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Commentary

Intracellular ABCB1 as a Possible Mechanism to Explain the Synergistic Effect of Hydroxychloroquine-Azithromycin Combination in COVID-19 Therapy

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Abstract. The co-administration of hydroxychloroquine with azithromycin is proposed in COVID-19 therapy. We hypothesize a new mechanism supporting the synergistic interaction between these drugs. Azithromycin is a substrate of ABCB1 (P-glycoprotein) which is localized in endosomes and lysosomes with a polarized substrate transport from the cell cytosol into the vesicle interior. SARS-CoV-2 and drugs meet in these acidic organelles and both basic drugs, which are potent lysosomotropic compounds, will become protonated and trapped within these vesicles. Consequently, their intra-vesicular concentrations can attain low micromolar effective cytotoxic concentrations on SARS-CoV-2 while concomitantly increase the intra-vesicular pH up to around neutrality. This last effect inhibits lysosomal enzyme activities responsible in virus entry and replication cycle. Based on these considerations, we hypothesize that ABCB1 could be a possible enhancer by confining azithromycin more extensively than expected when the trapping is solely dependent on the passive diffusion. This additional mechanism may therefore explain the synergistic effect when azithromycin is added to hydroxychloroquine, leading to apparently more rapid virus clearance and better clinical benefit, when compared to monotherapy with hydroxychloroquine alone.

KEY WORDS: azithromycin; hydroxychloroquine; ABCB1; lysosomes; COVID-19.

The co-administration of the antimalarial chloroquine or the anti-rheumatic hydroxychloroquine with the antibacterial azithromycin is presently proposed as a therapy amidst the COVID-19 pandemic (1). These aminoquinoline drugs and azithromycin are being repurposed for their clinical use in COVID-19, and they should be evaluated according to current practices of drug clinical trials for this indication. Although this process is normally time-consuming, population, media, and political pressures have propelled the use of this combined therapy on an emergency basis, thus opening controversial pro and con debates. Moreover, most of the different aminoquinoline clinical trials over the world are not comparable as they differ in terms of mono or bi-therapy choice, dosage regimen, and optimal time of administration versus the disease time course and severity. Several Chinese clinical trials have been based on the sole chloroquine or hydroxychloroquine administration and their clinical efficacies remain uncertain (2–4). Additionally, most of these methodological protocols suffer from major concerns in term of trial design quality such as inappropriateness in randomization, patient population size, and other limiting factors (5,6). The purpose of this commentary is to discuss the possible rationale for co-administering the antibiotic azithromycin with hydroxychloroquine, recognizing that azithromycin is not being used for its antibacterial activity but for its additive or synergistic effect on the antiviral action of the aminoquinolines (7). Moreover, several in vitro and in vivo studies support additional antiviral properties of azithromycin although the drug is not approved for the treatment of viral infections (8). Here, we provide comments and hypotheses to develop a rationale for this drug combination based upon sub-cellular pharmacokinetic and pharmacodynamic arguments.

Two recent works have detailed the targets and cellular pathways supporting the antiviral activity of chloroquine and hydroxychloroquine (4,9). In fact, the attempt to combine chloroquine and azithromycin started with the observation of resistance of *Plasmodium falciparum* to chloroquine. Gingras and Jensen were the first in 1992 to demonstrate in vitro that the association of azithromycin to chloroquine reversed the resistance of chloroquine-resistant strains of *P. falciparum* (10). Subsequent in vivo and clinical studies have validated this dual administration in malaria chemotherapy (11,12). An inverted association was also found effective to kill intracellular bacteria, such as *Staphylococcus aureus* when

