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ENVIRONMENTAL IMPACTS OF COVID-19 TREATMENT: TOXICOLOGICAL EVALUATION OF AZITHROMYCIN AND HYDROXYCHLOROQUINE IN ADULT ZEBRAFISH

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ABSTRACT

One of the most impact issues in recent years refers to the COVID-19 pandemic, the consequences of which thousands of deaths recorded worldwide, are still inferior understood. Its impacts on the environment and aquatic biota constitute a fertile field of investigation. Thus, to predict the impact of the indiscriminate use of azithromycin (AZT) and hydroxychloroquine (HCQ) in this pandemic context, we aim to assess their toxicological risks when isolated or in combination, using zebrafish (*Danio rerio*) as a model system. In summary, we observed that 72 h of exposure to AZT and HCQ (alone or in binary combination, both at 2.5 µg/L) induced the reduction of total protein levels, accompanied by increased levels of thiobarbituric acid reactive substances, hydrogen peroxide, reactive oxygen species and nitrite, suggesting a REDOX imbalance and possible oxidative stress. Molecular docking analysis further supported this data by demonstrating a strong affinity of AZT and HCQ with their potential antioxidant targets (catalase and superoxide dismutase). In the protein-protein interaction network analysis, AZT showed a putative interaction with different cytochrome P450 molecules, while HCQ demonstrated interaction with caspase-3. The functional enrichment analysis also demonstrated diverse biological processes and molecular mechanisms related to the maintenance of REDOX homeostasis. Moreover, we also demonstrated an increase in the AChE activity followed by a reduction in the neuromas of the head when zebrafish were exposed to the mixture AZT+HCQ. These data suggest a neurotoxic effect of the drugs. Altogether, our study demonstrated that short exposure to AZT, HCQ or their mixture induced physiological alterations in adult zebrafish. These effects can compromise the health of these animals, suggesting that the increase of AZT and HCQ due to COVID-19 pandemic can negatively impact freshwater ecosystems.

**Keywords:** water pollution; SARS-CoV-2; *Danio rerio*, ecotoxicity; antibiotic; antimalarial
1. INTRODUCTION

In the last decades, pharmacologically active compounds have been increasingly perceived in the aquatic ecosystem, representing a problem of great importance in environmental chemistry. However, the occurrence of these chemical compounds in nature is due to the release of industrial effluents and domestic sewage without adequate and effective treatment (Maasz et al., 2019). According to Salgado et al. (2021) the presence of drugs or its metabolized subproducts as result of body's excretion is an increasing concern of environmental contamination. It has been estimated that in 2030, the global consumption of antibiotics may be 200% higher than the 42 billion defined daily doses (DDD) estimated in 2015 (Klein et al., 2018). This disposal in the natural ecosystems can culminate in wide and unknown effects on the biota. Thus, ecotoxicologists around the world have made efforts to assess the toxicological risk impacts of drugs in non-target organisms, to understand how they can affect individuals and their populations. Several reports demonstrate on the ecotoxicity of different types of drugs (in various organisms), such as antidiabetics (Godoy et al., 2018; Godoy et al., 2019), analgesics and antipyretics (Nunes, 2020; Priyan et al., 2021), anti-inflammatory (Grandclément et al., 2020; Luongo et al., 2021), antihypertensive (Gallego et al., 2021), neuropsychiatric (Ramírez-Morales et al., 2021; Oliveira et al., 2021), and anticancer (Araújo et al., 2019; Mesak et al., 2019), which include biochemical, histopathological, genotoxic and mutagenic effects.

On the other hand, non-standard situations such as pandemic or endemic diseases, in which many patients receive specific medications, directly influence the use, excretion and disposal of drugs in the aquatic environment. One emblematic example is the significant increase in the use of azithromycin (AZT) and hydroxychloroquine (HCQ) in the context of the COVID-19 pandemic (Yazdany & Kim, 2020; Malik et al., 2020; Agarwal et al., 2020; Nasir et al., 2020; Mallhi et al., 2020; Quispe-Cañari et al., 2020). Their effectiveness, however, against SARs-Cov-2 infection is questioned by several studies (Ghazy et al., 2020; Jameleddine et al., 2020), but people are receiving these prescriptions or are self-medicating. AZT is a macrolide antibiotic that inhibits bacterial protein synthesis (Parnham et al., 2014). It has also been used to treat cancer and autoimmune and inflammatory diseases (Patel & Hashimi, 2020). HCQ is used in the prevention and treatment of malaria (Shippey et al., 2018) and is considered a therapeutic option in the treatment of rheumatoid arthritis (Lane et al., 2020), lupus erythematosus (Jakhar & Kaur, 2020), porphyria cutanea tarda (Malkinson & Levitt, 1980), Q fever (Cherry & Kersh, 2020) and photosensitive diseases (Millan & Quijano, 1957).

Therefore, the increase in the input and dispersion of these drugs in aquatic ecosystems is already a fact, especially due to the dumping of domestic sewage and hospital waste into rivers or streams or via leaching from landfills, which in many countries do not receive adequate treatment.
or the processes used are insufficient to remove these pollutants or are financially inaccessible (Khan et al., 2019). In cities with a high incidence of COVID-19, for instance, the dramatic increase in the production of hospital waste in health facilities has been an additional administrative challenge (Sarkodie & Owusu, 2020), in addition to amplifying the presumed concentrations of AZT and HCQ in the aquatic environment.

However, this evidence has not been sufficient for the systematic development of studies to evaluate the ecotoxicological effects of these drugs, whether in aquatic or terrestrial organisms [see review by Yang et al. (2020)]. Regarding macrolides, previous studies (in fish) addressed the toxic effects of erythromycin (Bills et al., 1993; Kiryu & Moffat, 2002; Ji et al., 2012; Rodrigues et al., 2016; Liu et al., 2017), roxithromycin (Zhang et al., 2017), clarithromycin (Sotto et al., 2017) tilmicosin (Yan et al., 2019). On the other hand, only the studies of Fairgrieve et al. (2005) e Shiogiri et al. (2016) evaluated the toxicological effects of AZT in fish. Fairgrieve et al. (2005) demonstrated that Chinook salmon Oncorhynchus tshawytscha exposed orally to AZT did not cause histopathologically significant lesions in gills, head and trunk, kidney, liver, spleen, heart, pyloric caeca, upper intestine, gonad, and brain. Shiogiri et al. (2017) reported only moderate damage in liver, minor histological changes in the gills and no lesions in the kidneys of tilapia Oreochromis niloticus exposed to AZT. A similar investigative scenario has been observed in the relation to studies involving antimalarials of the 4-aminoquinolines class (e.g.: HCQ). Research involving non-target organisms is restricted to groups of invertebrates (e.g. Daphnia magna - Lilius et al., 1994; Lilius et al., 1995; Zurita et al., 2005; Kumar et al., 2008; Rendal et al., 2011), microalgae (Chlorella vulgaris - Zurita et al., 2005), bacteria (Vibrio fischeri - Zurita et al., 2005) and plants (Salix viminalis - Jjemba, 2002; Rendal et al., 2011). In this interim, fish studies are limited to assessing the ecotoxicological effects of chloroquine (CQ), a compound structurally related to HCQ. In Ou et al. (2012), the authors did not report changes in hair cell death of D. rerio lateral line with increased duration of exposure to gentamicin combined with any of the quinoline derivatives (including CQ), unlike Ramesh et al. (2018), who reported enzymological/histopathological alterations in Cyprinus carpio exposed to QC. The study of Davis et al. (2020) is a pioneer in evaluating the in vivo effects of HCQ on freshwater fish. At the time, the authors observed a significant reduction (depending on the tested concentrations) in the number of surviving hair cells of D. rerio larvae exposed to HCQ and CQ.

