Research paper

Antiviral potential of diminazene aceturate against SARS-CoV-2 proteases using computational and *in vitro* approaches

Esley S. Santos [a,b], Priscila C. Silva [a], Paulo S.A. Sousa [c,d], Cristhyane C. Aquino [e], Gabriella Pacheco [a,b], Luiz F.L.S. Teixeira [a,d], Alyne R. Araujo [d], Francisca B.M. Sousa [a], Romulo O. Barros [f], Ricardo M. Ramos [f], Jefferson A. Rocha [c,d], Lucas A.D. Nicolau [a,d], Jand V.R. Medeiros [a,b,d,*]

[a] *Laboratory of Pharmacology of Inflammation and Gastrointestinal Disorders (LAFIDG), Post-graduation Program in Biotechnology, Parnaíba Delta Federal University, Parnaíba, PI, Brazil*
[b] *Medicinal Plants Research Center (NPPM), Federal University of Piauí, Teresina, Brazil*
[c] *Laboratory of Medicinal Chemistry and Biotechnology, QUIMEBIO, Federal University of Maranhão, São Bernardo, MA, Brazil*
[d] *Biodiversity and Biotechnology Research Center, BIOTEC, Post-graduation Program in Biotechnology, Parnaíba Delta Federal University, Parnaíba, PI, Brazil*
[e] *Postgraduate Program in Medical Sciences, Federal University of Ceará, Fortaleza, CE, Brazil*
[f] *Research Laboratory in Information Systems, Department of Information, Environment, Health and Food Production, Federal Institute of Piauí, LaPeSI/IFPI, Teresina, Piauí, Brazil*

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ABSTRACT

Diminazene aceturate (DIZE), an antiparasitic, is an ACE2 activator, and studies show that activators of this enzyme may be beneficial for COVID-19, disease caused by SARS-CoV-2. Thus, the objective was to evaluate the *in silico* and *in vitro* affinity of diminazene aceturate against molecular targets of SARS-CoV-2. 3D structures from DIZE and the proteases from SARS-CoV-2, obtained through the Protein Data Bank and Drug Database (Drubank), and processed in computer programs like AutodockTools, LigPlot, Pymol for molecular docking and visualization and GROMACS was used to perform molecular dynamics. The results demonstrate that DIZE could interact with all tested targets, and the best binding energies were obtained from the interaction of Protein S (closed conformation −7.87 kcal/mol) and M_{pro} (−6.23 kcal/mol), indicating that it can act both by preventing entry and viral replication. The results of molecular dynamics demonstrate that DIZE was able to promote a change in stability at the cleavage sites between S1 and S2, which could prevent binding to ACE2 and fusion with the membrane. In addition, *in vitro* tests confirm the *in silico* results showing that DIZE could inhibit the binding between the spike receptor-binding domain protein and ACE2, which could promote a reduction in the virus infection. However, tests in other experimental models with in vivo approaches are needed.

1. Introduction

The virus causing COVID-19, SARS-CoV-2; also named as Severe Acute Respiratory Syndrome virus - SARS, was initially identified in China as an enveloped positive-sense RNA virus capable of infecting human cells through binding of the spike protein to Angiotensin-Converting Enzyme II (ACE2) and other factors that aid virus entry [1–3].

The binding of Spike protein to ACE2, an enzyme that participates in the Renin-Angiotensin System (RAS) and has important physiological functions, such as regulation of water and electrolyte balance and blood pressure, can generate harmful effects [4]. Because ACE2 regulates the harmful effects of Ang II, cleaving it into Ang1-7, which binds to MAS receptors generating vasodilation, where the reduction in ACE2 caused by SARS-CoV-2 can lead to pro-inflammatory mechanisms and cardio-vascular injury [5–7].

SARS-CoV-2 commonly causes lung cell injury because this virus can encode various proteins that allow it to escape the immune system of the host. In addition, these proteins attack and overactivate more inflammatory and immune cells, inducing a cytokine storm, resulting in severe damage to infected tissues [8].

