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PII: S0048-9697(21)01621-1
DOI: https://doi.org/10.1016/j.scitotenv.2021.146553
Reference: STOTEN 146553
To appear in: Science of the Total Environment

Received date: 8 February 2021
Revised date: 11 March 2021
Accepted date: 13 March 2021

Please cite this article as: T.M. da Luz, A.P. da Costa Araújo, F.N. Estrela, et al., CAN USE OF HYDROXYCHLOROQUINE AND AZITHROMYCIN AS A TREATMENT OF COVID-19 AFFECT AQUATIC WILDLIFE? A STUDY CONDUCTED WITH NEOTROPICAL TADPOLE, Science of the Total Environment (2021), https://doi.org/10.1016/j.scitotenv.2021.146553

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ABSTRACT

The impacts on human health and the economic and social disruption caused by the pandemic COVID-19 have been devastating. However, its environmental consequences are poorly understood. Thus, to assess whether COVID-19 therapy based on the use of azithromycin (AZT) and hydroxychloroquine (HCQ) during the pandemic affects wild aquatic life, we exposed (for 72 hours) neotropical tadpoles of the species *Physalaemus cuvieri* to the water containing these drugs to 12.5 µg/L. We observed that the increase in superoxide dismutase and catalase in tadpoles exposed to AZT (alone or in combination with HCQ) was predominant to keep the production of NO, ROS, TBARS and H₂O₂ equitable between the experimental groups. In addition, the uptake of AZT and the strong interaction of AZT with acetylcholinesterase (AChE), predicted by the molecular docking analysis, were associated with the anticholinesterase effect observed in the groups exposed to the antibiotic. However, the unexpected increase in butyrylcholinesterase (BChE) in these same groups suggests its constitutive role in maintaining cholinergic homeostasis. Therefore, taken together, our data provide a pioneering evidence that the exposure of *P. cuvieri* tadpoles to AZT (alone or in combination with HCQ) in a predictably increased environmental concentration (12.5 µg/L) elicits a compensatory adaptive response that can have, in the short period of exposure, guaranteed the survival of the animals. However, the high energy cost for maintaining physiological homeostasis, can compromise the growth and development of animals and, therefore, in the medium-long term, have a general negative effect on the health of animals. Thus, it is possible that COVID-19 therapy, based on the use of AZT, affects wild aquatic life which requires greater attention to the impacts that this drug may represent.

Keywords: Pharmaceutical waste, amphibians, freshwater ecosystems, biochemistry, ecotoxicology.
1. INTRODUCTION

It is known that amphibians to comprise one of the most endangered groups (Beebee & Griffiths, 2005; Green et al., 2020), a fact that has been discussed for some time (Wake, 1991, Wake, 1998); but that, more recently, discussions have been more urgent (Green et al., 2020; Bolochio et al., 2020). This is because the increasing loss of natural habitats (Mayani-Parás et al., 2020; Semper-Pascual et al., 2021), increased UV-B irradiation (Lundsgaard et al., 2020; Morison et al., 2020), emergence of emerging diseases (Blaustein et al., 2018; Fisher & Garner, 2020; Brannelly et al., 2021), introduction of non-native species (Nunes et al., 2019), climate change (Bucciarelli et al., 2020) and the increase in pollution of freshwater ecosystems (Wesner et al., 2020; Meindl et al., 2020; Lent et al., 2020) has greatly intensified the reduction and distribution of various species of amphibians.

Regarding the impacts of pollutants on these animals, most studies have directed their designs to assess the effects of classic chemical compounds, such as pesticides and their degradation products, heavy metals, nitrogen-based fertilizer, among others [see review by Blaustein et al. (2003)]. However, fewer ecotoxicological studies addressing the impacts of emerging pollutants on amphibians are less [see reviews by McConnell et al. (2010) and Egea-Serrano et al. (2012)]. Such pollutants include synthetic or natural chemicals that are not part of the list of those included in routine (inter) national monitoring programs, but that have the potential to enter different environmental compartments and cause ecological and/or human health effects (Geissen et al., 2015; Calvo-Flores et al., 2018; NORMA, 2021).

