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In Silico and *in Vitro* Analysis of Pyrazolone Derivatives against Zika Virus and Identification of Potential NS5 Methyltransferase Inhibitors by Molecular Docking

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Abstract

Zika virus (ZIKV), a mosquito-borne flavivirus, has been associated with benign infections for decades. However, it has become a public health concern due to its association with severe fetal and neurological complications. Although many efforts have been made to control ZIKV infection, approved vaccines or antiviral drugs are still lacking. Consequently, the development of new effective anti-ZIKV agents is urgently needed. In this context, we investigated the antiviral potential of pyrazolone derivatives against ZIKV replication using *in silico* and *in vitro* methods. The four pyrazolone derivatives evaluated (**1a**, **1b**, **1c**, and **1d**) inhibited over 50% of ZIKV replication with low cytotoxicity. Among them, compound **1b** exhibited the most potent activity (EC_{50} = 4.3 μM) and the highest selectivity (SI = 342). Mechanism of action studies indicated that these compounds act at early stages of virus replication, and compound **1b** can also directly inactivate ZIKV particles. Molecular docking studies suggested that these compounds can bind to and block the activity of ZIKV NS5 methyltransferase. Finally, pharmacokinetic and

toxicological predictions have reinforced the safety and drug-like profiles of these derivatives. In conclusion, the pyrazolone scaffold proved to be valuable for anti-ZIKV drug development, and the derivatives studied deserve further investigation.

Keywords

Zika, Pyrazolone, NS5, Methyltransferase, Antivirals

1. Introduction

Zika virus (ZIKV) is an arbovirus of the Flavivirus genus, which belongs to the Flaviviridae family. Its transmission occurs mainly through the bite of infected *Aedes aegypti* mosquitoes [1]. In addition to mosquito bites, other transmission routes have also been reported, such as sexual and vertical transmission [2]. In addition, studies point to evidence that transmission can occur through blood products and breastfeeding [3]. The relationship between ZIKV and the autoimmune neurological Guillain-Barré syndrome, as well as the congenital Zika syndrome (CZS), aroused the interest of several researchers [4]-[8]. SCZ can produce spontaneous abortions, congenital malformations, and several situations that are harmful to long-term health, including psychiatric disorders during adult life [7] [9]-[11]. In 2021, Krenn and collaborators [12] demonstrated that viral infections caused by the Zika virus and Herpes simplex virus type 1 (HSV-1) were identified as the main causes of microcephaly in early pregnancy [12]. To date, 87 countries have reported epidemic outbreaks caused by ZIKV. These events characterize the ability of the virus to trigger large-scale infections [4] [13].

The ZIKV genome consists of a single-stranded positive-sense RNA [3] that assembles a polyprotein essential for the replicative cycle. Also, ZIKV has the untranslated region, 5' and 3' URTs that have an essential function for viral replication [14]. On the other hand, the ZIKV has only one open reading frame (ORF) that encodes three structural proteins (E, prM, and C) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [15] [16]. One of the central proteins of flavivirus replication is NS5 which is responsible for two distinct catalytic activities for virus replication [17]. The first domain is methyltransferase (MTase) and the second domain is an RNA-dependent RNA polymerase (RdRp) [18]. The NS5 MTase domain methylates the RNA coating to create N-7-methylguanosine and 2'-O-methyladenosine applying S-adenosyl-L-methionine (SAM) as the methyl giver [19]. On the other hand, the NS5 RdRp domain contributes to viral RNA synthesis through a *de novo* initiation mechanism [20]. Studies demonstrated that the performance of N-7-methylguanosine as well as the 2'-O-methyladenosine of the MTase of the non-structural protein NS5 are essential for viral replication [21]-[23]. Thus, the abolition of MTase activity seems to be a promising method for the development of anti-ZIKV agents.

Despite the efforts put into the development of control strategies, there are no antivirals and vaccines on the market yet. In this context, pyrazole derivatives emerge as an interesting scaffold for bioactive products. The pyrazole nucleus is an aromatic azole heterocycle with two adjacent nitrogen atoms. Pyrazole derivatives exhibit a broad spectrum of biological activities, including approved drugs such as celecoxib, antipyrine, phenylbutazone, rimonabant, and dipyrone, which contains the pyrazole ring [24]. In addition, the introduction of other heterocyclic systems resulted in new compounds with several biological and medicinal functions, such as antiviral, anti-inflammatory, anticancer, and antioxidant characteristics [25] [26]. Compounds bearing a pyrazole core have been highlighted in the search for new antivirals. For instance, our group have previously synthesized derivatives of 3H-benzo[b]pyrazole-[3,4-h]-1,6-naphthyridines and 3H-pyrido[2,3-b]pyrazole [3,4-h]-1,6-naphthyridines which inhibited HSV-1 replication [27]. However, to the best of our knowledge, there is no experimental evidence on the antiviral activity of other derivatives, such as pyrazolones, against ZIKV. Consequently, the aim of this work was to evaluate the cytotoxicity, antiviral activity, and mechanism of action of pyrazolone derivatives against a Brazilian ZIKV strain. Also, we aimed to analyze in silico the pharmacokinetic and toxicological properties of these compounds as well as their binding affinity and interactions with ZIKV NS5 MTase.

2. Methodology

2.1. Compounds

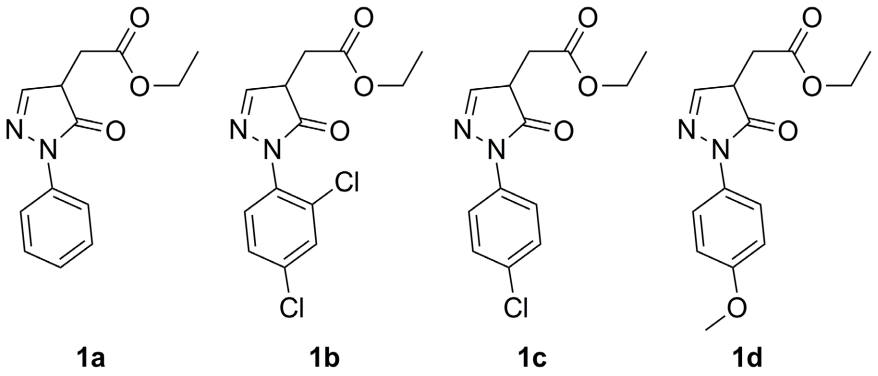

Figure 1. 2D structure of the pyrazolones derivatives evaluated in this work.