The critical stage during viral internalization process occurs when receptor binding domain (RBD) binds to ACE2. RBD is found on the virus

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ADT	AutoDock Tools
ATB	Automated Topology Builder
LS	Local search
RAS	Renin-Angiotensin System
RBD	Receptor binding domain
RMSF	Root mean square fluctuation
RMSD	Root Mean Square Deviation

surfacein the Spike glycoprotein and has two subunits, S1 and S2. Following RBD binding to ACE2, the spike protein is proteolytically cleaved between the two subunits, facilitating fusion with host cell membrane [9,10]. Additionally, Catepsina L (CatL), a cysteine protease, appears to be involved in the activation of protease S on membranous surface of host cell cells [11,12]. The cleavage of ACE2 is necessary for the fusion of SARS-CoV-2 in the cell membrane, a process performed by domain 17 metallopeptidase (ADAM-17), also known as TNF-α Converting Enzyme (TACE), resulting in the downregulation of ACE2 expression on cell surface generating an imbalance in the RAS. Additionally, an increase in ADAM 17 activity can lead to production of TNF-α and IL-6 and other pro-inflammatory cytokines, leading to the inflammatory process during SARS-CoV-2 infection [13,14]. Once inside cells, SARS-CoV-2 uses Mpro, a type 3C protease important in viral replication and transcription [15–17].

It is important to highlight that while ACE2 downregulation in the cell membrane plays a critical role in the pathology of SARS-CoV-2 infection, as it is associated with cardiovascular, pulmonary, and gastrointestinal tract problems; it can generate organ dysfunction in response to infection [13]. Interruption of Ang II degradation due to downregulation of ACE2 may be an important event in COVID-19, so drugs that activate and upregulate the RAS may have benefits in the pathogenesis of COVID-19 [7,18].

Diminazene Aceturate (DIZE: C14H15N7·2C4H7NO3) is an aromatic diamidine that was developed more than six decades ago as an anti-parasitic, is an ACE2 activator in several experimental models, contributing to the protective effect of RAS [6]. DIZE has a very rapid absorption rate and short elimination half-life, being metabolized in the liver and redistributed to peripheral tissues for renal excretion [19].

Activation of ACE2 by DIZE could attenuate pulmonary hypertension; in addition, it re-established the balance of the RAS and reduced the inflammatory cells in the peri-infarct área [20,21]. Furthermore, DIZE can reduce the pro-inflammatory cytokines IL-6, IL-12 and TNF-α [22], thus suggesting that it may produce therapeutic benefits in diseases that alter physiological conditions, especially pulmonary hypertension [23].

Even by considering the ability to upregulate ACE2 such as a complication factor due to this enzyme be the host cell entrance, the use of DIZE in the treatment of patients with COVID-19 seems to be interesting if molecular aspects are taken into account [24]. Moreover, others anti-parasitic agents have demonstrated, specially in the ambit of *in vitro* approaches, anti-viral potential [25]. Therefore, in this study we investigated the effect of DIZE on SARS-CoV-2 targets using a computational approach, e evaluated whether the drug could interfere with the binding of Spike protein and ACE2 (necessary for the virus entry into the cell) by *in vitro* methodology, which could reduce virus infection.

2. Material and methods

2.1. Molecular docking

The 3D structure of possible proteins from the new coronavirus (SARS-CoV-2) target were obtained from the Protein Data Bank (PDB) [26] with the code PDB ID: ACE2 (1R42), ADAM17 (2DDF), Cathepsin L (2XU3), RBD (6LZG), closed Protein S (6VXX), open Protein S (6VYB), Mpro (6Y2E) and TMPRSS2 (2OQ5). All docking procedures utilized the Autodock 4.2 package [27–29]. Residues of 3D protein structures were protonated using the online server H++ (http://biophysics.cs.vt.edu/hppdetails.php). Protein and ligands were prepared for docking simulations with AutoDock Tools (ADT) version 1.5.6 [30]. The receptor was considered rigid; each ligand was considered flexible. Gasteiger [31] partial charges were calculated after adding fall hydrogens. Non-polar hydrogen atoms of the protein and ligand were subsequently merged. A cubic box of 60 × 60 × 60 points spacing 0.35 Å between the grid points was generated for whole protein target. The affinity grid centers were defined. Global search Lamarckian genetic algorithm (LGA) [32] and the local search (LS) pseudo-Solis and Wets [33] methods were applied in the docking search. Each ligand was subjected to 100 independent runs of docking simulations [34]. Other docking parameters were set as the default values. The resulting docked conformations were clustered into families according to the RMSD. For a more detailed analysis, the coordinates of the selected complexes were chosen by criterion of lowest docking conformation of the cluster with the lowest energy combined with visual inspection.

