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INTERACTION OF HYDROXYCHLOROQUINE AND AZITHROMYCIN WITH ANTIFUNGAL IN ASPERGILLOSIS ASSOCIATED WITH COVID-19 (IN VIVO)

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Abstract

In vivo evaluation of the antifungal voriconazole in combination with antibiotics used to treat COVID-19 patients, hydroxychloroquine and azithromycin, was performed in mice. All twelves mice in the trials were injected with fungal suspension (*A.fumigatus* ,that isolated from covid-19 patients) to cause infection, and they were all checked twice a day until clinical symptoms appeared. Mice who were given the drug for 2 weeks to 4 weeks developed pathological symptoms included significant weakening of excitation, hypoactivity, loss of balance and activity, ataxia, tachypnea, and irritation of the upper respiratory tract. Each group then received either voriconazole (200 µg/ml), an antifungal, or a combination of azithromycin (500 µg/ml) and hydroxychloroquine (400 µg/ml) taken orally once daily for four weeks. Results show,the control group, which showed varying degrees of bronchitis, the abnormal group of mice had enlarged lungs upon examination. Contrarily, it was found that the signs were the same in the second group that was given the antifungal voriconazole alone, but they were less suffocated, less irradiated, and had lower mortality and shorter healing rates . On the other hand, in the groups treated with combination medications, there was increased mortality , and higher infection severity scores compared to the control groups with no outward signs and appeared normal.

Keywords: COVID19, Voriconazole, Aspergillosis, Hydroxychloroquine.

INTRODUCTION

Aspergillosis of the lungs and airways induced by SARS-CoV-2 is known as COVID-19-associated pulmonary Aspergillosis (CAPA). For those who suffer from invasive lung aspergillosis (IPA), the most deadly form of infection caused by Aspergillus spp., the frequency of CAPA in these cases is extremely high. Mortality rates among people infected with COVID-19 in ICU cohort studies range between 5 and 30 percent (Hawes & Permpalung, 2022; Xu *et al*., 2021). Conidia, or asexual spores, produced by the saprotrophic fungus *A. fumigatus* are inhaled and cause both short-term and long-term health problems in susceptible individuals. Indicating a persistent host-pathogen conflict in the upper respiratory tract and lower airways, *A. fumigatus* conidia of 2 to 3 mm in size may easily reach alveolar gaps. After being exposed to *A. fumigatus*, the immune system of a healthy person will eliminate the fungus from the lungs (Chong & Neu, 2021; Wassano *et al*., 2020).

Azole antifungal drugs are widely used to treat invasive Aspergillosis. The most common fungal sterol, ergosterol, is reduced in these medications because its production is blocked by blocking lanosterol 14a-demethylase (Fernandes *et al*., 2022). Hydroxychloroquine (HCQ) is approved for the treatment of coronavirus infection in a number of countries (COVID-19) The metabolism of HCQ is governed by the enzymes CYP2C8, CYP3A4/5, and CYP2D6. However, HCQ clearance may be slowed by the presence of CYP enzyme inhibitors and substrates (Sanders *et al*., 2020; Schrezenmeier & Dörner, 2020). Increased HCQ blood concentrations and major ADRs can result from substrate-inhibitor or substrate-substrate drug-drug interactions (DDIs), with the most severe DDIs of HCQ with azithromycin and

voriconazole in COVID-19 patients raising the risk of life-threatening Q and T wave (QT) prolongation. Two recent studies found that many people with COVID-19 suffer from cardiac arrhythmias and sudden cardiac death (Biswas & Roy, 2021).

Here, we hope to learn more about the in-vivo effects of combining the antifungal drug voriconazole with hydroxychloroquine and azithromycin.

MATERIAL AND METHOD

Fungal Isolate

Fungal isolate, that used in this study, was obtained from patients infected with Covid by taking samples of sputum and culturing them on medium SDA

Fungal Species Identification

Depending on the culture and microscopic properties of the fungus as stated in Kidd *et al*., (2022)

Antifungal Solution Preparation

50 mg of antifungal medicine was diluted in 5 ml of DMSO (dimethyl sulfoxide) in sterile screw-capped glass vials to create a stock solution with a concentration of 10000 g/ml. The drugs were then diluted extensively to attain their final concentrations (200 g/ml voriconazole, 500 g/ml azithromycin, 400 g/ml hydroxychloroquine) (Alradhi Falah, 2014).