Previously, our group synthesized and designed several aryl-substituted 1*H*-pyrazoles, which showed interesting biological properties [28]-[31]. Among pyrazoles, the pyrazolone moiety is endowed with a wide range of pharmacological activities, such as antimicrobial, neuroactive, anti-inflammatory, antioxidant, and antiviral [32]. Consequently, four aryl-substituted 5-pyrazolone derivatives (compounds **1a**-**1d**) (**Figure 1**) were obtained as described elsewhere [24] [33]. Among the substituents, we explored the introduction of electron-withdrawing (*e.g.* chlorine) and electron-donating (*e.g.*, methoxy) groups in the phenyl ring

attached to the pyrazolone nucleus. All derivatives were dissolved in sterile dimethylsulfoxide (DMSO) in 50 mM stock. On the other hand, chloroquine (Sigma-Aldrich, Brazil) was prepared in sterile water to a final concentration of 50 mM. All stocks were stored at −20˚C until use. The final concentration of DMSO was <0.1%.

2.2. Cells and Viruses

Vero cells (ATCC CCL-81; African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Brazil) at 37˚C, supplemented with 5% of fetal bovine serum (FBS) (Sigma-Aldrich, Brazil), 2 g/L of sodium bicarbonate, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 2.5 μg/mL of amphotericin B. The Brazilian strain of ZIKV KU49755 was kindly provided by Professor Dr. Ana Maria Pinto from the Biomedical Institute, Fluminense Federal University. This strain was isolated from amniotic fluid samples from a neonate with microcephaly and represents the Asian lineage. Viruses were propagated in Vero cells for five days at 37˚C and 5% CO_2 atmosphere and were harvested and stored at −80˚C until needed. Virus titers were defined by plaque reduction assay.

2.3. Cytotoxicity Assay

The cytotoxicity of the compounds on Vero cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described elsewhere [34]. Briefly, Vero cells were seeded onto 96-well culture plates at a density of 1×10^4 cells per well and were treated with increasing concentrations of compounds (50, 100, 200, 400, and 800 μM) for 72 h at 37˚C and humidified at 5% CO_2 atmosphere. Then, compounds were removed, and 50 μL of a 5 mg/mL solution of MTT (Sigma-Aldrich, Brazil) was added into each well and incubated for 4 h at 37˚C and humidified 5% CO_2 atmosphere. The MTT solution was then removed and 100 μL of DMSO was added to each well to dissolve formazan crystals. Finally, the resulting absorbance of each well was determined at 545 nm using a microplate reader (TP-Reader Thermo Plate). The relative cell viability was calculated as a percentage comparing the treated to untreated cells and the concentration that reduces cell viability by 50% (CC_{50}) was determined by a linear regression analysis of the dose-response curves. The experiment was undertaken three times independently in triplicates.

2.4. Antiviral and Plaque Reduction Assays

Vero cells were seeded onto 24-well plates at a density of 2×10^4 cells per well and were infected with ZIKV at a multiplicity of infection (MOI) of 1.0 for 3 h at 37˚C and humidified 5% CO_2 atmosphere. Then, the inoculum was discarded, and compounds were added. As an initial screening, compounds were added at a final concentration of 50 μM, while the ones that showed antiviral activity at this

stage were further evaluated at different concentrations (3.125, 6.25, 12.5, 25, and 50 μM). The treated plates were incubated for 20 h at 37˚C and humidified 5% CO_2 atmosphere. Finally, cells were lysed by three freezing and thawing cycles for viral titer determination by plaque reduction assays.

To determine virus titers, Vero cells maintained in 24-well plates at a cell density of 2×10^5 cells per well were infected with 10-fold serial dilutions of the collected samples for 3 h at 37˚C and humidified 5% CO_2 atmosphere. Afterward, the inoculum was removed, and cell monolayers were covered with DMEM supplemented with 3% of methylcellulose (Sigma-Aldrich, Brazil) and 2% of FBS for 7 - 8 days. Then, cells were fixed and stained with a solution of 0.2% crystal violet and 10% formaldehyde. Virus titers were expressed as the number of plaque-forming units per milliliter (PFU/mL) while inhibition of virus cytopathic effect was calculated comparing the infected treated and untreated cells. These assays were performed in triplicate. Finally, the concentration required to inhibit the virus cytopathic effect by half (EC_{50}) of the most potent compounds was calculated by linear regression.

2.5. Virucidal Assay

To assess the virucidal effect of the compounds, a cell-free system was employed. 1×10^7 PFU of ZIKV was diluted in DMEM medium in the presence or absence of the compounds (50 μM) and incubated for 2 h at 37˚C and humidified 5% CO_2 atmosphere. Then, Vero cells (2×10^5 cells/well) seeded in a 24-well plate were infected with the incubated virus for 1 h at 37˚C and humidified 5% CO_2 atmosphere. The inoculum was removed, and cell monolayers were covered with DMEM supplemented with 3% of methylcellulose and 2% of FBS for 7 - 8 days. Finally, cells were fixed and stained with a solution of 0.2% crystal violet and 10% formaldehyde. Viral titers were expressed as the number of PFU/mL.