2.2. Molecular dynamics (MD)

The MD simulations of the complexes selected after molecular docking (Spike_Dize) were performed using GROMACS 2018.1 software [35,36] following Arcanjo and collaborators [37]. Spike glycoprotein (S glycoprotein) model, code P0DTC2, was used from the SWISS-MODEL server [38]. The model is a trimer (A, B, and C chains), and each chain contains 1.162 amino acid residues. The protonation states of histidine residues of model were determined using the H++ [39]. The topologies of the ligands were generated from online repository Automated Topology Builder (ATB) version 3.0 [40]. To increase sampling, Spike_Dize complex MD simulations were run twice (02) for 10 ns using different starting atomic velocities assuming a Maxwellian distribution. Data generated during the last 4 ns of each simulation system, the period defined as production stage, were used for analysis. A total of 80 snapshots, each taken every 100 ps, were obtained during the production stage. The details of the interactions were calculated with LigPlot++ software [41]. A minimum of 50% contact (total of hydrophobic interactions and hydrogen bonds) in the analyzed frames was defined as a criterion of binding efficacy [42].

2.3. ACE2/SARS-CoV-2 spike inhibitor Screening Assay Kit

We tested DIZE (Sigma Aldrich; St. Louis, MO, USA) to inhibit the binding of SARS-CoV-2 spike protein to ACE2 using the SARS-CoV-2 Spike-ACE2 interaction inhibition Screening Assay Kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), according to the manufacturer's instructions. DIZE was used at the following concentrations 2.68; 8.04, 24.12, 76.6, 217.8, and 653.4 μM dilution in DMSO (0.17) or immunoassay buffer (for Spike inhibitor control), concentrations were from according to a published study on docking and *in vitro* experiments measuring the ability of DIZE to activate ACE2 [6]. Briefly, all reagents used were prepared following the kit recommendations. The assay uses a 96-well plate with a rabbit antibody labeled with the Spike S1 RBD protein of SARS-CoV2 that binds to a plate pre-coated with mouse and rabbit antibodies. Initially, Immunoassay Buffer C was added to the bottom wells; then, the test samples (DIZE or inhibitor control – item n° 402055) were added to all wells, followed by the addition of ACE2 inhibitor screening reagent (item n° 402057) to all wells except the blanks. Then Spike inhibitor screening reagent (item n° 402056) was added, except for blanks and background wells. The plate was incubated for 60 min at room temperature on the shaker. After incubation, the wells were washed, and the Anti-His-HRP conjugate (item n° 402054) was added to each well, except for the blank wells followed by 30 min incubation;

after this time, the TMB substrate solution (item n° 400074) was added to each well; finally, HRP stop solution (item n° 10011355) was added, and the absorbance was measured in a microplate reader (SpectraMax, Molecular Devices, USA) at 450 nm %Inhibition was calculated from: (Absorbance$_{control}$ − Absorbance$_{sample}$/Absorbance$_{control}$), following kit instructions. Where Absorbance$_{control}$ is equivalent to the reading of wells where no inhibitor is placed, for the interaction between spike protein and ACE2 to occur (100% initial activity according to Cayman assay brochure).

2.4. Statistical analysis

Data represent the means of the results in triplicate and are expressed as mean ± standard error of the mean. For statistically significant differences, it was considered * $p < 0.05$, analyzed by ANOVA and Tukey's post hoc, after verifying the normality of the data through the Shapiro-wilk test. From the absorbances, after normalization of data, a non-linear regression was performed to calculate the IC$_{50}$. GraphPad Prism software 8.0 used for statistical analysis.

3. Results

3.1. Docking analysis

The results concerning molecular docking of the diminazene ligand with the 1R42, 2DDF, 2XU3, 6LZG, 6VXX, 6VYB, 6Y2E, and 2OQ5 proteins are presented in Table 1. The best energies obtained from interaction between diminazene aceturate and proteases were with the closed protein S (6VXX) and Mpro (6YE2), the others being described in descending order. The affinity was observed with binding energy equal to −7.87 kcal/mol, and an inhibition constant of 1.71 μM (Table 1; Fig. 1). It was possible to observe the interaction with the site active amino acids Ile973, Leu517 and Ala520 via hydrogen bond in ligands. Furthermore, Mpro (6Y2E) had a molecular affinity with diminazene (Fig. 2), with binding energy equal to −6.23 kcal/mol, and an inhibition constant of 26.97 μM. Interaction with the amino acids Phe140, Glu166, His41, His164, Cys44, and Thr25 in active site was observed via a hydrogen bond.

