovascular risk are strictly monitored.

While there has been an anticipation to achieve clinically meaningful benefits from RCT data, in light of the scarcity of clinical data available, it appears difficult to reach any firm conclusion. In fact, although *in vitro* studies would initially suggest reasonable efficacy of chloroquine and hydroxychloroquine in COVID-19 patients, the evolution of clinical outcomes analysis was not consistent with the first positive reports [10, 41-44]. The first set of clinical trials conducted in France was inconclusive, for the reason that the design of the studies was biased with a limited sample , as well as the lack of either controls and randomization [45, 46]. Thus, to date, there is no evidence in support of the prescription of chloroquine and hydroxychloroquine in COVID-19 patients of any stage, based on the high uncertainty of outcomes, which discourages their use [47]. AIFA, the Italian Medicine Agency, withdrew the permission of off label prescription of the two drugs and restricted their use only to adequately powered clinical trials (aifa.gov.it).

Finally, further studies are now required in order to determine possible clinical advantages of chloroquine phosphate (or other salts) and hydroxychloroquine. Furthermore, it remains to be elucidated whether the efficacy of antimalarial therapy depends on the age class, the clinical presentation or the stage of the disease.

CONCLUSION

In conclusion, although either chloroquine or hydroxychloroquine, and the combination lopinavir/ritonavir are included in the COVID-19 treatment plan in many countries, actually, because of high uncertainty about their respective risk/benefit ratios, they should be authorized only within specifically designed clinical trials. Some trials evaluating these drugs for COVID-19 infection treatment, such as SOLIDARITY (WHO), RECOVERY (UK; NTC04381936) and DisCoVeRy (INSERM; NTC04315948) discontinued the hydroxychloroquine and lopinavir/ritonavir arms. In fact, the interim data showed that both hydroxychloroquine and lopinavir/ritonavir produced negligible or no reduction in the mortality of hospitalized COVID-19 patients *vs*. a given standard of care. In addition, many concerns have been raised about the possible toxicity of hydroxychloroquine, with special regard to cardiovascular AEs.

Finally, our data suggest that hydroxychloroquine may exhibit potential additional efficacy by inhibiting viral protease; however possible adverse reactions of antimalarial drugs and detrimental

effect on the immune system should be taken into account while using such drugs.

AUTHORS' CONTRIBUTIONS

"Conceptualization by S.R. and G.L.V.; methodology by S.R., R.D.M., R.B. and F.P.; formal analysis by S.R. and F.P.; writing-original draft preparation by G.L.V; writing-review and editing by S.R. And G.L.V. All authors have read and agreed to the published version of the manuscript.

LIST OF ABBREVIATIONS

CYP	=	Cytochrome P450 Enzymes
SARS	=	Severe Acute Respiratory Syndrome

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that supports the findings of this study is available from the corresponding author, [L.V.] upon reasonable request.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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