Preparation of fungal suspension

Inoculum solutions were prepared using freshly produced, mature cultures on Sabouraud agar slants (3-5 days old) as described by Gadzovska-Simic *et al*., 2012; Petrikkou *et al*., 2001.

Animals Inoculation

According to Alradhi Falah, 2014; Shafiq & Al-Joofy, 2010, 2010, a total of twelve mice participated in this experiment. Injections of a fungus slurry were given to three sets of four mice. Voriconazole (200 g/ml), an antifungal, was given to one group after infection, while azithromycin (500 g/ml) and hydroxychloroquine (400 g/ml) were given to another group as a combination VRC and taken orally once daily for four weeks.

Histopathological study : The researchers in this study adopted a procedure similar to that described by Scheuer & Chalk, (1986).

Statistical analysis

The data was analyzed statistically (with the Specialized Version 26 of the Statistics Scientific Packages) with the help of the X2-Sequre software, yielding a valuable Probability value of ≤ 0.05.

RESULTS AND DISCUSSION

Identification of isolate

According of (Alkhuzaie & Jasim, 2022; Efaq et al., 2019; Jr. Sciortino & Carmen, 2017; Kidd et al., 2022), identify Aspergillus fumigatus. Colony blue-green surface pigmentation on the culture media and conidiophore, felt-like, suede-like surface. Green, spherical or sub-spherical, rough-walled, or echinulate conidia

Animal Infectivity

Four weeks post-inoculation, Aspergillus fumigatus-infected animals showed clinical signs. We started scarifying it and took out its lungs. Compared to the control group, the lungs of the first set of mice clearly show signs of pneumonia, with congestion and red incurrence and the presence of bleeding patches in figure (1). Figure 2 shows the same signs in the second group, although they are less stifled and irradiated, while Figure 3 shows severe infection symptoms in the group treated with a combination of pharmaceuticals, and Figure 4 shows no outward symptoms or abnormalities in the control group (4).

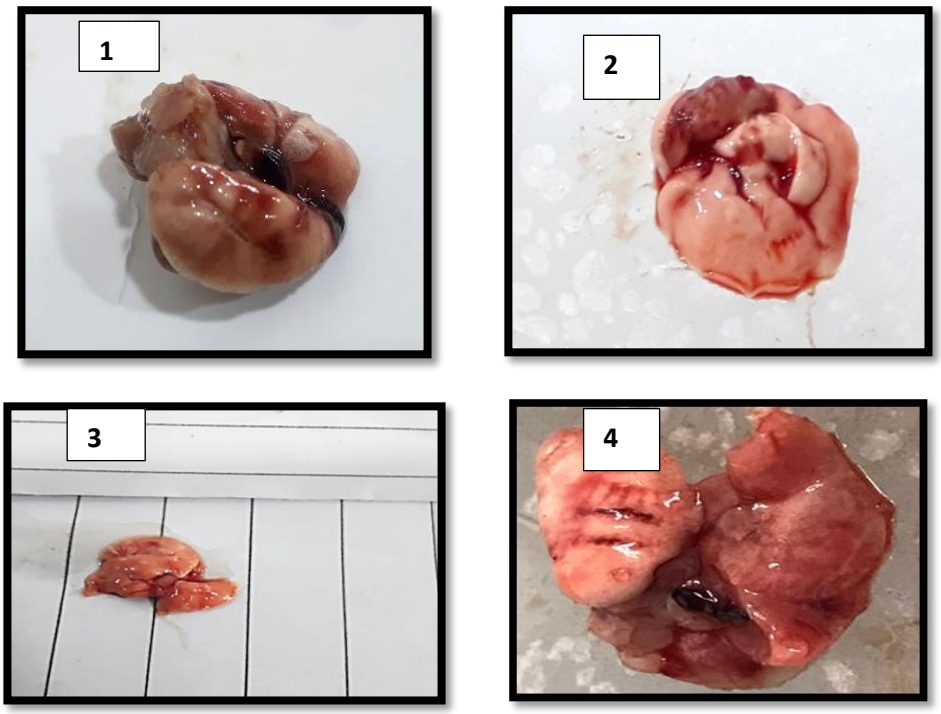

Figure (1): 1- infected lung with *A. fumigatus* by inhalation with congestion and red hepatitis after 14 days after injection 2- normal lung 3- lung after treatment with voriconazole after 28 days with treatment. 4- lung with combination Azithromycin, hydroxychloroquine and voriconazole