Two chemical compounds considered as emerging pollutants, whose impacts on amphibians have never been studied, refer to azithromycin (AZT) and hydroxychloroquine (HCQ) (Mendez et al., 2017; Dabić et al., 2019; Gomes et al., 2020). While AZT is a macrolide antibiotic which inhibits bacterial protein synthesis (Parnham et al., 2014) also used in the treatment of cancer and autoimmune and inflammatory diseases (Patel & Hashimi, 2020); HCQ is used in the prevention and treatment of malaria (Shippey et al., 2018) and as 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 (Hartzell et al., 2008; Cherry & Kersh, 2020) and photosensitive diseases (Millan & Quijano, 1957). Due to the COVID-19 pandemic (started in late 2019), the use of these drugs has increased considerably (Yazdany & Kim, 2020; Malik et al., 2020; Agarwal et al., 2020; Nasir et al., 2020; Mallhi et al., 2020; Quispe-Cañari et al., 2020), although their effectiveness against SARS-Cov-2 infection is questioned by several studies (Shuka et al., 2020; Ghazy et al., 2020; Jameleddine et al., 2020).

Therefore, the increase in the arrival and dispersion of these drugs in aquatic ecosystems is already a reality, especially due to the dumping of domestic sewage and hospital waste in rivers or
streams or via leaching from landfills, which in many countries do not receive adequate treatment (Ansari et al., 2019; Urban & Nakada, 2021) or the processes used are insufficient to remove these pollutants or are financially inaccessible (Ali et al., 2017; Khan et al., 2019). In cities with a high incidence of COVID-19, for example, 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. In India, for example, after the approval of the Indian Council of Medical Research for the empirical use of HCQ for prophylaxis of COVID-19, the stocks available in pharmacies have been reduced dramatically, especially when hospitals and health professionals began to prescribe the drug to their patients (Chauhan et al., 2020). The King Abdullah University Hospital in Jordan produced, at the height of the pandemic, ten times more medical waste compared to average production during the days before the spread of SARs-CoV-2 (Abu-Qdais et al., 2020). In Spain, an increase of more than 300% was observed (Klemes et al., 2020) and in Asia, it is estimated that the total of hospital waste generated exceeds 16.5 thousand tons/day, with India, followed by Iran, Pakistan, Saudi Arabia, Bangladesh and Turkey are the largest producers of this waste in the context of the COVID-19 pandemic (Sangkham, 2020).

In this sense, as discussed by Farias et al. (2020), questions about the impact that therapy against COVID-19 has on aquatic wildlife. Particularly in amphibians, how can this increase affect the health of these animals and the decline of their natural populations? Thus, to assume the ecotoxicological effects of these drugs on the natural populations of anurans, we exposed tadpoles of *Physalaemus cuvieri* (Anura, Leptodactylidae) to AZT and HCQ (alone or in combination). This species, in particular, occurs in several countries in South America (Mijares et al., 2011; De-Oliveira-Miranda et al., 2019) and, despite not being categorized as “unstable” and “worrying” by International Union for Conservation of Nature (version 2020-3) (IUCN, 2020), its wide geographical distribution and its large populations are characteristics that make them interesting translational models for anurofauna. From different biomarkers, we aim to test the hypothesis that short exposure to AZT and HCQ (in predictive environmentally relevant concentration) induces metabolic changes that alter REDOX homeostasis towards oxidative stress, as well as neurotoxic effects. In addition, through molecular docking analyzes we aim to predict possible interactions of drugs with important target molecules in the neurophysiological responses of animals. As far as our knowledge goes, this is the first report on the exposure of an amphibian species to AZT and/or HCQ.

2. MATERIAL AND METHODS

2.1. Drugs
Azithromycin (AZT) and hydroxychloroquine (HCQ) used in our study, [similar to the study by Amaral et al. (2019)] were intentionally acquired in common commercial facilities in order to bring our experimental design as close to the most realistic condition as possible. For the preparation of the AZT stock solution, we used AZT dihydrate dragees (500 mg) (Brainfarma Indústria Química e Farmacêutica S.A., Anápolis, GO, Brazil) and for the HCQ solution, HCQ sulfate dragees (400 mg), manufactured by Apsen Farmacêutica SA (São Paulo, SP, Brazil) were used. Both solutions were prepared by diluting the pills in acetonitrile solution (0.01 M), according to Shen et al. (2010). From these solutions, the aliquots added to the exposure waters were removed. Table 1 presents general information about the drugs used in our study.