The other proteases and energies obtained from the interaction with diminazene aceturate are described in the table below: open S protein −5,8 kcal/mol and interacting with Pro463, Asp428 (Fig. 3), TMPRSS2 connecting to Arg41, Ser195, and Asp189 with the energy of −6,08 kcal/mol (Fig. 4), ADAM17 −6.0 kcal/mol Gly362, Gly412 and Ser457 (Fig. 5), RBD -5.92 kcal/mol with Arg393, Glu402, Glu375 and Ala348

(Fig. 6), Cathepsin L −5.14 kcal/mol with Pro59, Gly58, Asn62 and Glu95 (Fig. 7) and ACE2 -4,06 kcal/mol Glu402, Tyr510 and Tyr515 (Fig. 8).

3.2. Molecular dynamics

Fig. 9 shows that DIZE interacts with the A and B chains of the spike model. DIZE exhibits hydrophobic and hydrogen bond interactions with four residues of the A chain (Tyr396, Phe515, Glu516, and Ser514). In the B chain, DIZE shows hydrophobic interactions with five residues (Tyr200 - 95%, Ile197 - 71%, Lys202 - 68%, Asp228 - 56% and Asp198 - 37%) and hydrogen bonds with four residues (Lys202 - 15%, Asp198 - 15%, Asp228 - 15% and Tyr200 - 2%) (Fig. 9).

Table 2 shows the Root Mean Aquare Fluctuation (RMSF) values for the residues that interacted with DIZE ligands (Fig. 9). It is observed that there was no significant difference in flexibility in the region of these residues. Fig. 10 shows greater flexibility for residues 682, 685, and 1161 (Fig. 10).

3.3. In vitro

We examined the ability of DIZE to inhibit S-RBD protein binding to ACE2 in tools 2.68, 8.04, 24.12, 72.6, 217.8, and 653.4 μM. As shown below, the results demonstrate that inhibition rates ranging from 3.82 ± 1.18 to 89.87 ± 7.19%. The highest inhibition rate was achieved at the two highest concentrations tested, 217.8 and 653.4 μM, about 80% (Fig. 11). After the analyses, IC50 was equal to 20.62 μM (95% CI = 10.37–37.82 μM).

4. Discussion

COVID-19 stands out as a disease of great concern to public health worldwide [1], having some phases, including the incubation period (up to 5 days), the onset of symptoms (6–7 days), painful breathing (8 days), respiratory distress syndrome (9 days) and severe cases - ICU (10 days) [43]. Since the beginning, several advances have been made to understand the pharmacological targets and therapeutic managements better; however, the suitable search for existing drugs seems valuable [44,45].

An alternative would be drug repositioning, as it is a new strategy for the treatment of emerging diseases. Time becomes crucial in discovering a therapeutic option, where the search for drugs already available on the market and that have new functions could reduce the time, the cost of developing and manufacturing a new therapy, benefiting from detailed information on pharmacology and toxicity which allows for faster trials
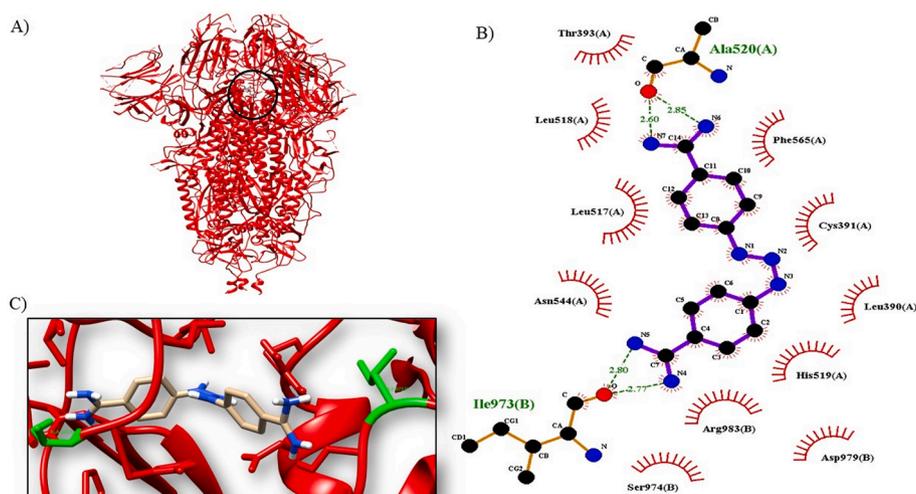

Fig. 1. 3D molecular docking of the protein–ligand complex with 6VXX protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).