The present study's results corroborate those Pawlinski *et al*., (2003) , who noted that A. fumigatus is an efficient opportunistic pathogen in wild rabbits, with lesions visible as numerous white nodules dispersed across the surface of the lung upon macroscopic inspection. Some of the elements of its ecophysiology that contribute to its pathogenicity are its morphological characteristics, exceptional stress-tolerance biology, capacity to breach host defenses and colonize/damage the host, and astounding ability to produce cell-available energy. Aspergillus species have been sequenced genome-to-genome, and many aspects of their stress metabolism, ecology, interactions with a wide range of animal hosts, clinical presentations, and treatment regimens are well-understood (Paulussen *et al*., 2017). the injected fungal solution had a noticeable effect on all of the animals. One possible explanation is that the metabolites of the fungus A.flavus caused vascular congestion in internal test members by increasing blood pressure, promoting TNF production (INFLAMATORY MEDIATOR), and increasing the permeability of capillary capillaries and the hemoglobin produced by red blood cells (Al-ameri, 2013; Zain, 2011). By transforming

into hyphae, A. fumigatus evades the immune system, as TLR-4 recognition is lost but TLR2-mediated IL-10 pathways are not, shifting the balance towards a permissive Th2-type profile. (Netea *et al*., 2003). Triazole antifungal medications like voriconazole block ergosterol formation by inhibiting the cytochrome P450-dependent enzyme 14-lanosterol demethylase.

Lana, A. J. D., Pippi, B., Carvalho, A. R., Moraes, R. C., Kaiser, S., Ortega, G. G., ... & Silveira, G. P. (2018). In Vitro additive effect on griseofulvin and terbinafine combinations against multidrug-resistant dermatophytes. *Brazilian Journal of Pharmaceutical Sciences*, *54*.

Methylated sterol accumulation has been shown to cause membrane stress and breakdown in fungi. These medications alter cell membranes and hence can interfere with vacuolar acidification and other functions of intracellular vacuoles. Researchers have demonstrated that voriconazole is effective against fungi at both the mycelial and fungal levels (Chryssanthou et al., 2008; Ghannoum & Rice, 1999; Rosowski et al., 2020; Vora et al., 1998; Zhang et al., 2010).

The antifungal drug treatment greatly reduced the severity of the gross pathological abnormalities in the lungs and had a beneficial effect on breathing, as demonstrated in our study. Interactions between hydroxychloroquine, zithromax, and voriconazole caused thrombosis and thrombosis, leading in mortality both immediately after the first dosage and after two weeks of treatment, particularly in the presence of severe symptoms of infection and substantial damage to the lungs. These findings show that inoculating hens with A. fumigatus through their thoracic intra-air sacs causes significant respiratory infection , activates TLR1 and TLR2 mediated immune responses, and elicits large production of pro-inflammatory cytokines like IL-1, Cxcl-8, and IFN- (Cornely et al., 2009). Conidial germination is widely recognized as a pivotal event in the development and spread of IPA. The alveolar macrophages in the host's immune system are the first line of defense against Aspergillus conidia. Lung resident macrophages in healthy people can quickly engulf and kill inhaled conidia, but they can't take in hyphal cells. However, in immunocompromised people, non-phagocytosed conidia produce germ tubes that elongate into hyphae in the lung tissue, causing a systemic infection due to their ability to invade blood vessels (Campione et al., 2021; Shoham & Levitz, 2005).

Clinical effectiveness of the three most common groups of antifungal medicines (polyenes, azoles, and echinocandins) is low. drugs' interactions with one another, and the all-important pharmacokinetic profile. Future research must focus on either finding new medications with the potential to interact with specific fungal targets, or improving the efficacy of currently available drugs against fungi while minimizing their undesirable side effects. (Kwon-Chung & Sugui, 2013).

Lung tissue samples from unaffected mice showed normal structure and no signs of pathology upon microscopic inspection. This included the presence of interalveolar barriers separating pulmonary alveoli from the rest of the lung tissue, alveolar cysts (groups of pulmonary alveoli that drain into a single alveolar duct), and pulmonary blood vessels (as show in figure 2).