Table 1. General information about the drugs used in our study

| Information | Azithromycin (AZT) | Hydroxychloroquine (HCQ) |
|-------------|-------------------|--------------------------|
| Drug class  | Macrolide antibiotic | Antimalarials |
| IUPAC name  | (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-11-[3,4,6-trideoxy-3-(dimethylamino)-8-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadec-13-yl 2,6-dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranoside | (RS)-2-{[4-[1-(7-chloroquinolin-4-yl)amino]pentyl}(ethyl)amino]ethanol |
| CAS number  | 83905-01-5 | 118-42-3 |
| Formula     | C₃₈H₇₂N₂O₁₂ | C₁₈H₂₆CIN₃O |
| Molar mass  | 748.996 g/mol | 335.87 g/mol |
| Excipient q.s. | Starch, microcrystalline cellulose, sodium lauryl sulfate, silica dioxide, povidone, croscarmellose sodium, magnesium stearate, titanium dioxide, macrogol, and hypromellose | Croscarmellose sodium, titanium dioxide, magnesium stearate, lactose monohydrate, povidone, starch, hypromellose, and macrogol |
| Manufacturer | Brainfarma Indústria Química e Farmacêutica S.A. (Anápolis, GO, Brazil) | Apsen Farmacêutica S.A. (São Paulo, SP, Brazil) |
| Register in the Brazilian Food and Drug Agency (ANVISA), Ministry of Health (Brazil). | 1.5584.0530 | 1.0118.0162 |
1Information provided by the manufacturer (AZT: https://www.bulas.med.br/p/laboratorios/laboratorio/bula/1366728/Azitromicina_di_hidratada__Comprimido_500_mg_.html) and HCQ (https://www.bulas.med.br/p/ulas-de-medicamentos/bula/7229/reuquinol.htm).

2.2. Model system and experimental design

To assess the aquatic toxicity of AZT and HCQ, we used tadpoles of the species *Physalaemus cuvieri* (Leptodactylidae) as a model system. Its wide geographical distribution in South America (Miranda et al., 2019), stability and population abundance in the areas that occur (Frost, 2017), in addition to good adaptability in the laboratory and early biological response to changes in its environment justify the choice of species in our study, as well as in other recent (eco) toxicological studies (Herek et al., 2020; Araújo et al., 2020ab; Rutkoski et al., 2020). All tadpoles used came from three ovigerous masses [containing approximately 1500 eggs/each, according to Pupin et al. (2010)] collected in a lentic environment (Urutai, GO, Brazil) surrounded by native vegetation from the Cerrado biome, under license no. 73339-1 of the Brazilian Biodiversity Information and Authorization System (SISBIO/MMA/ICMBio).

Upon arrival at the laboratory, the eggs were kept in an aquarium (40.1 cm x 45.3 cm x 63.5 cm) containing 80 L of naturally dechlorinated water (for at least 24 h), under controlled light conditions (cycles of 12 h of white light at 100 lux and 12 h of complete darkness), temperature (26 °C ± 1 °C - similar to the natural environment) and constant aeration (maintained by air compressors), being fed once a day (*ad libitum*) with commercial fish feed (guarantee levels: 45% crude protein, 14% ether extract, 5% crude fiber, 14% mineral matter and 87% dry matter). After the eggs hatched, the tadpoles remained in these conditions until they reached stage 26G, according to Gosner (1960) (body biomass: 70 mg ± 4.1 mg and total length: 20.1 mm ± 0.7 mm - mean ± SEM).

Then, 800 healthy tadpoles (i.e., with normal swimming movements and without morphological deformities or apparent lesions) were distributed into four experimental groups (n = 200 tadpoles/each). The “AZT” and “HCQ” groups were exposed to water containing 12.5 µg/L of both drugs (alone) and the animals in the “AZT + HCQ” group were exposed to water containing both AZT and HCQ, simulating the co-presence of drugs in the aquatic environment. The control group (“C”) was composed of tadpole kept in water containing only the vehicle solution (0.01 M acetonitrile solution) in an amount proportional to that added in the other experimental groups.
2.3. Exposure conditions and tested concentrations

All experimental groups were kept in glass containers containing 2 L of naturally dechlorinated water, in which the drugs were diluted, with an exposure period of 72 h, simulating an ephemeral exposure. During the exposure, the animals were fed once a day with commercial fish feed and the waters were not renewed (i.e., static system). The drug concentrations were based on previous studies that identified them in surface waters. Fernandes et al. (2020) reported that AZT concentration of up to 2.8 µg/L was detected in a river in northern Portugal and, in Olaitan et al. (2014), the median concentration of chloroquine (chemically similar to HQC, its derivative) identified in different water samples from Nigeria was 2.12 µg/L. Therefore, the concentration tested in our study (ie: 12.5 µg/L) simulates a potential increase (approximately 6 times) in AZT and HCQ concentrations in aquatic environments (associated with the COVID-19 pandemic), which can be considered a predictive environmentally relevant concentration.