Thus, taken together, it is evident that studies on the ecotoxicity of AZT and HCQ in aquatic organisms, especially in fish that inhabit potentially polluted freshwater environments are needed. Considering these facts, this study aims to evaluate the toxicity of these drugs, alone and in combination, using as an experimental model adult zebrafish (D. rerio) exposed to environmentally relevant concentrations of AZT and HCQ. Our hypothesis is that the uptake of these drugs by aquatic animals induces changes in different physiological parameters predictive of nutritional

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alteration, REDOX imbalance, and neurotoxicity. Furthermore, based on in silico analysis, we seek to identify putative mechanisms of action of the evaluated drugs. We believe that our study provides insights into the toxicity of AZT and HCQ in the animal model studied and predicts that an increase in the disposal and dispersion of these drugs in the environments could dramatically affect the freshwater ichthyofauna. Furthermore, considering that zebrafish is considered a good translational model for humans (Tal et al., 2020), this study provides some insight that can guide future studies in humans.

2. MATERIAL AND METHODS

2.1. Drugs

Azithromycin (AZT) and hydroxychloroquine (HCQ) used in our study, [similarly to study by Amaral et al. (2019)] were intentionally acquired in common commercial facilities to bring our experimental design as close to the most realistic condition as possible. However, for the preparation of the AZT stock solution, we used AZT dihydrate draggers (500 mg) (Brainfarma Indústria Química e Farmacêutica SA, Anápolis, GO, Brazil) and for the HCQ stock solution, HCQ sulfate draggers (400 mg), manufactured by Apsen Farmacêutica SA (São Paulo, SP, Brazil), were used. Both solutions were prepared by diluting the draggers in acetonitrile solution (0.01 M), according to Shen et al. (2010). From these solutions, the aliquots added to the exposure waters were removed.

The concentrations of AZT and HCQ tested in our study were based on the work of Fernandes et al. (2020) and Olaitan et al. (2014), respectively. Fernandes et al. (2020) reported that AZT was detected in a concentration up to 2.8 µg/L in a river at northern Portugal, while Olaitan et al. (2014) showed that the median concentration of chloroquine (chemically like HQC, its derivative) identified in different water samples from Nigeria was 2.12 µg/L. Therefore, the concentration tested in our study (i.e.: 12.5 µg/L) simulates a potential increase (approximately 6 times) in AZT and HCQ concentrations in aquatic environments, which can be a predictive environmentally relevant concentration, considering the COVID-19 pandemic.

2.2. Model system and experimental design

This study was carried out at the Biological Research Laboratory of Goiano Federal Institute - Urutaí Campus (GO, Brazil). To assess the aquatic toxicity of AZT and HCQ, we used adult zebrafish (D. rerio) at the age group of approximately 6 months presenting body biomass between 0.3 and 0.4 g with mixed sex. D. rerio is a tropical freshwater fish natural to rivers in Southern Asia, mainly in Northern India, Pakistan, Bhutan, and Nepal (Engeszer et al. 2007). This species has been used as model organism in studies about environmental toxicology and ecotoxicology worldwide (Magyary, 2018), besides being considered a translational model for humans (Tal et al., 2020).
Ninety-six healthy adults (i.e., with normal swimming movements and without morphological deformities or apparent lesions) were distributed into four experimental groups (n = 24 fish/group; 4 replicates tanks of six animals/each treatment group). The “AZT” and “HCQ” groups were exposed to water containing 12.5 µg/L of individual drugs, respectively, and the animals from the “AZT + HCQ” group were exposed to water containing both AZT and HCQ (at 12.5 µg/L/each). In the control group (“C”), adult zebrafish were kept in dechlorinated tap water naturally containing only the vehicle solution (0.01 M acetonitrile solution) in an amount proportional to that added in the other experimental groups. The period of exposure was 72 h (static condition). This exposure simulates the animals' ephemeral contact with drugs, since in the natural environment animals can migrate from contaminated places to places free of pollutants and, therefore, the exposure can be relatively short. The animals were kept in tanks (2.2 L), containing dechlorinated water and continuous aeration; and were fed once a day (ad libitum) with commercial fish feed. In addition, the room where the animals were kept had the temperature (24°C ± 2°C) and the luminosity controlled (12-12 h light-dark cycle).

2.3. Toxicity biomarkers

2.3.1. Quantification of drugs

2.3.1.1. Azithromycin

The AZT uptake by zebrafish was assessed according to the methodology adopted by Keskar et al. (2015), with little modifications. It was used 8 animals/group, weighing approximately 350 mg/animal, which were euthanized (immersion in ice-slurry) and subsequently macerated in 1 mL of phosphate buffered saline (PBS), and centrifuged at 13,000 rpm for 5 min (at 4°C). Aliquots of 30 µL of the sample supernatant were transferred to test tubes (previously sanitized) and mixed with 470 µL of acetonitrile solution (0.01 M), 500 µL of bromocresol green solution (0.0002 M) and 1.5 mL of acetonitrile-ethanol solution (1:1). Then, the samples were shaken and homogenized in a vortex shaker for 5 s and, sequentially, 200 µL of each sample were transferred to a 96-well microplate (in duplicate), for later reading at 630 nm, in an ELISA reader. In parallel, a standard curve was made using known concentrations of AZT (0, 0.03, 0.05, 0.0752, 0.1, 0.25, 0.4, 0.5, 0.6 and 0.7 mg/mL) and the equation of a straight line generated was used to determine the concentrations of the test samples. The background fluorescence of the control samples was determined and subtracted from the samples from the zebrafish exposed to AZT.

2.3.1.2. Hydroxychloroquine

The procedures used for the quantification of HCQ followed the recommendations of Bergqvist et al. (1985), with some modifications. The supernatant of the same 8 animals/group mentioned above was used. In that case, 200 µL aliquot of supernatant from each sample was
transferred to previously cleaned hygienic conical bottom microtubes and, sequentially, 400 µL of the bromothymol blue solution (0.65 mmol/L) and 600 µL of dichloromethane P.A. were added sequentially. Then, the solutions were homogenized in a vortex mixer (for 30 s) and centrifuged at 1,500 rpm, for 5 min, at 23°C. Subsequently, the aqueous phase of the mixture was discarded and 200 µL of the organic phase was transferred to a 96-well microplate, for later reading at 405 nm, in an ELISA reader. The concentrations of HCQ in the samples were determined from the equation of the straight line obtained by making a standard curve, using known concentrations of HCQ (0, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL). The background fluorescence of the control samples was also determined and subtracted from the samples from zebrafish exposed to HCQ.

2.4. Biochemical analyses

2.4.1. Sample preparation

Prior to biochemical assessments, the samples to be analyzed were prepared, similarly to Guimarães et al. (2021). Eight fish/group were also weighed (approximately 350 mg/animal), euthanized (immersion in ice-slurry), macerated in 1 mL of phosphate buffered saline (PBS), and centrifuged at 13,000 rpm for 5 min (at 4°C). The supernatant was separated into aliquots to be used in different biochemical evaluations. Entire bodies were used in the experiment due to difficulties on isolating certain organs from small animals. Organ “contamination” by organic matter and/or by other particles consumed by zebrafish can be bias at biochemical analysis applied to organs at dissection time (Lusher et al. 2017; Guimarães et al., 2021). Samples were stored in sterile conical bottom microtubes at 80°C for a maximum of 7 days.