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chloroquine was used to synergize the effect of the antibiotic azithromycin (13). Moreover, this drug combination was also extended to virus diseases with many clinical studies but most of them were moderately conclusive (14,15). Nevertheless, for each recent and new virus infections, the chloroquine-azithromycin combination strategy reappears as a possible miraculous solution such as in Ebola, SARS and MERS, HIV diseases, and now with SARS-CoV-2 in COVID-19 (16–19). In this last indication, hydroxychloroquine, mostly used for treating severe rheumatic diseases, is preferred to chloroquine as more effective in vitro and clinically safer than chloroquine (20). Moreover, a recent physiologically based pharmacokinetic (PBPK) model, combining in vitro data with published drug pharmacokinetics, was interestingly developed to predict the optimal dosage regimen of hydroxychloroquine in COVID-19 (21). Another recent mechanistic PK modeling study including efficacy with historical data on viral replication and the cardiotoxicity risk of QTc interval prolongation attempted to predict the highest hydroxychloroquine doses which are needed to achieve both safety and cure within 7 days (22).

Why could this couple of antimalarial and antibiotic drugs be efficient for such a broad number of infectious diseases caused by so various pathogens such as parasites and intracellular bacteria or viruses? That is the fundamental question.

Besides their anti-infectious properties, the aminoquinoline compounds are immunomodulators that could counteract the cytokine storm observed in the severe form of COVID-19 (4,9,16). Also, they are known to stop virus replication as in most of the clinical trials with both aminoquinolines; the virus load is considered as a critical biomarker endpoint (16). This direct effect on virus replication seems to be dependent on the fact that these drugs and viruses share common sub-cellular organelles trafficking routes involving either the *trans*-Golgi secretory pathway or the endosomal/lysosomal endocytotic pathway. On the one hand, the virus uses endosome formation as a cellular entry mechanism. Thus, following the binding of the CoV-2 spike protein to the angiotensin-converting enzyme 2 (ACE2) virus-receptor, SARS-CoV-2 travels inside the vesicular network including endosomes and lysosomes. On the other hand, several processes occur within the organellar lumen, like the glycosylation of CoV-2 proteins during their biogenesis and assembly as well as the action of several endosomal proteases able to cleave CoV-2 spike proteins for virus entry (4,9,16,20). Therefore, all these life-virus steps are dependent on the remarkable intra-organelle acidic conditions, with pH around 4–5. A vacuolar-type proton adenosine triphosphatase membrane (v-ATPase), pumping protons towards the vesicle interior, contributes to maintain this optimal pH medium for nearly 60 different hydrolytic lysosomal enzymes, such as some acid proteases which are implicated in the virus replication cycle (23) (Fig. 1).

In fact, viruses and drugs together meet in this common biophase of these sub-cellular organelles during their respective time-dependent distribution. These drugs share similar pharmacokinetic profiles with extensive tissue distributions characterized by elevated volumes of distribution, moderate plasma protein binding, and high intracellular distribution with tropism for lysosome-rich organs, such as liver and lung. They also exhibit low total body clearances which contribute to long body exposure (24–26). More interestingly, chloroquine, hydroxychloroquine, and azithromycin have also been described as potent lysosomotropic compounds as they are diprotic weak bases with pKa at 8.38–10.18, 8.27–9.67, and 8.74–9.45, respectively (4,9,27). These physicochemical criteria associated with elevated log P, ranged from 3.6 to 4.6, meet those defining a subgroup of lysosomotropic amines, known as cationic amphiphilic drugs (28). Thus, they will become protonated and trapped within the endosomal vesicles under these acidic conditions and their backward diffusion into the cytosol was hindered (29,30). Consequently, their intravesicular concentrations can reach hundredfold that of the cytosolic concentrations and attain or even surpass the low micromolar effective concentrations that are able to inhibit SARS-CoV-2 virus cycle (8,19,21). Concomitantly, the drug-induced lysosomotropic effect is completed by buffering the intravesicular acidity with pH increasing up to around neutrality. This last effect on the luminal pH is the most critical on virus development by decreasing several lysosomal enzyme activities responsible for either glycosylation of both ACE2 receptor and CoV-2 proteins or cleavage of Cov-2 spike proteins. Presently, this intracellular trapping mechanism is largely reported for sustaining the additive or synergistic effect on various pathogens following this drug co-administration (4,9,11).