2.6. Time of Addition Assay

We carried out a time of drug addition assay to investigate at which step of the viral life cycle the compounds act. Vero cells grown in 24-well plates at a cell density of 2×10^5 cells per well were infected with ZIKV at an MOI of 0.1. Compounds were added (50 μM) at different times, as follows: 3 hours before infection (−3 hpi; pretreatment), 0 h (at the time of infection), or at times post-infection, such as 3, 6, 9, and 12 hpi, and incubated for 72 h at 37˚C. After 72 h, the percentage of viral inhibition of each treated sample was calculated by comparing it with virus titers from untreated controls.

2.7. Prediction of Pharmacokinetic and Toxicological Properties

The theoretical pharmacokinetic and toxicological properties of the most active compounds (**1a**, **1b**, **1c**, and **1d**) and chloroquine were analyzed. The admetSAR 2 server [35] was employed to predict human intestinal absorption (HIA) and

carcinogenicity, whereas genotoxicity (based on the AMES test), hepatotoxicity, and cardiotoxicity (based on inhibition of hERG I and II) were predicted using the pkCSM server [36]. Additionally, the compounds were also evaluated according to industry rules, like Lipinski's "rule of five", GSK 4/400, and Pfizer 3/75 using the FAF-Drugs4 web server [37].

2.8. Molecular Docking

Molecular docking of the most potent compounds **1a**, **1b**, **1c**, and **1d** with the methyltransferase domain of NS5 (NS5 MTase) from ZIKV was carried out to evaluate their putative mechanism of antiviral action. The crystal structure of ZIKV NS5 methyltransferase complexed with its cofactor S-adenosylmethionine (SAM) was retrieved from the Protein Data Bank under the code 5KQR [38]. The three-dimensional structures of the pyrazolone derivatives were constructed and optimized using the Spartan'10 program (Wavefunction Inc., Irvine, CA, USA). Initially, structures were submitted to a conformational analysis in a vacuum using molecular mechanics and the MMFF force field. The lowest-energy conformer was subjected to a geometry optimization step using the semi-empirical RM1 method, followed by an ab initio calculation using the Hartree-Fock method and the 6-31G* basis set.

Molecular docking studies were performed using Autodock Tools 1.5.6 and AutoDock 4.2.6 [39]. First, the co-crystallized structure of SAM was redocked in NS5 methyltransferase to evaluate the docking software accuracy and validate the docking protocol (RMSD 0.61 Å). The protein was prepared by adding polar hydrogens and Gasteiger charges were assigned whereas polar hydrogens were added to the ligand and its rotatable bonds were determined automatically. During docking studies, the protein was kept rigid while the rotatable bonds of ligands were allowed to rotate freely. The grid box, with dimensions of 50 × 50 × 50 points (0.375 Å spacing), was centered on the SAM binding site. Lamarckian genetic algorithm was employed as a search engine with a population size of 50 while other parameters of the search algorithm were kept at default values. After validation, the same protocol was employed for docking studies with the pyrazolopyrimidine derivatives. Finally, the conformer with the lowest binding energy was selected for visual inspection and protein-ligand interaction analysis using Ligplot v. 4.5.3 [40] available at the European Bioinformatics Institute (EMBL-EBI) server.

2.9. Statistical Analysis

All the data shown represent the mean ± standard deviation (SD) from triplicates run three independent times. Analysis of variance (ANOVA) was used to analyze differences among group means, and the Tukey test was performed for post hoc analysis to differentiate among experimental groups. Statistical significance was considered when p-value < 0.05. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software Inc.).

3. Results

3.1. Cytotoxicity and Antiviral Activity of Pyrazolone Derivatives against ZIKV Replication

Prior to antiviral experiments, the toxicity of pyrazolone derivatives was evaluated in Vero cells. The CC_{50} of compounds **1a**, **1b**, **1c**, and **1d** were approximately 1353 μM ± 17.02, 1472 μM ± 40.42, 1130 μM ± 6.83, and 980 μM ± 18.12, respectively (Table 1). Since the compounds exhibited low cytotoxicity, their antiviral activity against ZIKV replication in Vero cells was assessed at a concentration of 50 μM. Among the four compounds, compound **1d** showed antiviral activity of 68.30%, while compound **1c** showed moderate activity (virus titer decreased by 50%). On the other hand, compounds **1a**, and **1b** exhibited stronger inhibitory activity and were able to reduce the virus titer by 71.20% and 79.30%, respectively. Similarly, chloroquine had a moderate inhibitory activity of 58%. Further, compounds were evaluated at different concentrations to determine their EC_{50} values. All derivatives showed dose-dependent antiviral activity and compound **1b** was the most active, with an EC_{50} value of 4.30 μM, followed by compounds **1a** (EC_{50} = 16.0 μM), **1d** (EC_{50} = 25.4 μM) and **1c** (EC_{50} = 46.63 μM). Compounds **1b** and **1b** were more potent than chloroquine (EC_{50} = 16.82 μM), a marketed drug with antiviral activity *in vitro* against ZIKV. Finally, the selectivity index (SI) of the compounds was calculated, being compound **1b** the most selective one with an SI of 342. Despite the lower antiviral activity in comparison to chloroquine (SI = 24.98), the pyrazolone derivatives showed comparable or even higher selectivity than this drug, which indicates the promising profile of these compounds.

Table 1. Cytotoxicity and antiviral activity of the four pyrazolone derivatives and the control drug chloroquine against ZIKV.

Compounds	CC_{50} (μM)[a]	Inhibition (%)[b]	EC_{50} (μM)[c]	SI[d]
1a	1353 ± 17.02	71.20	16 ± 0.99	84
1b	1472 ± 40.42	79.30	4.3 ± 1.25	342
1c	1130 ± 6.83	50.00	46.63 ± 0.87	24.21
1d	980 ± 18.12	68.30	25.40 ± 1.06	38.58
Chloroquine	420.2 ± 0.02	58.00	16.82 ± 0.92	24.98

[a]CC_{50}: Concentration that reduced the cytotoxicity concentration by 50%, [b]Inhibition rate (%) of ZIKV replication in Vero cells at 50 μM, [c]EC_{50}: Concentration that reduced 50% of ZIKV replication, [d]SI: Selectivity Index was defined as the ratio between CC_{50} and EC_{50}.