2.4. Toxicity biomarkers

2.4.1. Sample preparation

Prior to biochemical assessments, the samples to be analyzed were prepared, similarly to Guimarães et al. (2021). In this case, we used 96 tadpoles/group, distributed in eight samples composed of a pool of 12 animals/each. These animals were weighed (12.5 g ± 0.0004 - mean ± standard error) and subsequently macerated in 1 mL of phosphate buffered saline (PBS), 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 the hard time isolating certain organs from small animals. Unlike adult anurans, organ-specific biochemical assessment in tadpole requires highly accurate dissection due to their small size, which makes it difficult processing large numbers of samples under time constraint (Khan et al. 2015). Organ “contamination” by organic matter and/or by other particles consumed by tadpole can be bias at biochemical analysis applied to organs at dissection time (Lusher et al. 2017; Guimarães et al., 2021).

2.4.2. REDOX state

2.4.2.1. Oxidative stress biomarkers

The effects of exposure à AZT e HCQ (alone or in combination) on oxidative stress reactions were evaluated based on (i) indirect nitric oxide (NO) (via nitrite measurement) (Soneja et al. 2005); (ii) on thiobarbituric acid reactive species (TBARS), predictive of lipid peroxidation (De-Leon & Borges, 2020); (iii) production of reactive oxygen species (ROS), and on (iv) hydrogen peroxide (H₂O₂), which plays an essential role in responses to oxidative stress in different cell types (Sies, 2020;
Sies et al., 2020). The Griess colorimetric reaction [as described in Bryan et al., (2007)] was used to measure nitrite and the TBARS levels were determined based on procedures described by Ohkawa et al. (1979) and modified by Sachett et al. (2020). The production of H.O_2 and ROS was evaluated according to Elnemma et al. (2004) and Maharajan et al. (2018), respectively.

2.4.2.2. Antioxidant response biomarkers

The activation or suppression of antioxidant activity by treatments was evaluated by determining the activity of catalase and superoxide dismutase (SOD), which are considered first-line antioxidants important for defense strategies against oxidative stress (Ighodaro & Akinloye, 2018). While catalase activity was assessed according to Sinha et al. (1972) [see details in Montalvão et al. (2021)]; SOD levels were determined according to the method originally described by Del-Maestro & McDonald (1985) and adapted by Estrela et al. (2021).

2.5. 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)] and butyrylcholinesterase (BChE, also called serum cholinesterase or pseudocholinesterase) [according to the methodology described in Silva et al. (2020)].

2.6. Bioinformatics in silico analysis

Seeking to predict the binding mode and affinity of the bonds between AZT and HCQ used in our study and the protein structures of the enzymes AChE e BChE, we performed docking and chemoinformatic screens (Kolb et al., 2009). Protein structures and sequences of the *P. cuvieri* (i.e.: Leptodactyliidae) taxonomic family were not found in the biological structure databases. Therefore, we use as target structures those from the Pipidae (Xenopodinae) family, a family phylogenetically close to the group of Leptodactyliidae (Jetz & Pyron, 2018). The structures of AChE and BChE were obtained using the homology construction technique with similarity values of 95.48% and 97.14% to structures (targets) used for comparative modeling on the SWISS-MODEL server (https://swissmodel.expasy.org/). For molecular docking simulations, AutoDock tools (ADT) v4.2 (for preparing binders and targets) (Morris et al., 2009) and AutoDock Vina 1.1.2 (for calculations) were used (Trott & Olson, 2010). The binding affinity and interactions between residues were used to determine the best molecular interactions. The results were visualized using ADT, Biovia Discovery Studio v4.5 and UCSF Chimera X (Pettersen et al., 2021).
2.7. Azithromycin quantification

AZT uptake in tadpoles was assessed according to the methodology adopted by Keskar et al. (2015), with some modifications, using the supernatant of 10 samples/group (prepared according to item 2.4.1), composed of a pool of 5 animals/each (total of 50 animals/group). Initially, 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 the line generated (\( y = 1.2877x + 0.232; R^2 = 0.946 \)) 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 tadpoles exposed to AZT.

2.8. Quantification of hydroxychloroquine

The procedures used for the quantification of HCQ followed the recommendations of Bergqvist et al., (1985) (with some modifications), using 54 animals/group, distributed in 9 samples composed by a pool of 6 animals/each. Briefly, a 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 PA were added sequentially. this, 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 - equation: \( y = 15.953 + 0.0328; R^2 = 0.9942 \)). The background fluorescence of the control samples was also determined and subtracted from the samples from tadpoles exposed to HCQ.