Fig. 2. 3D molecular docking of the protein–ligand complex with 6Y2E protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).

Fig. 3. 3D molecular docking of the protein–ligand complex with 6VYB protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).
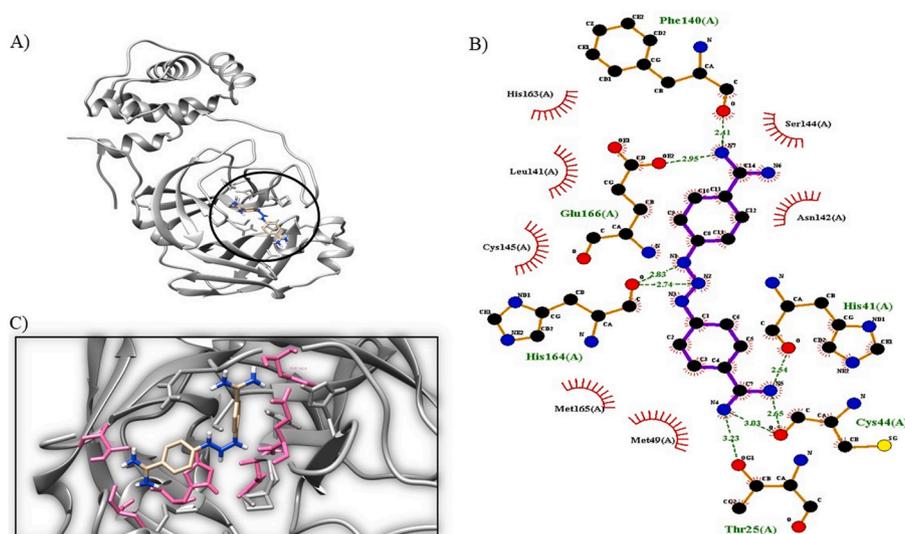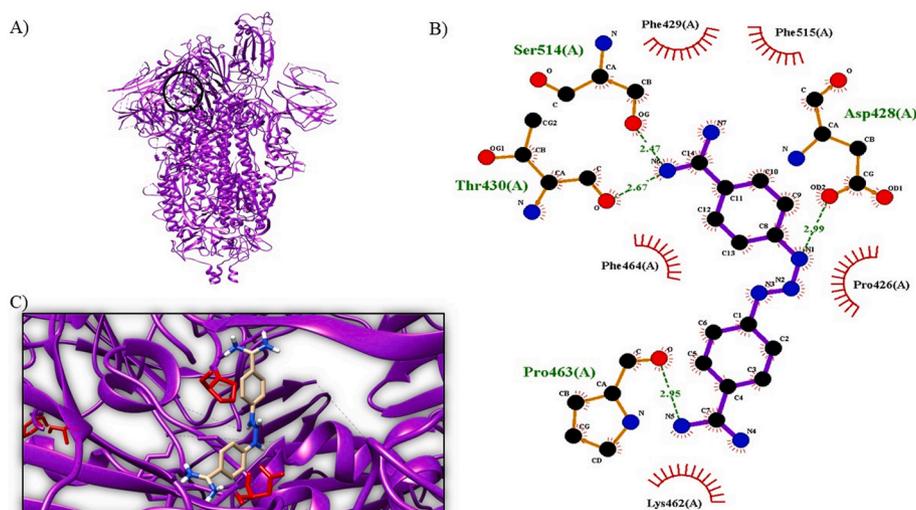
and clinical use [46,47].

DIZE, an ACE2 activator, has been described in several experimental models showing beneficial effects of RAS [23]. Despite being a veterinary drug, studies have shown that DIZE was used for the treatment of African trypanosomiasis in humans with positive results and minimal adverse effects [48,49], which could justify its use as a new therapeutic option in humans.