3.2. Evaluation of the Virucidal Activity

In order to analyze the inactivation of viral particles by the compounds, ZIKV suspensions were treated with the compounds at 50 μM for 2 h. Then, the remaining infectivity was evaluated by plaque reduction assays. Treatment of virus

particles with compounds **1a**, **1b**, **1c**, and **1d** reduced virus titers by 38%, 76%, 34%, and 27%, respectively, while chloroquine decreased virus titers by 42% (Figure 2(A)). The results obtained in the virucidal assays indicated that only compound **1b** has an interesting virucidal potential.

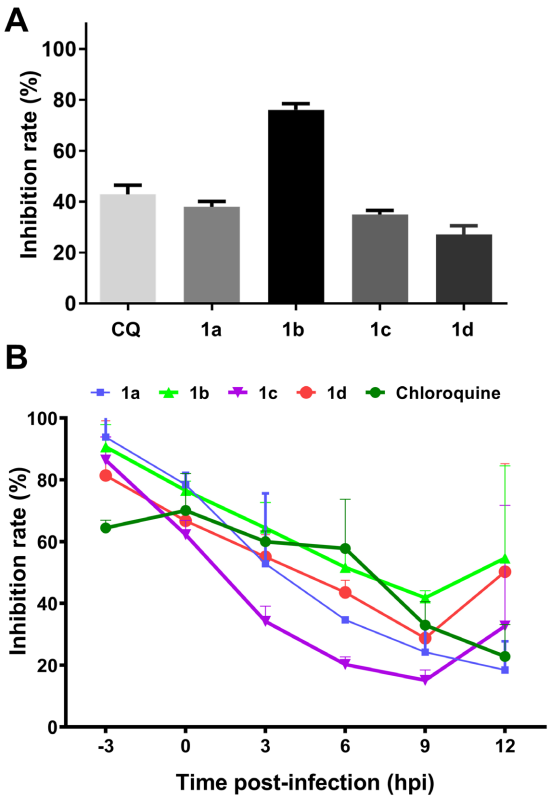

Figure 2. Mechanism of action evaluation of the pyrazolone derivatives (50 μM) and the control drug chloroquine (CQ) against ZIKV. (A) Virucidal activity; (B) Time-of-drug addition assay. Data are expressed as mean ± standard deviation (bars) from three independent assays.

3.3. Time of Addition Assay

We investigated the antiviral effects of the pyrazolone derivatives when added at different times during ZIKV replication to assess at which step of this process the compounds might act (Figure 2(B)). All compounds showed the highest antiviral activity when added 3 h before infection. Compounds **1a**, **1b**, and **1c** still presented high antiviral activity when added 3 h post-infection (higher than 50% inhibition), but their activity decreased significantly when they were added later on in the infection like the drug chloroquine. These findings suggested that all compounds mainly act at early stages of virus replication and may also act at host cells.

3.4. Molecular Docking of the Most Active Derivatives with ZIKV NS5 Methyltransferase

Based on the mechanism of action assays, we evaluated whether the most active

derivatives (**1a**, **1b**, **1c**, and **1d**) may bind ZIKV NS5 MTase and interfere with ZIKV replication (Figure 3). Compound **1b** showed the highest theoretical affinity with this enzyme, showing a binding energy of −6.23 kcal/mol, followed by compounds **1d** (−5.32 kcal/mol), **1c** (−4.49 kcal/mol), and **1a** (−4.47 kcal/mol). Overall, compounds showed extended binding modes and contacted similar residues (Figure 3). Compound **1b** established two hydrogen bond interactions with residues Lys105 and Val132 while the other compounds showed only one hydrogen bond interaction. For instance, compounds **1d** and **1c** interacted with His110 while compound **1a** interacted with Val132.