2.4.2. Assessment of nutritional status

Previous reports on the exposure of different aquatic organisms to different drugs can affect animals’ feeding behavior and change their energy metabolism (Mennigen et al., 2010; Burkina et al., 2015; Falfushynska et al., 2019; Barros et al., 2020). Thus, the influence of treatments on total proteins, triglycerides, and total soluble carbohydrate levels was herein assessed. Total proteins and triglycerides concentrations were determined by using commercial kits, based on the Lowry method (Lowry et al., 1951) (Ref. BT1000900; BioTécnica, Varginha, MG, Brazil) and on the enzymatic colorimetric method by using glycerol-3-phosphate oxidase (GPO) (Ref. BT1001000; BioTécnica, Varginha, MG, Brazil) (Sullivan et al., 1985), respectively. Total soluble carbohydrate levels were performed based on the methodology proposed by Dubois et al. (1956).

2.4.3. Oxidative stress biomarkers
The effects of exposure to AZT and HCQ (alone or in combination) on oxidative stress reactions were evaluated based on (i) indirect nitric oxide (NO) (via nitrite measurement; NO$_2^-$) (Soneja et al. 2005); (ii) thiobarbituric acid reactive substances (TBARS) [predictive of lipid peroxidation (De-Leon & Borges, 2020)]; (iii) production of reactive oxygen species (ROS), and (iv) hydrogen peroxide (H$_2$O$_2$) [which plays an essential role in responses to oxidative stress in different cell types (Sies, 2020)]. The Griess colorimetric reaction [as described in Bryan et al., (2007)] was used to measure NO$_2^-$ and the TBARS levels were determined based on procedures described by Sachett et al. (2018). The production of H$_2$O$_2$ and ROS was evaluated according to El nemma et al. (2004) and Maharajan et al. (2018), respectively.

2.4.4. Neurotoxicity

The possible neurotoxic effects induced by AZT and HCQ (alone and in combination) were evaluated by determining the activity of acetylcholinesterase (AChE) enzymes, according to the method of Ellman et al. (1961). In addition, to assess whether these drugs were able to alter the mechanosensory system of the fish, we performed the count of superficial neuromasts in exposed individuals. For this, we adopted the procedures described in Guimarães et al. (2021), in which, briefly, the live animals (n = 8/group) were placed (for 30 min) in a beaker containing 400 mL of water (with constant aeration) reconstituted with 5 mM of the fluorescent dye 4-(4-Diethylaminostyryl)-1-methylpyridinium iodide (4-Di-2-ASP), from stock solution (40 mg of 4-Di-2-ASP) diluted in 10 mL of dimethyl sulfoxide P.A. Then, the animals were carefully removed and transferred to a beaker containing dechlorinated water (without dye), and remained for 30 min, to remove excess of dye in the body. After that, the animals were euthanized (immersion in ice-slurry) and positioned horizontally on glass slides for later observation under a fluorescence microscope.

The number of positive neuromasts for 4-Di-2-ASP was determined in the region corresponding to the terminal neuromasts (T1, T2 and T3 - region highly conserved in zebrafish - Wada et al., 2008) of the lateral caudal line system of each animal, as well as in the region of the head (Figure 1). Quantification was done manually from sequential images captured in a camera attached to the microscope. Neuromasts located at the bottom of the head were excluded from the count, which generally contained significant amounts of nonspecific staining or because they were out of focus or absent due to the positioning of the animal under the microscope.

**Insert Figure**

**Figure 1.** (A) Head and (B) final portion of the tail of the adult zebrafish (*D. rerio*) where the neuromasts were quantified. T1 to T3: neuromasts’ nomenclature, based on Wada et al. (2008). The white arrows point to the neuromasts.
2.5. Bioinformatics *in silico* analysis

2.5.1. *In silico* chemogenomics-based ChemDIS system analysis

To assess the effects of potential interactions between AZT and HCQ and their possible targets in animals, we used a chemogenomics-based system called ChemDIS-Mixture (Tung et al., 2018), which is built using the previously introduced ChemDIS (Tung, 2015) and statistical p-tests combined with Venn diagram tools available by using the STITCH database (Szklarczyk et al., 2016). To enable the inference of chemical-induced effects, ChemiDIS-Mixture several databases are downloaded and integrated into a MongoDB database including STITCH 5, Reactome, SMPDB, miRTarBase, Ensemble, DOSE, DO.db, KEGG.db and org.Hs.eG.db. Currently, > 430,000 chemicals with > 15 million chemical–protein interactions can be analyzed using ChemDIS-Mixture (Tung & Wang, 2018). For each drug (AZT and HCQ) the possible interacting proteins were extracted, and the enrichment analysis was conducted based on a hypergeometric test for identifying the enriched GO (Gene Ontology) terms with an adjusted p-value < 0.05 using Benjamini-Hochberg multiple test correction.

2.5.2. Interaction networks analysis

To complement the analysis of the possible interactions between AZT and HCQ and their target molecules, we carried out an analysis of network building and functional annotation enrichments, through the STITCH 4.0 Resource (http://stitch.embl.de). The network of each individual drug and in combination was built to assess the possible modes of action of the drugs, considering the thickness of the network lines (thicker lines represent stronger associations). Furthermore, lines and, for directed edges, arrows of different colors stand for different edge types in the actions view: binding (blue), activation (green), inhibition (red), catalysis (magenta), same activity (cyan) and reaction (black) (Kuhn et al., 2008). Statistical significance was determined by corrected p-value < 0.05, using the Bonferroni test. We only considered the shortest paths (allowing no more than five interactions with the highest confidence score > 0.8 to ensure a high level of confidence for the interaction).

2.5.3. Molecular docking

To predict the binding sites and affinity of the bonds among AZT, HCQ and the protein structures of the enzymes AChE, BChE, SOD and CAT, we performed docking and chemoinformatic screens (Kolb et al., 2009). The ligands AZT (CSID: 10482163) and HCQ (CSID: 3526) were obtained from the virtual repository Chemspider (http://www.chemspider.com/) and optimized with force field type MMFF94 in Avogadro software (Hanwell et al., 2012). The protein
structures (targets) of the zebrafish were obtained by the homology construction technique by the SWISS-MODEL server (https://swissmodel.expasy.org/) with structural similarity values between 87.14% and 99.8%. The validation of the structures was verified with the SAVES v.6.0 server (https://saves.mbi.ucla.edu/). For molecular docking simulations, AutoDock tools (ADT) v4.2 were used to prepare binders and targets (Morris et al., 2009) and AutoDock Vina 1.1.2, to perform the calculations (Trott & Olson, 2010). The binding affinity and interactions between residues were used to determine the best molecular interactions. The results were visualized using ADT, Discovery Studio v4.5 and UCSF Chimera X (Pettersen et al., 2021).

2.5.4. Genomic similarity (zebrafish vs. humans)

The analyzes described above consider the genomic similarity between zebrafish and humans. As defined by Vilella et al. (2009), 71.4% of human genes have at least one zebrafish orthologist. Reciprocally, 69% of zebrafish genes have at least one human ortholog. Among orthologous genes, 47% of human genes have a one-to-one relationship with a zebrafish ortholog. The second largest class of ortholog contains human genes that are associated with many zebrafish genes (the “one-human-to-many-zebrafish” class) class), with an average of 2.28 zebrafish genes for each gene human [see details in Howe et al. (2013)].