Studies have shown that drugs that upregulate ACE2 could be harmful to the patient by stimulating the entry of the virus into cells and the severity of the disease [50,51]. Therefore, Pang; Cui; Zhu [52] do not recommend using DIZE for the treatment of COVID-19. Nonetheless, Nicolau and collaborators [7] propose that DIZE can be used as a new therapeutic strategy in the late stage of pulmonary complications of COVID-19 and that the reestablishment of ACE2 levels could restore homeostasis.

In addition, according to Rodríguez-puertas [53], the treatment of patients using ACE2 activators could bring two therapeutic benefits: i) preventing the binding of the spike protein of SARS-CoV-2 to ACE2 and ii) enabling protective effects of the enzyme in several organs, preventing fibrosis and lung injury. Based on that, we focused our interest

on a drug already available on the market and on proteases associated with the life cycle of SARS-CoV-2, because during the cycle, several processes occur that can be used as molecular targets for therapeutic solutions [54]. This study demonstrated for the first time that Diminazene Aceturate can interact with all of the SARS-CoV-2 proteases tested through molecular docking (Table 1). Our results corroborate with Matsoukas and collaborators [55]. These authors showed that, *in silico* docking and ligand interaction studies, DIZE can interact with some spike protein/ACE2 complex [55].

One of our tested targets was the spike protein of SARS-CoV-2 used in the closed conformation (6VXX) and in the open conformation (6VYB), thus showing that DIZE can bind to proteins regardless of their conformation; however, it demonstrated greater affinity with the closed protein S (−7.87 kcal/mol), thus suggesting that it may act even before SARS-CoV-2 binds to ACE2 and is cleaved by TMPRSS2. As described in the literature, the energies of lower bonds indicate a greater attraction of the ligand for the target thus the ligand with higher affinity can be used as a drug with greater capacity for clinical trials [56,57].

Another target tested was Mpro (6Y2E), a very important cysteine protease for viral multiplication, which makes it a target in an attempt to
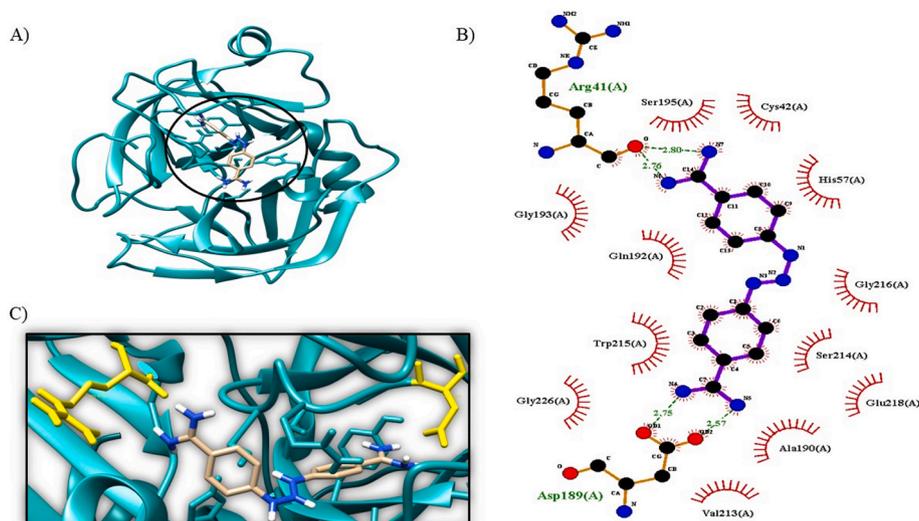
Fig. 4. 3D molecular docking of the protein–ligand complex with 2OQ5 protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).
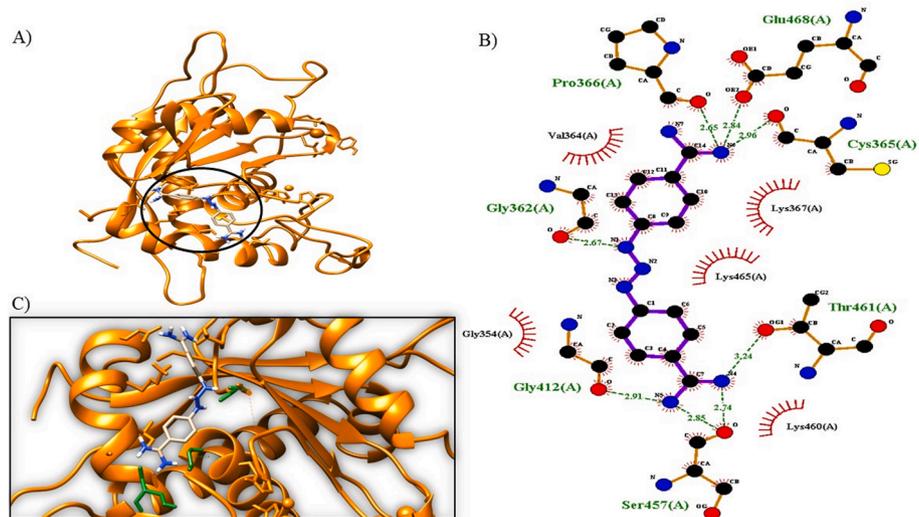