2.7. 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-Wilks test, and homogeneity of variance 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). When the means of only two groups were compared, we applied the Mann-Whitney test. Levels of significance were set at values of Type I error (p) less than 0.01, 0.001, 0.0001 or < 0.0001.

3. RESULTS

Initially, we did not register any deaths of animals exposed to any of the treatments, throughout the experiment. In addition, the concentrations of nitrite, ROS, TBARS and H.O. did not differ between the groups exposed to the drugs (Figure 1). On the other hand, the tadpoles’ co-exposure to AZT and HCQ, induced a significant increase in SOD activity (Figure 2A) and catalase activity increased in the “AZT” and “AZT + HCQ” groups. However, the “HCQ” group showed a significant reduction in relation to the control group (Figure 2B).
Figure 1. Boxplots of the production of (A) nitrite, (B) thiobarbituric acid reactive substances, (C) reactive oxygen species and (D) hydrogen peroxide in *P. cuvieri* tadpoles exposed or not to drugs (azithromycin and hydroxychloroquine), alone or in combination. (n = 96 tadpoles/group, distributed in eight samples containing a pool of 12 animals/each). C: control; AZT: azithromycin; HCQ: hydroxychloroquine, both at 12.5 µg/L.
Figure 2. Boxplots of (A) superoxide dismutase and (B) catalase activity in tadpoles of *P. cuvieri* exposed or not to drugs (azithromycin and hydroxychloroquine), alone or in combination. Asterisks indicate significant differences between the treated groups and the control group (n = 96 tadpoles/group, distributed in eight samples containing a pool of 12 animals each) [p value = 0.01 (*), 0.001 (**), 0.0001 (***), <0.0001 (****)]. C: control; AZT: azithromycin; HCQ: hydroxychloroquine, both at 12.5 µg/L.

Interestingly, we also observed that the treatments induced a differentiated effect on the animals' cholinesterase system. While AChE activity was reduced in the “AZT” and “AZT + HCQ” groups (Figure 3A); BChE concentrations increased in these same groups (Figure 3B). In addition, we observed that the animals' exposure period was sufficient to induce uptake of AZT and HCQ in the animals, which suggests that drugs dispersed in water were absorbed by the tadpoles. While the concentration of AZT was higher in animals exposed to the combination “AZT + HCQ” (Figure 4A), the uptake of HCQ was lower in those co-exposed to drugs, when compared to those in the “HCQ” group (alone) (Figure 4B). In addition, AZT concentrations (in the “AZT” and “AZT + HCQ” groups) were almost 70% higher than those of HCQ (in the “HCQ” and “AZT + HCQ” groups) (average = 68.47%).
Figure 3. Boxplots of (A) acetylcholinesterase and (B) butyrylcholinesterase activity in tadpoles of *P. cuvieri* exposed or not to drugs (azithromycin and hydroxychloroquine), alone or in combination. Asterisks indicate significant differences between the treated groups and the control group (n = 96 tadpoles/group, distributed in eight samples containing a pool of 12 animals/each) [p value = 0.01 (*), 0.001 (**), <0.0001 (***)]. C: control; AZT: azithromycin; HCQ: hydroxychloroquine, both at 12.5 µg/L.
Figure 4. Boxplots of the concentrations of (A) hydroxychloroquine and (B) azithromycin detected in tadpoles of *P. cuvieri*. Different lowercase letters indicate significant differences. For the quantification of each drug, we used 54 tadpoles/group, distributed in nine samples composed by a pool of 6 animals/each. AZT: azithromycin; HCQ: hydroxychloroquine; 1: 2.5 µg/L and 2: 12.5 µg/L. For both drugs, the background fluorescence of the control samples was also determined and subtracted from the samples from the tadpoles exposed to the treatments.

As for the analysis of molecular docking, our data predicted the affinity between the drugs and the enzymes AChE and BChE, as well as the existence of interactions with residues from all tested moorings. Figure 5 shows that the binding energies required for AZT to bind to AChE (-8.8 ± 1.12 kcal/mol) and BChE (-9.1 ± 0.65 kcal/mol) were comparable, similar to that observed for HCQ and its molecules target [HCQ / AChE (-6.9 ± 0.97 kcal/mol) and HCQ/BChE (-6.2 ± 0.69 kcal/mol)] (Figure 5B). However, the activation energies required for the connection between AZT and the evaluated enzymes were lower than those required for HCQ (Figure 5), which suggests greater stability of the AZT-AChE and AZT-BChE complex and, consequently, the most likely to be formed in the evaluated biological system.
Figure 5. Graphical representation of binding energies (∆G, in kcal/mol) of molecular docking between the ligands [azithromycin (AZT) and hydroxychloroquine (HCQ)] and targets [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] calculated by AutoDock Vina® software.