2.6. Statistical analysis

GraphPad Prism Software Version 8.0 (San Diego, CA, USA) was used to perform the statistical analysis. Initially, data were checked for deviations from normality of variance and homogeneity of variance before analysis. Normality of data was assessed by use of the Shapiro-Wilk test, and homoscedasticity was assessed by use of Bartlette’s test. Multiple comparisons were performed using a one-way ANOVA and Tukey’s post-hoc analysis (for parametric data) or Kruskal-Wallis test, with Dunn’s post-hoc (for non-parametric data). Correlation analyses were performed through Pearson tests (for parametric data) or Spearman tests (for non-parametric data). Significance level adopted for all analyses was alpha = 0.05

3. RESULTS AND DISCUSSION

3.1. AZT and HCQ detection (uptake)

Our data revealed that the exposure to AZT and HCQ, even in a short period (72 h), allowed their absorption by adult zebrafish (Figure 2). The concentrations of AZT in the body tissues of the zebrafish were higher than those of HCQ in individuals exposed to the drugs alone and in combination (Figure 2). In the “AZT” and “AZT + HCQ” groups, AZT concentrations were 84.7% and 80.9% higher than those of HCQ detected in the “HCQ” and “AZT + HCQ” groups,
respectively (Figure 2). In addition, we observed that the exposure to the combination of drugs did not influence the uptake of AZT (Figure 2). Similar results were found in tadpoles (Luz et al., 2021). According to Luz et al. (2021), Physalaemus cuvieri tadpoles (stage 26G) that were exposed to AZT, HCQ and the combination of these two drugs (72 h; 12.5 µg/L of both drugs) showed an AZT concentration almost 70% higher than those of HCQ (in the HCQ and AZT + HCQ groups). When compared to other drugs such as erythromycin, AZT also showed a higher accumulation. In Fall Chinook salmon (Oncorhynchus tshawytscha) (exposed to azithromycin 30 mg/kg fish, for 14 days), this accumulation was 95% higher in fry, and 4.4% higher in smolts (Fairgrieve et al., 2005). In addition, AZT had greater tissue persistence (> 76 d after treatment ceased) than erythromycin (21 d post-treatment) (Fairgrieve et al., 2005). These authors did not find any histopathological changes in the trunk kidney or other organ tissues and attributed this prolonged retention of azithromycin in *O. tshawytscha* to an increase in the efficacy of that antibiotic. However, it has been reported that macrolide antibiotics, such as AZT, can promote hepatotoxicity in larval zebrafish, such as liver degeneration, alterations in liver size and hepatic steatosis (Zhang et al., 2020).

Hand & Hand (2002) reported that AZT can accumulate much more in human polymorphonuclear leukocytes than other antibiotics. These authors evaluated specific characteristics and mechanisms of AZT interactions with human polymorphonuclear leukocytes and demonstrated that an extracellular antibacterial activity of drugs is related to the release of this intra-phagocyte drug at the sites of infection. Therefore, AZT is highly accumulated and slowly released. This may justify the long time that this drug remain in the *Oncorhynchus tshawytscha* organs as reported by Fairgrieve et al. (2005). Furthermore, it helps us to understand our results of higher uptake of AZT in relation to HCQ. Interestingly, Klempner & Styrt (1983) demonstrated that some drugs, including chloroquine, caused an alkalinization of the intralysosomal pH, which resulted in the inhibition of neutrophil degranulation. Similar results were also found by Dey & Bishayi (2015), in a study of murine peritoneal macrophages. This may indicate that HCQ can further assist in the accumulation of AZT.

**Figure 2.** Concentrations of azithromycin (AZT) and hydroxychloroquine (HCQ) in the body tissues of *D. rerio* adults, after 72 hours of exposure. The bars represent the mean ± SEM, the data was submitted to one-way ANOVA, with Tukey's post-test, at 5% probability. AZT: group exposed to azithromycin (12.5 µg/L); HCQ: group exposed to hydroxychloroquine (12.5 µg/L); AZT (MIX) and HCQ (MIX): represent the animals exposed to the binary combination of drugs, with the individual quantification of each compound. n = 8 fish/group.

### 3.2. Biochemical effects
We also observed that the uptake of drugs by adult zebrafish was not able to increase significantly or reduce tissue levels of total soluble carbohydrates (Figure 3A). However, drug exposures caused a reduction in total protein levels (Figure 3B). For triglyceride levels, it was possible to observe a reduction only in the “AZT + HCQ” group, compared to the animals in the control group (Figure 3C). On the other hand, we observed an increase in the production of TBARS, H₂O₂, ROS and NO⁻ (Figure 4A-D, respectively) in zebrafish exposed to all treatments. These data suggest that the oxidative stress processes in these animals were enhanced by both AZT and HCQ, without a synergistic, additive, or antagonistic effect of the combined exposure. This result was corroborated by Cook et al. (2006) showing a possible pharmacokinetic interaction between AZT and CQ (chloroquine) in healthy volunteers. Their results indicated no clinically relevant effect of one drug on the other, suggesting that AZT and CQ do not exhibit any direct pharmacokinetic interaction (Cook et al., 2006). However, triglyceride data demonstrated synergistic negative effect of the two drugs on the triglyceride values (Figure 3C). Altogether, these data suggest that combination of two drugs can influence energy metabolism in adult zebrafish. To our knowledge, there are not many reports in the literature about the influence of AZT in reducing triglyceride levels. Interestingly, HCQ generally has protective actions against dyslipidemia (high blood lipid levels). This can lead to a reduction in cardiovascular diseases, systemic lupus erythematosus and rheumatic diseases (Cairoli et al., 2012; Masui et al., 2019; Morris et al., 2011). However, the consequences of the synergistic negative effect of AZT and HCQ on triglyceride levels still need to be further studied.

The TBARS, H₂O₂, ROS and NO⁻ levels in zebrafish differed between the groups exposed to the drugs. Additionally, our analyses show a positive and significant correlations between these different biomarkers (Figure 5). However, the same treatments did not produce similar effects in P. cuvieri tadpoles (Luz et al., 2021). This result suggest a species-specific type of response. Since some species such as Daphnia magna and Dicentrarchus labrax also show an increase in biomarkers of oxidative stress, while other species such as Oreochromis niloticus, these markers were not affected (Li et al., 2020; Mhadhbi et al., 2020; Shiogiri et al., 2017). It is essential to note that studies that assess biomarkers of oxidative stress induced by HCQ in aquatic organisms are extremely limited. Therefore, it is important that the impacts of HCQ on the aquatic environment are evaluated, especially when this drug is associated with other drugs of indiscriminate use.

**Insert Figure**

**Figure 3.** (A) Total soluble carbohydrates, (B) total proteins and triglycerides levels in body tissues of *D. rerio* adults exposed or not to azithromycin (AZT) and hydroxychloroquine (HCQ). The bars represent the mean ± SEM, and the data were submitted to one-way ANOVA, with Tukey’s post-test, at 5% probability. Different lowercase letters indicate differences among experimental
groups. C: control group; AZT: group exposed to azithromycin (12.5 µg/L); HCQ: group exposed to hydroxychloroquine (12.5 µg/L); AZT + HCQ: represent animals exposed to the binary combination of drugs. n = 8 fish/group.