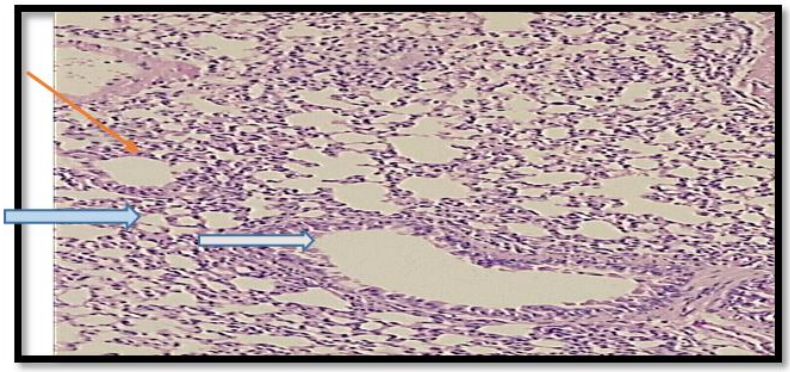

Figure (2): across section of normal tissue showing the presence of pulmonary vein(⇨)And typical normal structure of the interaalveolar alveoli (➡) And cell lining the bronchioles (⇨)

Pathological tissue changes were evident in the lungs of infected mice, including a significant attenuation of lung structure due to decomposition or the appearance of severe hyperplasia in the epithelial layer lining the bronchi in a distorted manner due to injury, as well as inflammatory cellular infiltration and edematous fluid between the cells forming the pulmonary alveolar wall. Epithelial cells lining the bronchioles in the lungs detonated and shed their outer layers, and microscopic analysis revealed the infectious agent in its characteristic form, a conidial carrier culminating in a conidial head and surrounded by infiltrates of inflammatory cells. Figure (3) illustrates this point. The severity of the damage sustained by the lungs of the mice resulted in emphysema, plasma protein blood fluid infiltration due to intravascular congestion, and severe bleeding (figure 3).

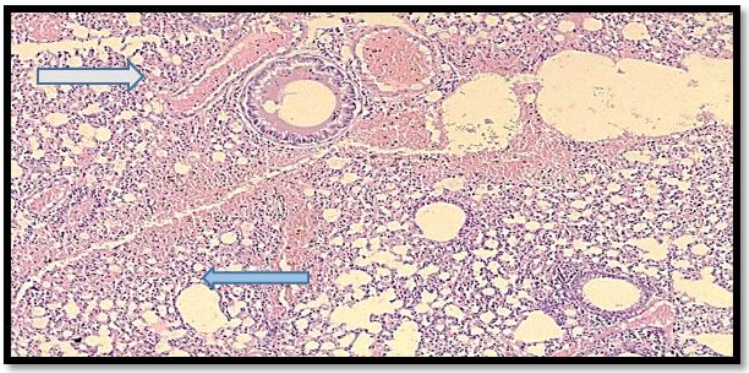

Figure (3): Section of lung infected with fungus there is completely loss of the typical structure of lung tissue due massive infiltration of inflamation cell, hyperplasia and hemorrhage (⇨) Bleeding fluid infiltration congestion in the lung and sloughing of the epithelial layer lining the bronchioles, infiltration of inflamatory cell complete necrosis of the alveolie and their fusion (➡) And laceration of alveolar wall

Voriconazole-treated mice displayed less severe clinical and histological abnormalities than their infected counterparts, including less emphysema and a minor hyperplasia in the epithelial layer lining the bronchi, which was observed as very apparent bumps (figure 4).