The interaction analysis showed that AZT interacted with AChE through different types of bonds, involving asparagine (Asn254), tyrosine (Tyr430), tryptophan (Trp549) and two prolines (Pro256 and Pro426) (Figure 6A). With BChE, interactions of the conventional hydrogen bond type were prevalent [glutamine (Gln71), serine (Ser72) and glycine (Gly116)], in relation to those of the carbon-hydrogen type (Asn68), Pi-Sigma (Tyr332) and Pi-Alkil [phenylalanine (Phe329)] (Figure 6B). Regarding the receptor-ligand complex formed between HCQ and AChE, we observed the presence of interactions of the Pi-Alkil type (Phe314 and Phe355), conventional hydrogen bond type (Ser310) and the Pi-Pi T-Shaped type (Tyr358) (Figure 6C). On the other hand, HCQ-BChE interactions occurred through different amino acid residues of the enzyme [Leu286, Phe398 and Phe329, Trp82 and Trp231, Gly116 and Gly117 and glutamic acid (Glu197)], involving predominantly Alkil and Pi type interactions -Sigma (Figure 6D). The three-dimensional structures of the docked complexes with solid surfaces are shown in Figure 7.
Figure 6. Molecular interactions of the (A-B) azithromycin and (C-D) hydroxychloroquine with acetylcholinesterase and butyrylcholinesterase. Receptor amino acids are represented by spheres of different colors around the structure. Asn: asparagine, Tyr: tyrosine, Trp: tryptophan, Pro: proline, Gln: glutamine, Ser: serine, Gly: glycine, Phe: phenylalanine, Leu: leucine. The numbering indicated in the colored circles refers to the position of the amino acids in the primary structure of the protein.
4. DISCUSSION

It is a consensus among different researchers that the identification and characterization of the effects caused by pollutants on the biota constitutes an essential step for decision-making and proposing mitigation measures or pollution remediation, allowing us to cease impacts and/or prevent the occurrence of even more extensive damage to organisms (Lacy et al., 2017). In this sense, when we demonstrated for the first time that the presence and dispersion of AZT and HCQ in surface waters induce physiological changes in tadpoles, we launched trumpets about the dangerousness of these drugs in non-target organisms of wild aquatic fauna.

Initially, we evaluated whether the tadpoles' exposure to AZT and HCQ (alone or in combination) could induce an increase in oxidative processes, from different biomarkers. However, no difference was observed between the experimental groups, regarding the concentrations of nitrite, TBARS, ROS and H₂O₂ (Figure 1). These results are interesting, as they disagree with some previous reports and corroborate the findings of others. In the study by Li et al. (2020), for example, the increased production of ROS in Daphnia magna exposed to AZT (after 96h of feeding Chlorella pyrenoidosa exposed to AZT) was related to changes in feeding behavior, nutritional status, and digestive physiology of these animals. Similarly, Mhadhbi et al. (2020) reported that exposure to AZT (0.05 and 0.08 mg/L, during 4 and 14 days) caused an increase in oxidative processes and peroxidative damage in the gills and liver of juvenile Dicentrarchus labrax. On the other hand,
similarly to our findings, Shiogiri et al. (2017) found no evidence of increased oxidative stress in *Oreochromis niloticus* (juveniles) exposed for 14 days to different concentrations of AZT (1, 50 and 100 mg/L). In relation to HCQ, studies involving aquatic organisms have not evaluated biomarkers of oxidative stress, despite having already observed effects of chloroquine on the enzymatic and histopathological physiology of *Cyprinus carpio* fish (Ramesh et al., 2018), immobilization of *D. magna* (Zurita et al., 2005; Rendal et al., 2010), reduction of lysosomal function in *Poeciliopsis lucida* fish cells, inhibition of luminescence in *Vibrio fischeri* bacteria and inhibition of the growth of *Chlorella vulgaris* algae (Zurita et al., 2005), as well as transpiration inhibition in *Salix viminalis* plants (Rendal et al., 2010). Therefore, this scenario denounces not only the lack of studies focusing on the ecotoxicological effects of AZT and HCQ, but also shows that the biological response to drugs is dependent on the species, period and concentrations used in the exposures.