Insert Figure

Figure 4. (A) Production of thiobarbituric acid reactive substances (TBARS), (B) hydrogen peroxide (H₂O₂), (C) reactive oxygen species (ROS) and (D) nitrite (NO₂⁻) in body tissues of D. rerio adult exposed or not to azithromycin (AZT) and hydroxychloroquine (HCQ). The bars represent the mean + SEM (in “A, B and D”), data were submitted to one-way ANOVA, with Tukey’s post-test (in “A, B and D”) and to Kruskal-Wallis test, with Dunn’s post-test (in “C”), both at 5% probability. Different lowercase letters indicate differences among experimental groups. C: control group; AZT: group exposed to azithromycin (12.5 µg/L); HCQ: group exposed to hydroxychloroquine (12.5 µg/L); AZT + HCQ: represent animals exposed to the binary combination of drugs. n = 8 fish/group.

Insert Figure

Figure 5. Spearman correlation matrix of the biomarkers "hydrogen peroxide (H₂O₂)", "oxygen reactive species (ROS)", "nitrite (NO₂⁻)" and "thiobarbituric acid reactive substances (TBARs)". Correlation coefficients (r) appear on the bottom triangle (beige), and a graphical display of these values appears on the top triangle (white). The number of asterisks denote the significance of the correlation: * denotes p value <0.03, ** p value <0.01, *** p value <0.001, and **** p value <0.0001. Blue-tinted ellipses represent positive correlations. The boldness of the color and shape of the circle represent the strength of the relationship between variables, with stronger correlations having bolder colors and narrower circles.

3.3. Oxidative stress and molecular docking

We performed different in silico analyzes to comprehend the mechanisms of action that led to increased oxidative stress in adult zebrafish exposed to drugs. Initially, we evaluated through molecular docking the plausibility of the interactions between AZT and HCQ with the molecular structure of the enzymes superoxide dismutase (SOD) and catalase, both considered in the frontline of antioxidant defense. As it can be seen in Figure 6, our analyzes predicted a strong affinity between the drugs and their potential antioxidant targets, as well as the existence of interactions with residues from all tested moorings. The binding energies required for AZT and HCQ to bind to catalase were -
8.1 ± 0.71 kcal/mol and -6.6 ± 0.36 kcal/mol (mean ± SD), respectively. The energies expected for the binding between drugs and SOD were -7.1 ± 0.7 kcal/mol (for AZT and SOD) and -6.8 ± 0.19 kcal/mol (for HCQ and SOD) (Figure 6). In addition, the analysis of the interactions showed that AZT reacted with the catalase by means of conventional and carbon hydrogen bond, involving the amino acids Asn338, Gln415 and Thr381 (Figure 7A-B) and the interactions between HCQ and catalase were of the type of conventional hydrogen bond, Pi-Pi Stacked and Pi-Alkyl, involving the amino acids Phe356 and Asp157 (Figure 7C-D). In relation to SOD, the interaction with AZT occurred through conventional and carbon hydrogen bond (Arg170, Gly168 and Asn166) (Figure 7E-F) and with HCQ, through interactions of the conventional hydrogen bond and Pi- Alkyl (Ala179, Gln180 and Lys30) (Figure 7G-H).

Insert Figure

**Figure 6.** Graphical representation of the binding energies (in kcal/mol) of molecular docking between azithromycin (AZT) and hydroxychloroquine (HCQ) with their potential antioxidant targets such as catalase and superoxide dismutase (SOD). Values were calculated by the software AutoDock Vina.

Insert Figure

**Figure 7.** Two-dimensional/three-dimensional representation and residues of interaction between azithromycin (AZT) and hydroxychloroquine (HCQ) with their potential antioxidant targets. (A-B) AZT-catalase, (C-D) HCQ-catalase; (E-F) AZT-SOD and (G-H) HCQ-SOD. SOD: superoxide dismutase.

The pharmacokinetics of AZT are characterized by exceptionally low serum concentrations and wide distribution in tissues (Hand & Hand, 2002). A high concentration of AZT has been proceeded in murine and human phagocytic cells by several authors (Bonnet & Van der Auwera, 1992; Fietta et al., 1997; Gladue et al., 1989; Meyer et al., 1993; Rakita et al., 1994; Stamler et al., 1994). When macrophages trigger an explosion of respiratory activity, there is an increased production of ROS, such as the superoxide anion and H.\textsubscript{2}O\textsubscript{2} that can damage lipids, proteins, and nucleic acids (Dey & Bishayi, 2015). However, some authors have reported that AZT is not able to induce oxidative stress by attenuating the membrane destabilizing effect of bioactive phospholipids (Anderson et al., 1996; Dey & Bishayi, 2015). In fact, some species such as tilapias (O. niloticus) and tadpoles (P. cuvieri) did not show changes in ROS levels when exposed to AZT (Luz et al., 2021; Shiogiri et al., 2017). However, our data revealed that in zebrafish, AZT was able to generate ROS and we also demonstrated through molecular docking that AZT and HCQ also interact with
antioxidant enzymes such as SOD and catalase. Similar results were demonstrated by Yan et al. (2019), in which zebrafish embryos were exposed to macrolide antibiotics, including AZT. Their results indicated severe toxicities in the development of this species, in addition to increased oxidative stress, decreased SOD activities and increased MDA content. This indicates that antibiotics such as AZT can cause damage to the zebrafish and this needs to be further investigated through biochemical and molecular biological investigations.

Notwithstanding, CQ acts in the production of H.O.₂ and superoxide anion, demonstrating its bactericidal effect in terms of ROS production more accentuated than AZT (Abrantes et al., 2008). These results corroborate our data and all together indicate that these two drugs may have different mechanisms of action due to oxidative stress. In addition, it is likely that there is a failure in the response of antioxidants, since, in this study, the oxidative stress generated by AZT and HCQ was not well orchestrated.

3.4. Interaction network

We also explored the putative pathways, integrating the investigated drugs with different proteins in a metabolite-protein interaction network. According to the STITCH interaction network, AZT and HCQ were linked to different metabolic pathways that may also explain the increase in oxidative stress observed in the evaluated animals. AZT showed a strong interaction with different cytochrome P450 family members, family 3, subfamily A (CYP3A5, CYP3A4 and CYP3A7) (Figure 8A) and HCQ to caspase-3 (Figure 8B). It has been shown that in fish, as in other animals, xenobiotic biotransformation carried out by liver cytochromes P-450 and antioxidant defense system play an important role in maintaining cellular homeostasis (Burkina et al., 2015; Westphal, 2000). Thus, CYP450 activity is a crucial factor determining the detoxification abilities of living organisms.

The activation of caspase 3 by HCQ is very well reported in the literature. According to Boya et al. (2003), HCQ causes mitochondrial release of cytochrome c and activates caspase-3. The same effect was reported in bladder cancer cells treated with HCQ (Lin et al., 2017), in malignant B cells of 20 patients with chronic B lymphocytic leukemia treated with HCQ (Lagneaux et al., 2001; Lagneaux et al., 2002) and in culture of rheumatoid synoviocytes, suggesting that HCQ can exert its anti-rheumatic effect on rheumatoid joints through these mechanisms (Kim et al., 2006).

In this regard, for both drugs, functional enrichment analysis demonstrated that the binding of AZT and HCQ and their target molecules involved different biological processes and molecular mechanisms in the cytosol, including ROS metabolism and regulation of nitric-oxide synthase activity, in addition to other enzymes and proteins that participate in REDOX homeostasis (Table 1).

Insert Figure
Figure 8. Network analysis results using the Search Tool for Interactions of Chemicals (STITCH) to explore the interaction between azithromycin (AZT) and hydroxychloroquine (HCQ) with their different target molecules (A) AZT and (B) HCQ assessed separately. (C) AZT and HCQ assessed together. Splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Small nodes: protein of unknown 3D structure. Large nodes: some 3D structure is known or predicted. Colored nodes: query proteins and first shell of interactors. White nodes: second shell of interactors.