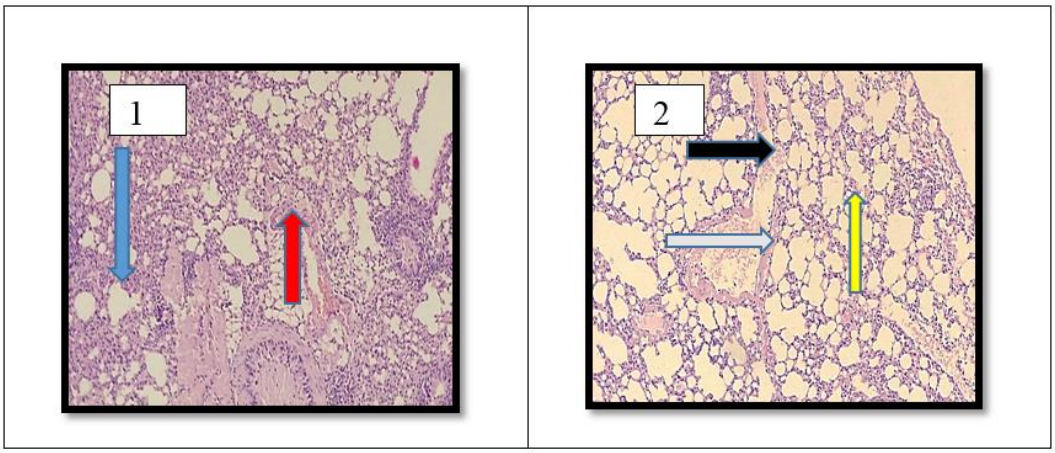

Figure (4): section of mice lung infected with fungus and oral treatment with voriconazole (200µg/ml) it appears restoration of the alveoli to their normal shape (➡) Restoration of the alveolar septum to their usual thickness, and return of the pulmonary histological structure to its normal state, normal bronchioles(⇨)and the presence of normal alveoli with mild emphysema (⬅)with the presence of columnar epithelial lining of the pulmonary bronchioles (⇨)with little inflammatory cells into interstitial tissue between the alveoli (➡)

The analysis of lung tissue that has been treated with medicine results in an assign of inflammation as a marker. Numerous pathological tissue changes were observed in the lungs of infected mice, including a dramatic attenuation of lung structure due to decomposition or the appearance of severe hyperplasia in the epithelial layer lining the bronchi in a distorted manner due to injury, as well as inflammatory cellular infiltration and edematous fluid between the cells forming the pulmonary alveolar wall. Microscopically, the infectious agent was found in its characteristic form, a conidial carrier culminating in a conidial head and surrounded by infiltrates of inflammatory cells, in the epithelial cells lining the bronchioles of the lungs, which had undergone a process of molting and explosion (figure 5).

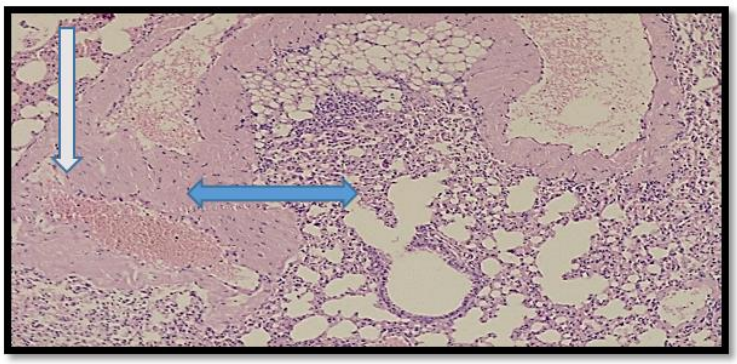

Figure (5): Section of lung infected with fungus there is completely loss of the typical structure of lung tissue due massive infiltration of inflammation cell, hyperplasia and hemorrhage (⇨) Bleeding fluid infiltration congestion in the lung and sloughing of the epithelial layer lining the bronchioles , infiltration of inflamatory cell complete necrosis of the alveolie and their fusion (⟹)And laceration of alveolar wall

Overall, the results of this study demonstrated a substantial reduction in normal lung structure due to tissue decomposition, the appearance of severe hyperplasia in the epithelial layer lining the bronchi, or the thickening that occurred between the cells as a result of edema (fluid infiltration) as an inflammatory response of the lymphocytes clearly on and around the bronchi. Emphysema, distorted and desquamated alveoli, a reduction in the number of terminal bronchioles, and phospholipid-containing alveoli on the lamellar bodies were just some of the pathological abnormalities that the severity of the damage induced in the lungs of the mice. The Clara cells, which are unique to the terminal bronchioles, are long columnar cells with granular peaks. The effect was different in various tissues. The alveoli had become completely deformed, with thickened walls and small voids visible under a histological microscope (a condition known as cleavage). These adjustments greatly outnumbered the phagocytic cells, which also multiplied. This confirms the injury to the alveolar wall and its infiltration into the alveoli, and the presence of many areas covered with large amounts of bleeding with bloody congestion is evidence that the causative agent possesses the enzyme hemolysin, which decomposes red blood cells and their infiltration outside the blood vessel during the inflammatory process by a process called exudation. Erythropoietin production is triggered when the pulmonary alveoli are damaged and oxygen levels drop chronically.