In our study, it is possible to attribute the absence of oxidative effect induced by AZT and HCQ to the action of the enzymes SOD and catalase. Although only the group co-exposed to the drugs showed a significant increase in SOD activity (Figure 2A), in the groups exposed to AZT and HCQ (alone) the enzyme activity increased by 34.9% and 30.6% (respectively) compared to the control group, which biologically may have been preponderant to inhibit the increase of cellular oxidative processes. Similar reasoning can be used to increase catalase in the “AZT” and “AZT + HCQ” groups (Figure 2B), since both enzymes are important for antioxidant defense against free radicals. While SOD converts the superoxide anion radical to H₂O₂, catalase converts H₂O₂ into H₂O and O₂ molecules (Lee et al., 2018; Ransy et al., 2020; Damiano et al., 2020). As for the reduction of catalase activity in animals exposed to HCQ (alone), it is possible that it has been compensated for by the performance of other peroxisomal enzymes (which also aid in the decomposition of H₂O₂ and other reactive oxygen and nitrogen species) to maintain the oxidative homeostasis in this group. Such enzymes include, for example, peroxiredoxin 5, glutathione S-transferase kappa, 'microsomal' glutathione S-transferase, and epoxide hydrolase 2 (Fransen et al., 2012).

On the other hand, our data show AZT’s anticholinesterase effect on the studied tadpoles, marked by a significant reduction in AChE activity in the “AZT” and “AZT + HCQ” groups (Figure 3A), like the reports by Mhadhbi et al. (2020), in which juveniles *D. labrax* exposed to AZT (0.05 and 0.08 mg/L, during 4 and 14 days) also showed a reduction of this enzyme in the gills and liver. As discussed by Massoulié et al. (1993), AChE is one of the most prominent constituents of central cholinergic pathways. It ends the synaptic action of ACh through its hydrolysis and produces the choline portion necessary for recycling the neurotransmitter. Therefore, any changes in the activity of this enzyme can lead to important neurological consequences. In our study, it is plausible to assume that the uptake of AZT in animals (Figure 4) and, especially, its greater affinity with AChE (in relation to HCQ, Figure 5) were preponderant for the occurrence of the observed anticholinesterase effect.
As suggested by molecular docking, this effect may have been due to the probable “AZT-AChE” interaction, via connections involving different amino acid residues (Tyr430, Asn254, Pro256, Pro427 and Trp549 - Figure 6). Although these residues are not part of any AChE active or catalytic site [see details in Harel et al. (1993), Silman & Sussman, (2005), Johnson & Moore (2006) and Chen et al. (2017)], it is possible that AZT acted as an allosteric modulator, changing the conformation of the enzyme and decreasing its activity, which would not have occurred in the “HCQ-AChE” interaction. In this case, in addition to the binding energy for this interaction being higher than that required for the “AZT-AChE” interaction (Figure 5), it is possible that such connections did not cause sufficient conformational changes to alter the activity/functionality of the enzyme or that the uptake concentration of HCQ (Figure 4) was insufficient to induce changes in the enzyme or even if biologically (ie: in vivo) such connections did not occur. As discussed by Kitchen (2004), the greater the free binding energy predicted in molecular docking, the less likely to occur.

On the other hand, interestingly, we found an increase in BChE concentrations in the same groups in which AChE activity was reduced (ie: “AZT” and “AZT + HCQ” groups; Figure 3), which suggests an adaptive (compensatory) response to break down excess ACh in synaptic clefts caused by reduced AChE. Despite being encoded by different genes, the enzymes AChE and BChE have high structural homology, differing in their affinity for substrates and sensitivity to inhibitors. While AChE is an esterase that hydrolyzes predominantly ACh (Soreq & Seidman, 2001), BChE hydrolyzes different types of choline esters, including butyrylcholine (BCh), succinylcholine (SCh) and ACh (Darvesh et al., 2003; Nurulain et al., 2020). However, considering that these enzymes have different Km values (Michaelis-Menten constant), they are expected to have different kinetic responses to ACh concentrations in synaptic clefts. According to Silver (1974), at low concentrations of ACh, AChE is highly efficient but BuChE is much less efficient. However, at higher ACh concentrations BuChE's efficiency in the hydrolysis of ACh is significantly increased. Thus, this evidence, associated with other studies that have already reported the compensatory support role of BChE in response to the absence or decrease of AChE, reinforces the hypothesis about the occurrence of a physiological adaptation to maintain cholinergic homeostasis in tadpoles exposed to AZT (alone or in combination with HCQ) (Norel et al., 1993; Li et al., 2000; Xie et al., 2000; Mesulam et al., 2002ab). Alternatively, we cannot rule out the hypothesis that the interaction between AZT and BChE (molecular docking; Figure 6-7) has also caused changes in the normal activity of the enzyme. However, contrary to the effects of the “AZT-AChE” interaction, such changes would have favored the enzyme’s activity, with AZT acting as a positive allosteric modulator.