Nevertheless, I believe that a supplementary mechanistic explanation may be missing in the literature for supporting the additive or synergistic interaction when these drugs are combined. The first reason is that azithromycin has been shown, both in vitro and in vivo, to be a substrate of ABCB1 (P-glycoprotein) (31–36). ABCB1, a member of the ATP-binding cassette (ABC) transporter superfamily, is mainly known as being expressed on the cellular plasma membrane in various tissues and can limit the cellular uptake of a large number of drug substrates like azithromycin (37). A second reason is linked to the ABCB1 localization in intracellular compartments, such as the endoplasmic reticulum and Golgi (site of ABCB1 synthesis), the endosomes for ABCB1 trafficking and recycling, and lysosomes for its degradation (38,39). Finally, the ABCB1 substrate transport direction on endosome and lysosome membranes is a very critical property. The polarized transport direction occurs from the cell cytosol, where the neutral pH allows the ATP catalytic cycle of ABCB1, into the interior of the vesicle. This transport direction is the opposite from that mediated at the plasma membrane surface (Fig. 1).

Based on these overall information, we might hypothesize that ABCB1 could play a possible role for the additive or most likely, the synergistic effect of azithromycin on hydroxychloroquine. At this stage, the question on how the ABCB1-dependent synergy might occur is still open. Based on known pharmacological modulations mediated by ABCB1, we could imagine two possible scenarios.

The first one is related to the inverted influx transport of ABCB1, meaning that the azithromycin molecules, in addition to their passive diffusional uptake, are actively captured and trapped inside the vesicles, even if the vesicular intraluminal pH increases and the percentage of protonic drug molecules decreases enabling the backward diffusion of neutral species to the cytosol (Fig. 1). Therefore, this could

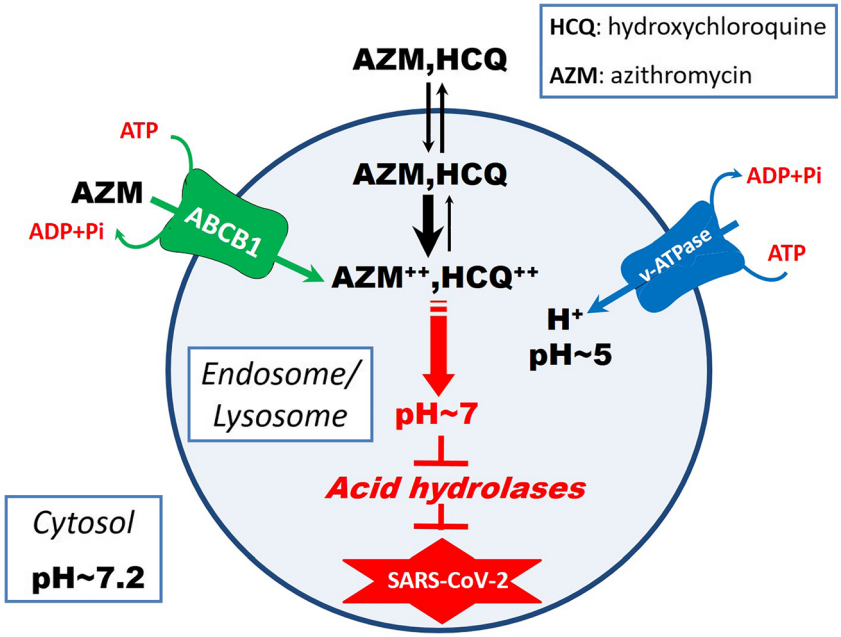

Fig. 1. A proposed model of the possible role of ABCB1 to synergize the lysosomotropic effect of azithromycin and hydroxychloroquine and to alter SARS-CoV-2 replication cycle via the endolysosomal pathway. The diagram shows how the unionized drugs can readily diffuse across lysosomal membranes. Due to the acidic environment of the lysosome, the equilibrium between charged and uncharged drug species shifts in favor of the ionized species, thus limiting their backward diffusion into the cytosol. As a substrate of ABCB1, the active uptake of azithromycin contributes to the enhancement of this trapping effect and to the neutralization of the acidic pH. This last effect contributes to a cascade of anti-inflammatory and antiviral activities