Fig. 5. 3D molecular docking of the protein–ligand complex with 2DDF protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).

interrupt the coronavirus life cycle [58,59]. The results suggest that the binding energy found (−6.23 kcal/mol) can block viral replication and prevent the continuation of the virus life cycle. The active site of Mpro is located on the amino acid His41/Cys145 of the catalytic dyad, and Glu166 in molecule A is important to keep the enzyme in its active conformation [12,17]. Our results showed that DIZE acts on the respective amino acids of the active site and that the sensitivity of Mpro to structural disturbances is located around the catalytic site Cys145 [60]. Thus, we propose that DIZE can present satisfactory results in inhibiting this protease, interrupting the proliferation of infectious viral particles.

TMPRSS2, a transmembrane serine protease that also participates in the SARS-CoV-2 infection cycle, was tested, and the results obtained demonstrate that DIZE can interact with the protease (−6.08 kcal/mol); according to the report of Chikhale and collaborators [61], camostat mesylate, a TMPRSS2 inhibitor, obtained energy of −6.23 kcal/mol and according to Gyebi and collaborators [62], it acts on the following amino acids: Gln 192, Arg 41, Ser 195, Trp 215, Ala 190, and Asp 189; however, DIZE had similar energy to the inhibitor and acts on the same and other amino acids: Arg41, Ser195, Asp189 and Gln192, His57, Ser214,

Trp215, Glu218, Gly216, Gly226, Ala190, Val213, Cys42, and Gly193. Camostat mesylate could inhibit SARS-CoV-2 infection in human lung cells [63]. It is then suggested that DIZE can also inhibit it, as it has binding energy similar to the inhibitor and acts on the same amino acids; thus, such action would prevent the virus from internalizing.

Cleavage of ACE2 by ADAM 17(2DDF) generates downregulation of ACE2, so its inhibition could exhibit beneficial effects in patients with COVID-19 [64,65]. Furthermore, the energy obtained from the interaction of DIZE with ADAM 17 (−6.0 kcal/mol) demonstrates the possibility of promoting its inhibition. Murumkar and collaborators [66] report that Gly349, Leu 348, and Glu406 are essential active site amino acids and Borah and collaborators [67] demonstrated that IK682, a selective ADAM inhibitor 17, acts on the same amino acids mentioned; however, DIZE was unable to act on any of the amino acids, thus suggesting that the affinity mechanism may occur otherwise and should be investigated.