Table 1. Functional enrichment analysis for investigating the biological processes involved in the interaction between azithromycin (AZT) and hydroxychloroquine (HCQ) with their different target molecules.

| Pathway ID | Pathway description                                                                 | Count in gene set | False discovery rate |
|------------|--------------------------------------------------------------------------------------|-------------------|----------------------|
| GO:0072593 | Reactive oxygen species metabolic process                                           | 12                | 1.3 x 10^{-11}       |
| GO:0046209 | Nitric oxide metabolic process                                                      | 8                 | 3.4 x 10^{-9}        |
| GO:0050999 | Regulation of nitric-oxide synthase activity                                        | 6                 | 4.68 x 10^{-7}       |
| GO:0006979 | Response to oxidative stress                                                        | 10                | 8.08 x 10^{-6}       |
| GO:0000302 | Response to reactive oxygen species                                                 | 8                 | 1.57 x 10^{-6}       |
|            | **Molecular function (GO)**                                                         |                   |                      |
| GO:0004601 | Peroxidase activity                                                                | 6                 | 6.36 x 10^{-6}       |
| GO:0016209 | Antioxidant activity                                                               | 7                 | 6.36 x 10^{-6}       |
| GO:0016491 | Oxidoreductase activity                                                             | 13                | 6.36 x 10^{-6}       |
| GO:0004602 | Glutathione peroxidase activity                                                     | 5                 | 8.43 x 10^{-6}       |
| GO:0020037 | Heme binding                                                                       | 7                 | 1.57 x 10^{-6}       |
|            | **Cellular component (GO)**                                                        |                   |                      |
| GO:0005829 | Cytosol                                                                            | 19                | 0.000465             |
|            | **Hydroxychloroquine**                                                             |                   |                      |
| GO:0072593 | Reactive oxygen species metabolic process                                           | 11                | 7.96 x 10^{-11}      |
| GO:0046209 | Nitric oxide metabolic process                                                      | 8                 | 1.1 x 10^{-9}        |
| GO:0050999 | Regulation of nitric-oxide synthase activity                                        | 6                 | 3.97 x 10^{-7}       |
| GO:0001666 | Response to hypoxia                                                                | 8                 | 1.32 x 10^{-6}       |
| GO:0032496 | Response to lipopolysaccharide                                                      | 8                 | 1.32 x 10^{-6}       |
|            | **Molecular function (GO)**                                                         |                   |                      |
| GO:0004517 | Nitric-oxide synthase activity                                                     | 3                 | 7.5 x 10^{-7}        |
| GO:0034617 | Tetrahydrobiopterin binding                                                        | 3                 | 9.99 x 10^{-7}       |
| GO:0034618 | Arginine binding                                                                   | 3                 | 9.99 x 10^{-7}       |
| GO:0003958 | NADPH-hemoprotein reductase activity                                                | 3                 | 9.74 x 10^{-7}       |
| GO:0050661 | NADP binding                                                                       | 4                 | 0.000205             |
|            | **Cellular component (GO)**                                                        |                   |                      |
| GO:0005829 | Cytosol                                                                            | 13                | 0.00906              |
|            | **Azithromycin AND Hydroxychloroquine**                                            |                   |                      |
3.5. Chemical-chemical interaction (via ChemDIS-Mixture)

To deepen the prediction of possible mechanisms of action responsible for the effects observed in our study, we performed an analysis of chemical-chemical interaction (involving the tested drugs and different molecules). In addition, we evaluated the potential specific biological endpoint resulting from these interactions. We identified from the ChemDIS-Mixture tool a total of 446 proteins that can interact with AZT or HCQ. Of these, 255 were specific for AZT, 178 for HCQ and 13 proteins are shared between drugs (Figure 9). We also observed that among the top ten most significant hits for the targets responsible for the effect of interaction with AZT (i.e., with a score ≥ 0.8), 70% are proteins directly or indirectly related to oxidative stress (catalase, cytochrome P450 family 3 subfamily A member 4, glutathione S-transferase alpha 3, glutathione S-transferase alpha 1, glutathione S-transferase alpha 4, glutathione S-transferase alpha 2, cytochrome P450 family 3 subfamily A member 5, cytochrome P450 family 3 subfamily A member 7) (Figure 9A), which is similar to what was observed in the interaction network analysis above. In relation to HCQ, the main targets (i.e., score ≥ 0.8) included caspase 3 and toll like receptors (Figure 9B), thus covering the pathways by which the drug may have induced an increased in oxidative stress. Among the protein targets shared by both AZT and HCQ, our analysis showed interleukin 6 (IL-6) as a target in which the scores for both drugs were higher than 0.825 (Figure 9).

The anti-inflammatory effects of HCQ already discussed in this article, such as interference with lysosomal acidification and inhibition of phospholipase absorption, are also accompanied by the inhibition of toll-like receptor signals, inhibition of T and B cell receptors and, mainly, the decreased production of macrophage cytokines such as interleukin (IL) -1 and IL-6 (Ben-Zvi et al., 2012). In this
manner, HCQ controls the inflammatory response since inhibition of cytokines such as IL-6 decreases tissue damage and endothelial inflammation (Moudgil & Choubey, 2011).

The antioxidant effects of AZT alone and combined with HCQ was observed in *P. cuvieri* tadpoles. In this species, SOD and catalase were increased when exposed to these drugs and possibly acted to maintain the basal production of NO, ROS, TBARS and H.O₂ (Luz et al., 2021). The antioxidant effects of AZT alone and combined with HCQ was observed in *P. cuvieri* tadpoles. In this species, SOD and catalase were increased when exposed to these drugs and possibly acted to maintain the basal production of NO, ROS, TBARS and H₂O₂ (Luz et al., 2021). Already the increase in ROS presented in our article is suggestive of a failure in the antioxidant response that can be attributed to the interaction of drugs with the main antioxidant enzymes, SOD, and catalase. However, further studies must be conducted to elucidate this hypothesis.

**Figure 9.** Venn diagram comparing the protein-protein interaction with azithromycin or hydroxychloroquine or both. (A-C) Summarized information on the most significant results for the targets responsible for the effect of interaction with (A) azithromycin (AZT), (B) hydroxychloroquine (HCQ) and (C) AZT/HCQ.

**3.6. Gene ontology**

To provide an overview of the main processes, molecular mechanisms, and cellular localization of proteins with potential interaction with AZT and/or HCQ, we conducted an ontology (GO) analyze gene. In this analysis, 674 genes responsive to drugs were identified, 293 to AZT, 264 to HCQ and 117 shared between AZT and HCQ. Biological process analysis indicated that proteins with a strong interaction with AZT act mainly in processes related to glutathione metabolism and cellular oxidant detoxification; acting on molecular mechanisms involving the activity of various enzymes, especially glutathione transferase, which expands the findings of molecular docking. In addition, our analysis revealed that these proteins are in different cytoplasmic elements/structures, such as in the mitochondrial matrix and in the NADPH oxidase complex. Figure 10 shows the GO prediction of the biological process, molecular mechanism and cellular compartment of proteins that interact with AZT, highlighting the number of genes involved (Figure 10A) and the increasing order of significance observed (Log10 p value) (Figure 10B).