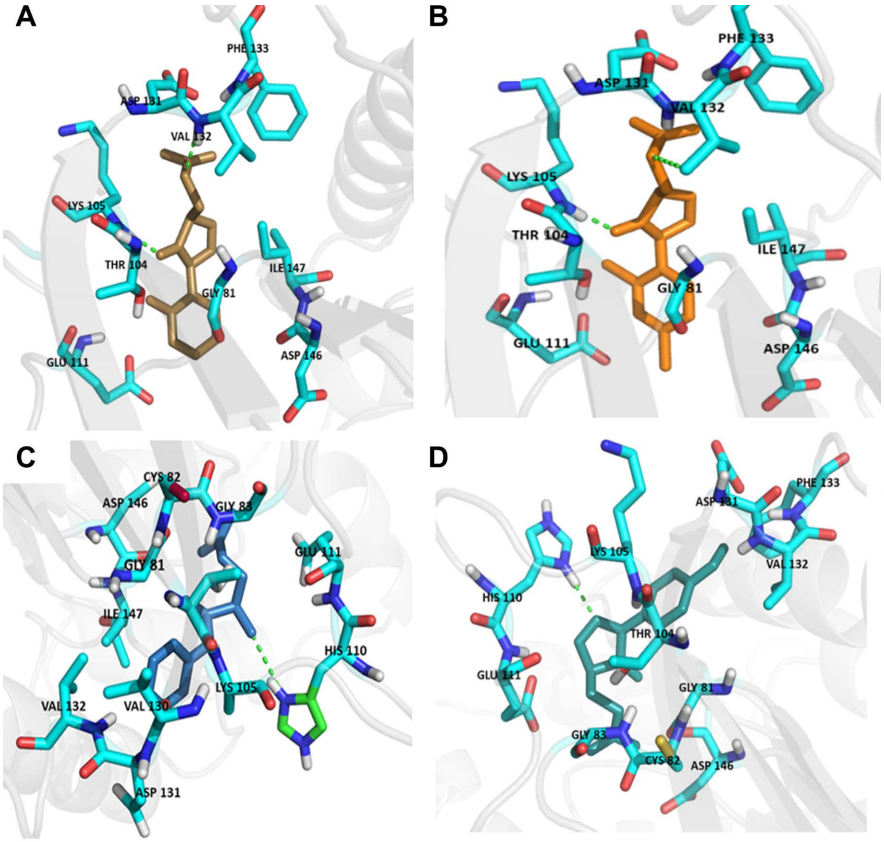

Figure 3. Molecular docking of pyrazolone derivatives with ZIKV NS5 MTase (PDB code 5KQR). Binding mode of compounds (A) **1a**, (B) **1b**, (C) **1c**, and (D) **1d** are shown. Hydrogen bonds are represented as dashed lines. Created with The PyMOL Molecular Graphics System, Schrödinger, LLC.

3.5. *In Silico* Analysis of the Pharmacokinetics and Toxicological Properties of the Pyrazolone Derivatives

To evaluate the potential of the pyrazolone derivatives as drug candidates, we evaluated their pharmacokinetic and toxicological properties using computational tools (Table 2). All compounds showed good human intestinal absorption and fulfilled Lipinski's "rule of five", indicating good oral bioavailability. In addition, these compounds were approved on the GSK 4/400 rule, indicating satisfactory pharmacokinetic and toxicological properties. However, a warning was

raised on the Pfizer 3/75 due to their low polar surface area. Yet, they were not reproved, suggesting they exhibit a low probability of showing pre-clinical toxicity.

Table 2. Comparison of theoretical pharmacokinetic and toxicological properties and classification of pyrazolone derivatives and the control drug chloroquine regarding the drug-like rules developed by pharmaceutical industries (Lipinski's "rule of five", GSK 4/400, and Pfizer 3/75). Prediction probabilities are shown in parentheses.

Compound	1a	1b	1c	1d	Chloroquine
Human intestinal absorption	Yes (0.98)	Yes (0.98)	Yes (0.97)	Yes (0.98)	Yes (1.00)
Lipinski's "rule of five" violation	0	0	0	0	0
GSK/400	Pass	Pass	Pass	Pass	Pass
Pfizer 3/75	Warning	Warning	Warning	Warning	Warning
Carcinogenicity	No (0.64)	No (0.64)	No (0.66)	No (0.74)	No (0.83)
Hepatotoxicity	No	Yes	No	Yes	No
Cardiotoxicity	No	No	No	No	Yes

Moreover, we evaluated other toxicity risks for these compounds, such as carcinogenicity, hepatotoxicity, and cardiotoxicity (Table 2). None of the compounds showed carcinogenic and cardiotoxic risks, but compounds **1b** and **1d** showed increased risks for hepatotoxicity. Interestingly, compounds **1a** and **1c** showed a safer profile than chloroquine, which is a marketed drug, with low toxicity risks for all endpoints evaluated.

4. Discussion

ZIKV is an emerging arbovirus that has rapidly spread in recent years and caused unprecedented large human outbreaks, which, for the first time, revealed an etiological link with severe neurological complications, including Guillain-Barré syndrome and fetal microcephaly. According to the Centers for Disease Control and Prevention, it is estimated that between 2007 and 2019, 87 countries were affected by the ZIKV [4] [41]. In the Americas, 38 countries reported the presence of the virus [42]. The absence of an effective vaccine leads to the development of new drugs that are both active as antivirals and have low systemic and cellular toxicity.

Herein, we investigated the antiviral potential of pyrazolone derivatives against ZIKV replication in Vero cells. All the compounds analyzed have CC_{50} values in the range of 980 μM and 1472 μM, proving their low cytotoxicity profile, even when compared to the marketed drug chloroquine (CC_{50} = 420 μM)

selected as a positive control for *in vitro* anti-ZIKV assays as described earlier [43]. Consequently, the four pyrazolone derivatives were screened for their inhibitory activity against ZIKV replication at 50 μM. Interestingly, they strongly reduced virus replication with inhibition rates of 71.2% for compound **1a**, 79.3% for compound **1b**, 50.0% for compound **1c**, and 68.3 % for compound **1d**. Most of these derivatives showed higher activity than chloroquine (58% inhibition) which is a known endocytosis-blocking agent.

We further calculated their EC_{50} values from dose-response curves. The unsubstituted compound **1a** presented an EC_{50} of 16 μM, but the introduction of a methoxy group at the para position of the phenyl ring yielded a less active derivative (**1d**; EC_{50} = 25.4 μM). Yet, the introduction of a chlorine atom at the same position resulted in an even less active derivative (compound **1c**; EC_{50} = 46.63 μM). Surprisingly, the presence of two chlorine atoms at both *ortho* and *para* positions of the phenyl group significantly improved its antiviral activity (compound **1b**; EC_{50} = 4.3 μM) against ZIKV. These results indicated that the anti-ZIKV activity of these derivatives is favored by the addition of electron-withdrawing groups at both *ortho* and *para* positions, but the introduction of electron-donating groups also leads to good antiviral agents. Design, synthesis, and evaluation of other derivatives should be conducted in the future to obtain a robust structure-activity relationship analysis since the unsubstituted derivative showed great activity. For instance, the introduction of electron-withdrawing groups, such as other halogens (fluorine, bromine, and iodine) or nitro groups, or electron-donating groups, such as hydroxyl or amine groups, at similar positions of the phenyl ring will provide valuable information on this matter.