represent an explanation for the synergistic effect when azithromycin is added to hydroxychloroquine or chloroquine. At all these intracellular stages, ABCB1 can be a possible enhancer by confining the substrate azithromycin more extensively as expected when the trapping mechanism is solely limited to the protonated-diffusion explanation. This first hypothesis could be supported by results obtained from a clinical study where increasing amounts of azithromycin (from 500 mg to 2 g) combined with a fixed dose of 600 mg chloroquine showed a clear dose-response relationship with the maximum clearance rates of *P. falciparum* observed with the highest dose of azithromycin (11). Moreover, in vitro experiments using Vero E6 cells infected with SARS-CoV-2 virus have also reported concentration and time-dependent effects of hydroxychloroquine on the virus cytotoxic EC50 values, which decreased with longer incubation times (20,21). This suggests that time is required for drug accumulation or/and drug cytotoxicity kinetics. These observations could be supported by the long-lasting ABCB1-mediated exposure effect of azithromycin. This azithromycin trapping effect induced by ABCB1 could also be extended to the aminoquinoline compounds to the extent that they could interact with ABCB1. This could most likely occur with chloroquine which was classified in several vesicular and cellular in vitro models as a moderate inhibitor and a non-substrate of ABCB1 (40–43). Unfortunately, hydroxychloroquine has been demonstrated neither as a substrate nor as inhibitor of ABCB1 with the usual in vitro models. Nevertheless, this aminoquinoline has been reported in human pharmacokinetics to interact with digoxin and nelfinavir, two known ABCB1 substrates, by moderately increasing their oral bioavailability (44,45). Thus, all these data suggest that hydroxychloroquine and chloroquine could have a moderate inhibition effect on the azithromycin ABCB1-mediated intravesicular influx. They also point to the need to further investigate the inhibitor/substrate status of hydroxychloroquine towards ABCB1. Additionally, it is noteworthy that the antibiotic and fluoroquinolone ciprofloxacin which is also both a lysosomotropic compound with pKa 8.7 and substrate of ABCB1 behaves like azithromycin to synergize the anti-infectious effect of chloroquine (27). This last observation appears to demonstrate that the lysosomotropic drug property is certainly a key factor in the synergistic effect of this drug combination.

The second scenario could be similar to the well known reversal of multidrug resistance, the MDR effect, observed with anticancer drugs such as doxorubicin or daunomycin which are also actively trapped inside lysosomes under ABCB1 active influx. The administration of ABCB1 inhibitors, like valspodar or elacridar, tends to displace the anthracyclines from the lysosomal lumen into the cytosol making doxorubicin available for its nucleus targets (46). The translation of this MDR reversal to azithromycin might suggest its ability to inhibit ABCB1, and therefore to increase aminoquinoline cytosolic concentrations which could be active for other biological targets and cellular pathways impacting the virus development (4,9,16). Therefore, this second scenario seems unlikely as hydroxychloroquine and chloroquine are not substrates of ABCB1.

Nevertheless, this drug release from the vesicle lumen to the cytosol could proceed from the deleterious effects of these cationic amphiphilic drugs on lysosomal viability. As a result of their over-accumulation in the endolysosomal lumen which could be exacerbated by the azithromycin effect on ABCB1, they could permeabilize the lysosomal membranes to protons, Cl^- and water causing enlargement of the lysosomes and phospholipidosis (47). Interestingly, azithromycin, chloroquine, and hydroxychloroquine have been all reported as responsible of these types of endolysosomal damages which can affect vesicular trafficking by inhibiting the surface expression of receptors, the fusion to autophagosomes, and lipid metabolism which indirectly contributes to the inhibition of the cytokine production. All these impairments could contribute to the panel of cytotoxic mechanisms affecting the SARS-CoV-2 replication cycle as well as the risks of organ-toxicity like cardiomyopathy following this drug combination (29,48,49). They also highlight a critical need for optimal dosing of both drugs using PBPK and PKPD modelings to achieve both safety and efficacy (21,22).