The proteins that interacted strongly with HCQ act mainly in biological processes related to glucuronidation (one of the phase II reactions of elimination of xenobiotics through biotransformation) and with flavonoid biosynthetic process, through molecular mechanisms that include, especially, related to ligand binding and glucuronosyltransferase activity (Figure 11).
addition, cellular compartment prediction confirmed that these proteins are identified especially in the intracellular environment, including autophagosomal and endocytic vesicles, as well as in organelle membranes (Figure 11). On the other hand, the proteins shared between AZT and HCQ act in processes that involve nucleophagy, macroautophagy, immune response (from the induction of inflammatory response), as well as in oxidation-reduction process, especially through ligand binding mechanisms that involve the activity of different enzymes (Figure 12). Such proteins are found, especially in the part of the cytoplasm that does not contain organelles, but which does contain other particulate matter, such as protein complexes (cytosol), lipid bilayer surrounding the endoplasmic reticulum and extracellular exosome, i.e., vesicle that is released into the extracellular region by fusion of the limiting endosomal membrane of a multivesicular body with the plasma membrane (Figure 12).

**Insert Figure**

**Figure 10.** Gene Ontology (GO) classification of differentially expressed genes related exclusively to azithromycin. The differentially expressed genes are grouped into three hierarchically structured terms: biological process, cellular component, and molecular function. In “A” the number of genes is presented and in “B” the increasing significance (Log10 P value) of each GO annotation.

**Insert Figure**

**Figure 11.** Gene Ontology (GO) classification of differentially expressed genes related exclusively to azithromycin. The differentially expressed genes are grouped into three hierarchically structured terms: biological process, cellular component, and molecular function. In “A” the number of genes is presented and in “B” the increasing significance (Log10 P value) of each GO annotation.

**Insert Figure**

**Figure 12.** Gene Ontology (GO) classification of differentially expressed genes related exclusively to azithromycin. The differentially expressed genes are grouped into three hierarchically structured terms: biological process, cellular component, and molecular function. In “A” the number of genes is presented and in “B” the increasing significance (Log10 P value) of each GO annotation.

### 3.7. Neurotoxicity (acetylcholinesterase/molecular docking and neuromasts)

Regarding the evaluation of AChE activity, we observed that the combination “AZT + HCQ” induced a cholinesterasic effect in the adult zebrafish, as indicated by the increased enzyme activity, as compared to the control group (Figure 13A). In agreement, molecular docking analyzes predicted a strong affinity between drugs and AChE [binding energy required for AZT and AChE binding: \(-8.2 \pm 0.48\) kcal/mol and binding energy required for binding of AZT and AChE: HCQ and AChE: \(-6.9 \pm\)
0.36 kcal/mol (mean ± SD) (Figure 13B). These interactions involved conventional bonds, carbon-hydrogen bond and π-Alkyl, involving the amino acids Leu590, Leu587, Trp583, Asn584, Asp331, Thr260, His387 (for AZT; Figure 13C) and Thr541 and Arg533 (for HCQ; Figure 13D). When evaluating the number of neuromast, treatments did not affect their number in the tail of the zebrafish (Figure 14A). In contrast, a reduction was observed in the head of animals exposed to AZT (alone) or in combination with HCQ (Figure 14B). This result seems to indicate that AZT alone and associated with HCQ can destroy hair cells in zebrafish. These hair cells are mechanosensorial cells existing within neuromasts and have similarities to the cells present in the mammalian ear. Both in the inner ear of mammals and in the lateral line of the zebrafish, these cells are sensitive to drugs (Harris et al., 2003; Hernández et al., 2006; Murakami et al., 2003; Nakashima et al., 2000; Ton & Parng, 2005; Williams & Holder, 2000).

Our results also indicate an extraordinarily strong interaction between AZT and HCQ with AChE. However, its effects on AChE occurred only when the drugs were combined. In fact, AZT appears to have an inhibitory effect on AChE in European sea bass (Dicentrarchus labrax) and tadpoles (P. cuvieri) (Luz et al., 2021; Mhadhbi et al., 2020). The same can be observed for HCQ (Luz et al., 2021). Interestingly, Luz et al. (2021) demonstrated that the association of the drugs AZT and HCQ decreases the levels of AChE in tadpoles. Our data show the opposite for zebrafish. The combination of AZT and HCQ induced an increase in AChE and this increase indicates a consequence of environmental exposure to neurotoxic pollutants (Senger et al., 2011; Van Dyk & Pletschke, 2011), as well as the combination of AZT and HCQ in zebrafish.

**Insert Figure**

**Figure 13.** (A) Activity of the enzymatic acetylcholinesterase (AChE) in the body tissues of *D. rerio* adults, exposed or not to drugs. The bars represent the mean±SEM, and the data were submitted to one-way ANOVA, with Tukey's post-test, at 5% probability (n=8 fish/group). (B) Graphical representation of the binding energies (in kcal/mol) of molecular docking between the ligands azithromycin (AZT) and hydroxychloroquine (HCQ) and the target “acetylcholinesterase”, calculated by the AutoDock Vina software. (C-D): Two-dimensional/three-dimensional representation and residues of interaction between the ligands (C) azithromycin (AZT) and (D) hydroxychloroquine (HCQ) with the target “acetylcholinesterase”.

**Insert Figure**

**Figure 14.** Number of neuromasts identified in (A) tail and (B) head of *Danio rerio* adults, exposed or not to drugs. The bars represent the mean ± SEM, and the data were submitted to one-way ANOVA, with Tukey's post-test, at 5% probability. In “B”, different lowercase letters indicate
significant differences between the experimental groups. C: control group; AZT: group exposed to azithromycin (12.5 µg/L); HCQ: group exposed to hydroxychloroquine (12.5 µg/L); AZT+HCQ: represent animals exposed to the binary combination of drugs. n=8 fish/group.

4. CONCLUSION

To sum up, our study confirms the hypothesis that 72 h of exposure to AZT, HCQ or their combination was sufficient to allow the uptake of drugs by zebrafish and induce the reduction of total protein levels, as well as predictive changes in oxidative stress (inferred by TBARS, H.O., ROS and NO levels) and neurotoxicity (sustained by the observation of increased AChE and reduced number of superficial neuromasts). In addition, in silico analyzes suggested that the observed effects are related to different physiological and molecular mechanisms. Thus, future investigations that focus on the effects of the molecular bindings between AZT and HCQ on the kinetics of SOD, catalase, and AChE, as well as on the functions of different cytochrome P450 molecules, caspase-3 and on the glutathione-mediated biotransformation will be useful for confirming predictions provided by the bioinformatic analyzes performed. In addition, assessments related to the biochemical and molecular expression and signals of toll-like receptors and IL-6 will provide new insights into how AZT and HCQ affect the zebrafish immune system. Finally, it is paramount to emphasize that our study is not exhaustive and, therefore, our results are only the “tip of an iceberg” that represents the ecotoxicological effects arising from the tested drugs. Therefore, we strongly recommend that further investigations should be carried out to understand the magnitude of the impact of the indiscriminate use of AZT and HCQ, especially in the context of the COVID-19 pandemic, whose environmental concentrations are certain to increase.

5. ACKNOWLEDGMENTS

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6. COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare no conflict of interest.

Ethical approval: All experimental procedures were carried out in compliance with ethical guidelines on animal experimentation. Meticulous efforts were made to assure that animals suffered the least possible and to reduce external sources of stress, pain and discomfort. The current study did not exceed the number of animals necessary to produce trustworthy scientific data. This article does not refer to any study with human participants performed by any of the authors.
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Figure 2

[AZT molecule structure]

HCQ

Azithromycin and hydroxychloroquine concentration (µg/mL)

0.00 0.01 0.02 0.03

AZT group
HCQ group
AZT (MIX) group
HCQ (MIX) group

F(3,28) statistic = 3.389; p value = 0.0018

a

b
Figure 3

(A) Total carbohydrates (mg/g ash) for different treatments: C, HCQ, AZT, AZT+HCQ. The F_{(3,20)} statistic is 1.992, and the p-value is 0.3686.