Interestingly, the pyrazolone derivatives evaluated in this work showed a more potent anti-ZIKV activity than other compounds reported, such as sofosbuvir with an EC_{50} value higher than 50 μM [44]. Another important parameter in antiviral drug discovery is the selectivity index (SI), which expresses the therapeutic window of a given compound. It is suggested that the ideal value for SI is 2 or higher for antiviral agents [45]. Excitingly, all compounds showed SI values comparable to or even higher than the control drug chloroquine (SI = 24.98), with compound **1c** presenting the lowest selectivity (SI = 24.21). By contrast, compound **1b** exhibited a significantly higher selectivity (SI = 342). These results point to the good *in vitro* efficacy and safety as antivirals.

Consequently, we evaluated the mechanism of action of these compounds. Only compound **1b** had direct effects on ZIKV particles and reduced approximately 75% of virus replication. We further investigated at which step of the ZIKV replication cycle these compounds might act. We noticed that compounds exhibited the highest antiviral activity when added up to 3 hpi, indicating that they act at early steps of virus replication, including attachment, entry, and replication.

Accordingly, we used computational tools to investigate whether the ZIKV

NS5 MTase could be a target of these compounds. Molecular docking demonstrated that the four pyrazolone derivatives interacted directly with key amino acids for NS5 MTase activity, through hydrogen bonding or van der Waals contacts. The most active derivative **1b** showed the highest affinity with the enzyme (binding energy of −6.23 kcal/mol) and interacted with residues such as Lys105, His110, and Val132, which are important residues for the activity of this enzyme from other flaviviruses as well. These results suggest that the antiviral activity of these derivatives may be triggered by the inhibition of ZIKV NS5 MTase.

Finally, according to our in silico studies, we observed that these compounds showed satisfactory pharmacokinetic properties, according to industry rules. Also, these derivatives showed good intestinal absorption and oral bioavailability, indicating that they are suitable for oral delivery. As well, they showed a comparable or safer profile than the marketed drug chloroquine, especially the unsubstituted derivative (**1a**), which, in turn, highlights this scaffold as promising for the development of novel drugs to tackle ZIKV infections.

5. Conclusion

In the present study, we have identified four pyrazolone derivatives (**1a**, **1b**, **1c**, and **1d**) with significant *in vitro* antiviral activity against ZIKV and good selectivity. The mechanism of action evaluation showed that these compounds act at early steps of virus replication while compound **1b** also showed virucidal activity. These compounds showed a high theoretical affinity with ZIKV NS5 Mtase and interacted with important residues from the protein's active site, pointing to this protein as a feasible target for the antiviral activity of these derivatives. Finally, the pharmacokinetic and toxicological predictions reinforced the promising potential of these compounds as drug candidates which deserve further investigations to fight ZIKV infections.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Elliott, K.C. and Mattapallil, J.J. (2024) Zika Virus—A Reemerging Neurotropic Arbovirus Associated with Adverse Pregnancy Outcomes and Neuropathogenesis. *Pathogens*, **13**, Article 177. https://doi.org/10.3390/pathogens13020177

[2] Foy, B.D., Kobylinski, K.C., Foy, J.L.C., Blitvich, B.J., Travassos da Rosa, A., Haddow, A.D., *et al.* (2011) Probable Non—Vector-Borne Transmission of Zika Virus, Colorado, USA. *Emerging Infectious Diseases*, **17**, 880-882. https://doi.org/10.3201/eid1705.101939

[3] Kuno, G. and Chang, G.-J. (2007) Full-Length Sequencing and Genomic Characterization of Bagaza, Kedougou, and Zika Viruses. *Archives of Virology*, **152**, 687-696. https://doi.org/10.1007/s00705-006-0903-z

[4] Centers for Disease Control and Prevention (2019) Zika Virus—Statistics and Maps. https://www.cdc.gov/zika/index.html

[5] Pan American Health Organization (2017) Zika—Epidemiological Update: Situation Summary in the Americas.

[6] França, G.V.A., Schuler-Faccini, L., Oliveira, W.K., Henriques, C.M.P., Carmo, E.H., Pedi, V.D., *et al.* (2016) Congenital Zika Virus Syndrome in Brazil: A Case Series of the First 1501 Livebirths with Complete Investigation. *The Lancet*, **388**, 891-897. https://doi.org/10.1016/s0140-6736(16)30902-3

[7] Jitsatja, A., Ramphan, S., Promma, P., Kuadkitkan, A., Wikan, N., Uiprasertkul, M., *et al.* (2020) Comparative Analysis of a Thai Congenital-Zika-Syndrome-Associated Virus with a Thai Zika-Fever-Associated Virus. *Archives of Virology*, **165**, 1791-1801. https://doi.org/10.1007/s00705-020-04667-7

[8] Schwartz, D.A. (2016) Autopsy and Postmortem Studies Are Concordant: Pathology of Zika Virus Infection Is Neurotropic in Fetuses and Infants with Microcephaly Following Transplacental Transmission. *Archives of Pathology & Laboratory Medicine*, **141**, 68-72. https://doi.org/10.5858/arpa.2016-0343-oa

[9] de Araújo, T.V.B., Ximenes, R.A.d.A., Miranda-Filho, D.d.B., Souza, W.V., Montarroyos, U.R., de Melo, A.P.L., *et al.* (2018) Association between Microcephaly, Zika Virus Infection, and Other Risk Factors in Brazil: Final Report of a Case-Control Study. *The Lancet Infectious Diseases*, **18**, 328-336. https://doi.org/10.1016/s1473-3099(17)30727-2

[10] Kazmi, S.S., Ali, W., Bibi, N. and Nouroz, F. (2020) A Review on Zika Virus Outbreak, Epidemiology, Transmission and Infection Dynamics. *Journal of Biological Research-Thessaloniki*, **27**, Article No. 5. https://doi.org/10.1186/s40709-020-00115-4

[11] Ventura, C.V., Maia, M., Ventura, B.V., Linden, V.V.D., Araújo, E.B., Ramos, R.C., *et al.* (2016) Ophthalmological Findings in Infants with Microcephaly and Presumable Intra-Uterus Zika Virus Infection. *Arquivos Brasileiros de Oftalmologia*, **79**, 1-3.