Anyway, regardless of the mechanisms that explain our findings, it is important that new studies expand the understanding of the intrinsic factors involved in the physiological response of
tadpoles exposed to different treatments. Although our data strongly suggest that BChE could potentially replace AChE in the context of tadpole's exposure to AZT and HCQ, as well as playing a constitutive role (rather than just back-up) in the hydrolysis of ACh, there is no way to guarantee (in our study) that this compensatory action has, in fact, regulated the concentrations of this neurotransmitter in the synaptic clefts. As is well known, both the increase and the decrease in the amounts of ACh in the synaptic clefts can induce effects on different physiological functions in the organisms, which include a wide spectrum of clinical manifestations (eg: dysfunctional gland disorders, respiratory processes, and disorders in the functioning of the central nervous system). In this case, in vivo evaluations to determine the concentrations of ACh in the tadpoles exposed to the treatments (AZT and HCQ), constitute interesting future investigative perspectives. Equally important will be the conduct of in vivo and in vitro studies to confirm the mechanisms of action predicted by molecular docking and, once confirmed, whether the interactions of drugs with the target molecules are reversible or irreversible.

Finally, taken together, our data point to an unexpected response from *P. cuvieri* tadpoles to exposure to drugs, in which the REDOX and cholinergic imbalance induced by AZT would have been counterbalanced by the compensatory increase in enzyme activity that neutralized production excessive free radicals and apparently reestablished the central cholinergic pathways affected by the reduction in AChE. As discussed by Biagianti-Risbourg et al. (2013), this type of response constitutes an individual-level physiological adaptation and, therefore, can (in the short term) increase animal survival and maintain the highest possible fitness under stressful conditions (Hoffman, 1995; Collier et al., 2019). However, this physiological adaptation is energetically expensive for the organism and, depending on the nature and intensity of environmental stress, can trigger a physiological trade-off might, including the reduction of life expectancy or the reproductive success of individuals (Wilson & Franklin, 2002; Wood & Harrison, 2002; Farwell et al., 2012; Loria et al., 2019). Therefore, when transposing these considerations to the context of our study, we cannot guarantee that the prolonged exposure of tadpoles to drugs will have its harmlessness sustained by the physiological tolerance observed in the short exposure. Considering that the metamorphosis of amphibians, in itself, consists of a high energy cost phase (Pfab et al., 2020), the reallocation of energy from other processes (eg: growth and development) to maintain physiological homeostasis, will have a general negative effect on animal health. In this sense, it will be essential to assess how much the biological responses observed in our study will be able to guarantee the survival of the tadpoles until their complete metamorphosis, without affecting their reproductive performance.

5. CONCLUSIONS
In conclusion, our study demonstrated that the short exposure of *P. cuvieri* tadpoles to AZT and HCQ (alone or in combination) unexpectedly induced an adaptive physiological response marked by increased activity of the enzymes SOD and catalase (for the maintenance of homeostasis oxidative) and by increasing BChE (to possibly counteract the anticholinesterase effect induced by AZT). In addition, the uptake of AZT in tadpoles and the strong link between this drug and AChE, suggested by molecular docking, were preponderant in triggering the animals' physiological response. When considering the pioneering nature of the present study, our results constitute only the “tip of the iceberg” that can represent the physiological effects of COVID-19 therapy based on AZT/HCQ in animal physiology. Therefore, we strongly suggest that studies of this nature be continued.

6. ACKNOWLEDGMENT

The authors are grateful to the Brazilian National Research Council (CNPq) (proc. N. 426531/2018-3) and to Instituto Federal Goiano for the financial support (Proc. No. 23219.000077.2021-62). Malafaia G. holds productivity scholarship from CNPq (Proc. No. 307743/2018-7).

7. 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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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests:
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Graphical abstract
CAN USE OF HYDROXYCHLOROQUINE AND AZITHROMYCIN AS A TREATMENT OF COVID-19 AFFECT AQUATIC WILDLIFE? A STUDY CONDUCTED WITH NEOTROPICAL TADPOLE

Highlights

1) *P. cuvieri* tadpole exposed to AZT and HCQ show greater activity of superoxide dismutase and catalase
2) Molecular docking suggests interaction between AZT and the enzymes AChE and BChE
3) Exposure of *P. cuvieri* tadpoles to AZT reduces AChE and increases BChE activity.
4) AZT (alone or in combination with HCQ) elicits a compensatory adaptive response in *P. cuvieri* tadpole.