(B) Total protein levels (g/mL) for different treatments: C, HCQ, AZT, AZT+HCQ. The F_{(3,20)} statistic is 15.18, and the p-value is < 0.0001.

(C) Triglycerides levels (mg/dL) for different treatments: C, HCQ, AZT, AZT+HCQ. The F_{(3,20)} statistic is 5.175, and the p-value is 0.0057.
Figure 4

(A) Thiol-hydroxyl radical reactive substances (nmol/L g protein)
- Strata: C, HCQ, AZT, AZT+HCQ
- Statistic value: F_{[20]} statistic = 5.150; p value = 0.0038

(B) Hydrogen peroxide production (nmol/L g protein)
- Strata: C, HCQ, AZT, AZT+HCQ
- Statistic value: F_{[20]} statistic = 9.292; p value = 0.0001

(C) ROS production (fluorescence intensity/ protein)
- Strata: C, HCQ, AZT, AZT+HCQ
- Statistic value: Kruskal-Wallis statistic = 18.81; p value = 0.0003

(D) Nutric levels (nmol/L g protein)
- Strata: C, HCQ, AZT, AZT+HCQ
- Statistic value: F_{[20]} statistic = 3.585; p value < 0.0001
Figure 5
Figure 6

ΔG, in kcal/mol

AZT/CAT  HCQ/CAT  AZT/SOD  HCQ/SOD
Figure 7

Interactions:
- Green: Conventional hydrogen bond
- Gray: Carbon-hydrogen bond
- Blue: Pi-Pi Stacked
- Pink: Pi-Alkyl
The diagram illustrates complex interactions between various proteins and molecules, including:

- **Proteins/Protein Molecules**: XIAP, BIRC2, CASP3, IL6R, IL6ST, HSP90AA1, CAV1, NOS1, NOSTRIN, NOS1AP, and more.

- **Molecules/Compounds**: malondialdehyde, oxygen, hydrogen peroxide, nitric oxide, and arginine.

The **Action Types** and **Action Effects** are indicated in the legend:

- **Action Types**:
  - Activation
  - Binding
  - Phenotype
  - Reaction
  - Inhibition
  - Catalysis
  - Posttranslational modification
  - Transcriptional regulation

- **Action Effects**:
  - Positive
  - Negative
  - Unspecified
Figure 9

### Table A

| Protein       | Gene Symbol | Entrez gene ID | Gene name                                  | Score |
|---------------|-------------|----------------|--------------------------------------------|-------|
| ENSP00000295246 | HPGDS       | 27306          | Hematopoietic prostaglandin D synthase     | 0.843 |
| ENSP00000244152 | CAT         | 847            | Catalase                                   | 0.843 |
| ENSP00000337914 | NF-25       | 1576           | Cytochrome P450 family 3A4 family A member 4 | 0.830 |
| ENSP00000140148 | GTH2        | 2929           | Glutathione S-transferase alpha 2          | 0.825 |
| ENSP00000352998 | GSTA1       | 2941           | Glutathione S-transferase alpha 4          | 0.825 |
| ENSP00000333620 | GSTA1       | 2958           | Glutathione S-transferase alpha 1          | 0.825 |
| ENSP00000291179 | GSTA3       | 2949           | Glutathione S-transferase alpha 3          | 0.825 |

### Table B

| Protein       | Gene Symbol | Entrez gene ID | Gene name | Score |
|---------------|-------------|----------------|-----------|-------|
| ENSP00000311932 | SCA-1       | 836            | Caspase-3  | 0.912 |
| ENSP00000279034 | TLR7        | 51284          | Toll like receptor 7 | 0.828 |
| ENSP00000338374 | TLR9        | 54106          | Toll like receptor 9 | 0.825 |

### Table C

| Protein       | Gene Symbol | Entrez gene ID | Gene name | AZT | HCQ |
|---------------|-------------|----------------|-----------|-----|-----|
| ENSP00000295743 | BSE-2       | 3369           | Involved in 6   | 0.842 | 0.838 |
Figure 10
Figure 12

| Molecular mechanisms | Biological processes | Cellular component | Description |
|----------------------|----------------------|-------------------|-------------|
| Inflammatory response | Oxidation-reduction process | Pre-autophagosomal structure | AZITHROMYCIN AND YDROXYCHLOROQUINE |
| Nucleophagy           | Regulation of cytokinesis | Extracellular exosome | |
| Macrophtagy           | Macrophtagy           | Endoplasmic reticulum membrane | |
| Cytokine activity     | Nucleophagy           | Cytokine | |
| Steroid binding      | Nucleophagy           | Protein heterodimerization activity | |
| Oxidoreductase activity | Oxidation-reduction process | Nitric-oxide synthase regulator activity | |
| Cytokine activity     | Inflammatory response | Nitric-oxide synthase regulator activity | |
| Inflammatory response | Oxidation-reduction process | Nitric-oxide synthase regulator activity | |
| Nucleophagy           | Regulation of cytokinesis | Nitric-oxide synthase regulator activity | |

| Molecular mechanisms | Biological processes | Cellular component | Description |
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| Inflammatory response | Oxidation-reduction process | Pre-autophagosomal structure | AZITHROMYCIN AND YDROXYCHLOROQUINE |
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| Macrophtagy           | Macrophtagy           | Endoplasmic reticulum membrane | |
| Cytokine activity     | Nucleophagy           | Cytokine | |
| Steroid binding      | Nucleophagy           | Protein heterodimerization activity | |
| Oxidoreductase activity | Oxidation-reduction process | Nitric-oxide synthase regulator activity | |
| Cytokine activity     | Inflammatory response | Nitric-oxide synthase regulator activity | |
| Inflammatory response | Oxidation-reduction process | Nitric-oxide synthase regulator activity | |
| Nucleophagy           | Regulation of cytokinesis | Nitric-oxide synthase regulator activity | |
Figure 13

(A) Acetylcholinesterase activity (nmol/min/mL) for different treatments: C, HCQ, AZT, AZT-HCQ. The F_{(3,29)} statistic is 5.473, with a p value of 0.0043.

(B) ΔG, in kcal/mol, for different combinations: AZT/AChE, HCQ/AChE.

(C) Interactions illustrated for different molecular structures.

(D) A detailed view of interactions with specific markers indicating conventional hydrogen bonds, 1 Carbon hydrogen bonds, and Pi-Alkyl interactions.
ENVIRONMENTAL IMPACTS OF COVID-19 TREATMENT: TOXICOLOGICAL EVALUATION OF AZITHROMYCIN AND HYDROXYCHLOROQUINE IN ADULT ZEBRAFISH

GRAPHICAL ABSTRACT
ENVIRONMENTAL IMPACTS OF COVID-19 TREATMENT: TOXICOLOGICAL EVALUATION OF AZITHROMYCIN AND HYDROXYCHLOROQUINE IN ADULT ZEBRAFISH

HIGHLIGHTS

✓ AZT and HCQ dispersed in the water are uptake by zebrafish
✓ Zebrafish exposed to AZT and HCQ show REDOX imbalance
✓ AZT and HCQ induces changes in total protein levels of zebrafish
✓ Combined exposure to AZT + HCQ induces cholinesterase activity in zebrafish
✓ Superficial neuromats are affected by AZT and HCQ
✓ Mechanisms of action of drugs are proposed by *in silico* analysis