[12] Krenn, V., Bosone, C., Burkard, T.R., Spanier, J., Kalinke, U., Calistri, A., *et al.* (2021) Organoid Modeling of Zika and Herpes Simplex Virus 1 Infections Reveals Virus-Specific Responses Leading to Microcephaly. *Cell Stem Cell*, **28**, 1362-1379.E7. https://doi.org/10.1016/j.stem.2021.03.004

[13] Musso, D. and Gubler, D.J. (2016) Zika Virus. *Clinical Microbiology Reviews*, **29**, 487-524. https://doi.org/10.1128/cmr.00072-15

[14] Bernardo-Menezes, L.C., Agrelli, A., Oliveira, A.S.L.E.d., Moura, R.R.d., Crovella, S. and Brandão, L.A.C. (2022) An Overview of Zika Virus Genotypes and Their Infectivity. *Revista da Sociedade Brasileira de Medicina Tropical*, **55**, e0263-2022. https://doi.org/10.1590/0037-8682-0263-2022

[15] Calvet, G.A., Santos, F.B. and Sequeira, P.C. (2016) Zika Virus Infection: Epidemiology, Clinical Manifestations and Diagnosis. *Current Opinion in Infectious Diseases*, **29**, 459-466. https://doi.org/10.1097/qco.0000000000000301

[16] Sironi, M., Forni, D., Clerici, M. and Cagliani, R. (2016) Nonstructural Proteins Are Preferential Positive Selection Targets in Zika Virus and Related Flaviviruses. *PLOS Neglected Tropical Diseases*, **10**, e0004978. https://doi.org/10.1371/journal.pntd.0004978

[17] Elshahawi, H., Syed Hassan, S. and Balasubramaniam, V. (2019) Importance of Zika Virus NS5 Protein for Viral Replication. *Pathogens*, **8**, Article 169. https://doi.org/10.3390/pathogens8040169

[18] Egloff, M.-P. (2002) An RNA Cap (nucleoside-2'-O-)-Methyltransferase in the Flavivirus RNA Polymerase NS5: Crystal Structure and Functional Characterization. *The EMBO Journal*, **21**, 2757-2768. https://doi.org/10.1093/emboj/21.11.2757

[19] Zhang, Q., Sun, K., Chinazzi, M., Pastore y Piontti, A., Dean, N.E., Rojas, D.P., *et al.* (2017) Spread of Zika Virus in the Americas. *Proceedings of the National Academy of Sciences*, **114**, E4334-E4343. https://doi.org/10.1073/pnas.1620161114

[20] Gutiérrez-Bugallo, G., Piedra, L.A., Rodriguez, M., Bisset, J.A., Lourenço-de-Oliveira, R., Weaver, S.C., *et al.* (2019) Vector-Borne Transmission and Evolution of Zika Virus. *Nature Ecology & Evolution*, **3**, 561-569. https://doi.org/10.1038/s41559-019-0836-z

[21] Züst, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B.W., Ziebuhr, J., *et al.* (2011) Ribose 2'-*O*-Methylation Provides a Molecular Signature for the Distinction of Self and Non-Self mRNA Dependent on the RNA Sensor Mda5. *Nature Immunology*, **12**, 137-143. https://doi.org/10.1038/ni.1979

[22] Züst, R., Dong, H., Li, X., Chang, D.C., Zhang, B., Balakrishnan, T., *et al.* (2013) Rational Design of a Live Attenuated Dengue Vaccine: 2'-O-Methyltransferase Mutants Are Highly Attenuated and Immunogenic in Mice and Macaques. *PLOS Pathogens*, **9**, e1003521. https://doi.org/10.1371/journal.ppat.1003521

[23] Dong, H., Chang, D.C., Xie, X., Toh, Y.X., Chung, K.Y., Zou, G., *et al.* (2010) Biochemical and Genetic Characterization of Dengue Virus Methyltransferase. *Virology*, **405**, 568-578. https://doi.org/10.1016/j.virol.2010.06.039

[24] Faria, J.V., Vegi, P.F., Miguita, A.G.C., dos Santos, M.S., Boechat, N. and Bernardino, A.M.R. (2017) Recently Reported Biological Activities of Pyrazole Compounds. *Bioorganic & Medicinal Chemistry*, **25**, 5891-5903. https://doi.org/10.1016/j.bmc.2017.09.035

[25] Anuradha, S. (2014) Antiviral Agents and Treatment of Viral Infections. *International Medical Sciences Academy*, **27**, 191-192.

[26] Bernardino, A.M.R., Ferreira, V.F., Fontoura, G.A.T., Frugulhetti, I.C.P.P., Lee, M.Y., Romeiro, G.A., *et al.* (1996) Synthesis of 4-Anilino-1*H*-Pyrazolo [3,4-*b*] Pyridine Derivatives and Their *in vitro* Antiviral Activities. *Journal of the Brazilian Chemical Society*, **7**, 273-277. https://doi.org/10.5935/0103-5053.19960055

[27] Bernardino, A.M., Azevedo, A.R., Pinheiro, L.C., Borges, J.C., Paixão, I.C., Mesquita, M., *et al.* (2012) Synthesis and Anti-HSV-1 Evaluation of New 3*H*-Benzo[*b*]Pyrazolo[3,4-*h*]-1,6-Naphthyridines and 3*H*-Pyrido[2,3-*b*]Pyrazolo[3,4-*h*]-1,6-Naphthyridines. *Organic and Medicinal Chemistry Letters*, **2**, Article No. 3. https://doi.org/10.1186/2191-2858-2-3