It, in turn, drives production of red cells beyond normal limits, leading to a semi-tumor state of the constituent tissue, as seen Lai *et al*., (2007), that the blood rushing through your veins and capillaries is to blame for this. These results corroborate the conclusion reached by Hohl & Feldmesser, (2007) , namely, that the fungus spreads through human tissues or the respiratory system (the bronchi and the pulmonary cavity). congruent with the hypothesis that the host is infected with this fungus, as the presence of fungus filaments within the host tissues is seen to be conclusive proof of infection (Rolle *et al*., 2016). In addition, the opportunistic infections Aspergillus fumigatus and A. flavus are most often described as causative agents of CAPA (Lai & Yu, 2021).

When comparing the effects of voriconazole and hydroxychloroquine and azithromycin on the histological structure of the lungs of infected mice, microscopic research showed that voriconazole had a more positive effect. Voriconazole-treated mice had blood vessels that looked to be more regular in the wall and less congested, and that were located next to the airways. This contrasts with the appearance of blood vessels in overlapping-treatment mice, where the interior is in the form of transparent folds around a lumen. These results are consistent with other research demonstrating that antibiotics effectively reduce sickness symptoms (Arastehfar et al., 2020), For the prevention of invasive aspergillosis (AI), voriconazole is the drug of choice. CAPA has seen widespread usage of voriconazole; however, cardiac events have been linked to the use of voriconazole in combination with medicines used to treat COVID-19 (hydroxychloroquine, azithromycin, and protease inhibitors like lopinavir/ritonavir) (Giudicessi *et al*., 2020; Jenks *et al*., 2019; Varshneya *et al*., 2021). The Role of CYP450 Enzymes in Inflammation and Infection Because of decreased expression and/or activity of hepatic and extrahepatic CYP enzymes, drug metabolism, and drug transporters, inflammation and infection can alter the bioavailability of oral medications, however these factors are rarely explored in pharmacogenetic investigations. (Morgan, 2009). (Stavropoulou et al., 2018). While azithromycin can cause QT prolongation, it has been successfully given alongside

hydroxychloroquine without adding any more QT prolongation when well monitored (Gautret *et al*., 2020).

Patients with infectious and inflammatory processes, such as COVID-19, can be more likely to experience adverse medication reactions if they get standard dosages. Concurrently, inflammation-related decrease of P450 activities may diminish the therapeutic efficacy of pro-drugs that are triggered by metabolism. This suggests that inflammatory markers and immune response genes might be taken into account when assessing the wide range of responses to COVID-19 treatment.

In this article we discuss several pharmacogenetic biomarkers associated with the metabolic pathway of medicines used for COVID-19 treatment. Consistent with prior findings (Babayeva & Loewy, 2020; Takahashi *et al*., 2020; Zubiaur *et al*., 2020). Therefore, pharmacogenetic investigations should reveal the precise influence of genetic variations in the COVID-19 treatment by identifying the impact of drug-drug interactions and the inflammation and infection processes in patient response to drugs (Shah, 2017; Smit et al., 2018). In order to effectively treat CAPA, numerous clinical trials are being conducted on novel antifungals. Opelconazole, Fosmanogepix, Ibrexafungerp, Olorofim, and Rezafungin are only few of the antifungals that can be used (Feys *et al*., 2021; Mohamed *et al*., 2020).

SARS-CoV infected patients are especially vulnerable to invasive fungal infections. Corticosteroids, sedatives, and remdesivir all interact with voriconazole. Because CYP3A4 is genetically polymorphic, its usage requires the implementation of therapeutic drug monitoring (Chumbita *et al*., 2021). Some unproven medicines are used in the treatment of COVID-19. The following treatments involve medicines known to interact: hydroxychloroquine, atanzavir, and lopinavir/ritonavir (Hoenigl, 2021). Azithromycin is another medication that could cause problems (Lai & Yu, 2021). Voriconazole was previously thought to influence the pharmacokinetics of azithromycin, however a research with healthy volunteers disproved this (Purkins *et al*., 2003). Voriconazole-related QT-interval lengthening is an additional concern. Patients infected with COVID-19 are at a higher risk (Lai & Yu, 2021).

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