To sum up, the intracellular ABCB1 could represent a potent target for enhancing the antiviral and anti-inflammatory activities of the aminoquinolines when lysosomotropic ABCB1 substrates like azithromycin or ciprofloxacin are combined. This could explain why the association strategy leads to apparent rapid virus clearance and better clinical benefit vs. aminoquinoline use alone (1). Moreover, these observations justify the bi-therapy administration in the early stage of the disease or for prophylactic use, i.e., when the virus distributes within the disease target cells, such as the pulmonary epithelial cells. Experimental assessments of this hypothetical ABCB1 role could be easily investigated using both in vitro cellular models currently used with SARS-CoV-2 and widely developed for ABCB1 transport (19,20,46,50). Moreover, the reported co-localization of ABCB1 with other ABCs on the lysosomal membranes, such as ABCG2, ABCC1, and ABCC2 could also expand this experimental field of investigation as to their possible involvement in the activity of these lysosomotropic drugs (51).

By taking into consideration this possible role of ABCB1 in the synergistic combination of these two drugs, the question of the modulation of ABCB1 transport could be raised for preventing risks of a lysosomotropic effect decrease on virus dynamics and of interindividual variability in the clinical use of this drug combination and with possible additional drug co-therapy. These risks should be considered at both the intracellular level and at the drug disposition level where ABCB1 has been extensively reported as mediating drug-drug interactions (DDI) (52). First, the intracellular DDI risk can be anticipated from previous studies showing that lysosomal ABCB1 can be a DDI target (46,53). In addition to the previous MDR reversal example, ABCB1-mediated lysosomal competition between two weak bases has been elegantly shown in a study using positron emission tomography imaging where the radiotracer [^{11}C] *N-desmethyl*-loperamide was displaced from lysosomes by tariquidar and other ABCB1 competitors like cyclosporine or verapamil (54).

Finally, current pharmacokinetic considerations indicate that these drugs are characterized by a low safety margin and by cardiotoxicity risks (55,56). Therefore, risks of inter and intra-individual variabilities have to be considered. Presently, no systemic pharmacokinetic interactions have been observed between chloroquine and azithromycin (24). Azithromycin is known for interindividual variabilities in drug response and among ethnic groups and could be a DDI perpetrator candidate. Azithromycin is not metabolized by cytochromes P450 and not an inducer/inhibitor of these hepatic enzymes. The dominant azithromycin excretion pathway occurs at the biliary and intestinal levels via ABCB1 and ABCC2 (MRP2) active transport which could represent DDI targets (36). However, a few and insignificant clinical DDIs have been observed between azithromycin and co-prescribed drugs (24).

Lastly, the most intriguing interpopulation variability could result from the azithromycin sensitivity to ABCB1 genetic polymorphism. Two clinical studies have described that heterozygous and mutant *ABCB1* genes in Chinese Han and Pakistani subjects have decreased rate and extent of azithromycin absorption when compared to the wild-type subjects (34,35). This suggests that the ABCB1-dependent effectiveness of azithromycin at the intracellular level could be variable according to the patient ABCB1 genetic status.

In conclusion, we hypothesize that the intracellular ABCB1 may serve as a possible new target for improving the effects of the aminoquinolines when co-administered with a lysosomotropic drug, such as azithromycin or ciprofloxacin in COVID-19 chemotherapy. In vitro experiments could readily confirm or contradict this hypothesis. The proposed hypothesis might stimulate further investigations and experimental validation for the clinical relevance of this possible treatment. Moreover, this case example highlights that molecular and cellular pharmacokinetics should be considered in the future for the understanding of drug interaction at the intracellular level. *Postscript.* While this manuscript was under review, a paper describing similar lysosomal accumulation of the two drugs via ion-trapping mechanism was published (57).

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The author declares that he has no conflict of interest.

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