[28] dos Santos, M.S., Oliveira, M.L.V., Bernardino, A.M.R., de Léo, R.M., Amaral, V.F., de Carvalho, F.T., *et al.* (2011) Synthesis and Antileishmanial Evaluation of 1-Aryl-4-(4,5-Dihydro-1*H*-Imidazol-2-yl)-1*H*-Pyrazole Derivatives. *Bioorganic & Medicinal Chemistry Letters*, **21**, 7451-7454. https://doi.org/10.1016/j.bmcl.2011.09.134

[29] dos Santos, M.S., Gomes, A.O., Bernardino, A.M.R., de Souza, M.C., Khan, M.A., de Brito, M.A., *et al.* (2011) Synthesis and Antileishmanial Activity of New 1-Aryl-1*H*-

Pyrazole-4-Carboximidamides Derivatives. *Journal of the Brazilian Chemical Society*, **22**, 352-358. https://doi.org/10.1590/s0103-50532011000200022

[30] Quejada, L.F., de Almeida, R., Fazolin Vegi, P., Silva dos Santos, M., Maria Rolim Bernardino, A., Afonso Vericimo, M., *et al.* (2022) Rotenone Enhances Antifungal Activity of Novel Pyrazoles against *Candida* Spp. *European Journal of Medicinal Chemistry Reports*, **5**, Article 100045. https://doi.org/10.1016/j.ejmcr.2022.100045

[31] Terra, L., de Jesus Viegas, D., Bernardino, A.M.R., Faria, J.V., Vegi, P.F., de Paula Pinto, R.G.D.M., *et al.* (2020) Synthesis of 5-Amino-1-Aryl-3-Methyl-1H-Pyrazole-4-Carbonitriles, Antifungal Activity and in Silico Analysis. *Letters in Organic Chemistry*, **17**, 779-787. https://doi.org/10.2174/1570178617666200210105246

[32] Zhao, Z., Dai, X., Li, C., Wang, X., Tian, J., Feng, Y., *et al.* (2020) Pyrazolone Structural Motif in Medicinal Chemistry: Retrospect and Prospect. *European Journal of Medicinal Chemistry*, **186**, Article 111893. https://doi.org/10.1016/j.ejmech.2019.111893

[33] Das, N., Verma, A., Shrivastava, P.K. and Shrivastava, S.K. (2008) Synthesis and Biological Evaluation of Some New Aryl Pyrazol-3-One Derivatives as Potential Hypoglycemic Agents. *Indian Journal of Chemistry*, **47B**, 1555-1558.

[34] Mosmann, T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, **65**, 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

[35] Yang, H., Lou, C., Sun, L., Li, J., Cai, Y., Wang, Z., *et al.* (2018) Admetsar 2.0: Web-Service for Prediction and Optimization of Chemical ADMET Properties. *Bioinformatics*, **35**, 1067-1069. https://doi.org/10.1093/bioinformatics/bty707

[36] Pires, D.E.V., Blundell, T.L. and Ascher, D.B. (2015) pkCSM: Predicting Small-Molecule Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures. *Journal of Medicinal Chemistry*, **58**, 4066-4072. https://doi.org/10.1021/acs.jmedchem.5b00104

[37] Lagorce, D., Bouslama, L., Becot, J., Miteva, M.A. and Villoutreix, B.O. (2017) FAF-Drugs4: Free ADME-Tox Filtering Computations for Chemical Biology and Early Stages Drug Discovery. *Bioinformatics*, **33**, 3658-3660. https://doi.org/10.1093/bioinformatics/btx491

[38] Coloma, J., Jain, R., Rajashankar, K.R., García-Sastre, A. and Aggarwal, A.K. (2016) Structures of NS5 Methyltransferase from Zika Virus. *Cell Reports*, **16**, 3097-3102. https://doi.org/10.1016/j.celrep.2016.08.091

[39] Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., *et al.* (2009) AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *Journal of Computational Chemistry*, **30**, 2785-2791. https://doi.org/10.1002/jcc.21256

[40] Wallace, A.C., Laskowski, R.A. and Thornton, J.M. (1995) LIGPLOT: A Program to Generate Schematic Diagrams of Protein-Ligand Interactions. *Protein Engineering, Design and Selection*, **8**, 127-134. https://doi.org/10.1093/protein/8.2.127

[41] Silva, E.M., Conde, J.N., Allonso, D., Nogueira, M.L. and Mohana-Borges, R. (2013) Mapping the Interactions of Dengue Virus NS1 Protein with Human Liver Proteins Using a Yeast Two-Hybrid System: Identification of C1q as an Interacting Partner. *PLOS ONE*, **8**, e57514. https://doi.org/10.1371/journal.pone.0057514

[42] Song, B., Yun, S., Woolley, M. and Lee, Y. (2017) Zika Virus: History, Epidemiology, Transmission, and Clinical Presentation. *Journal of Neuroimmunology*, **308**, 50-64. https://doi.org/10.1016/j.jneuroim.2017.03.001

[43] Shiryaev, S.A., Mesci, P., Pinto, A., Fernandes, I., Sheets, N., Shresta, S., *et al.* (2017) Repurposing of the Anti-Malaria Drug Chloroquine for Zika Virus Treatment and Prophylaxis. *Scientific Reports*, **7**, Article No. 15771. https://doi.org/10.1038/s41598-017-15467-6

[44] Sacramento, C.Q., de Melo, G.R., de Freitas, C.S., Rocha, N., Hoelz, L.V.B., Miranda, M., *et al.* (2017) The Clinically Approved Antiviral Drug Sofosbuvir Inhibits Zika Virus Replication. *Scientific Reports*, **7**, Article No. 40920. https://doi.org/10.1038/srep40920

[45] Tsuchiya, Y., Shimizu, M., Hiyama, Y., Itoh, K., Hashimoto, Y., Nakayama, M., *et al.* (1985) Antiviral Activity of Natural Occurring Flavonoids *in Vitro*. *Chemical and Pharmaceutical Bulletin*, **33**, 3881-3886. https://doi.org/10.1248/cpb.33.3881