The RBD (6LZG) represents an important step for SARS-CoV-2 infection, as it is found in protein S and binds to ACE2 present on the cell surface, allowing viral entry and replication [68]. Our results indicate that DIZE can interact with RBD (−5.92 kcal/mol). It was recently
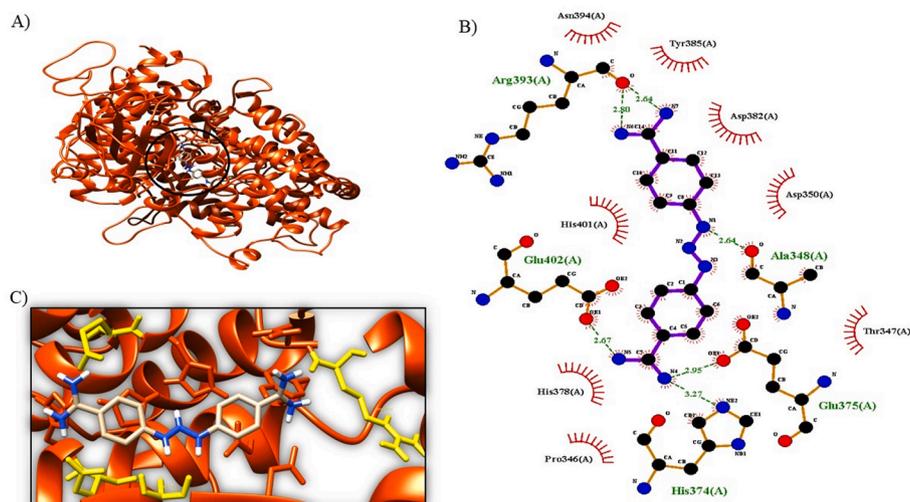
Fig. 6. 3D molecular docking of the protein–ligand complex with 6LZG protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).
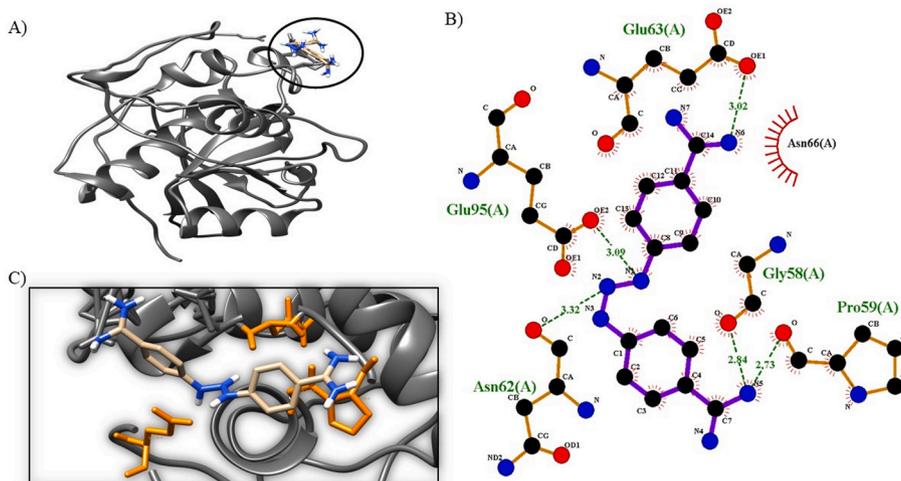
Fig. 7. 3D molecular docking of the protein–ligand complex with 2XU3 protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).
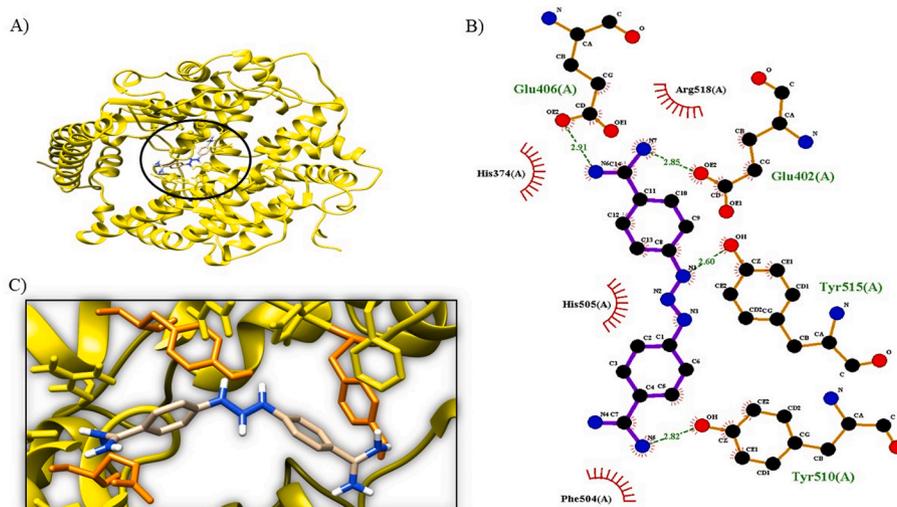
Fig. 8. 3D molecular docking of the protein–ligand complex with 1R42 protein and ligand diminazene illustrating the active binding site (A) with the respective hydrogen bridge interactions (B and C).

Fig. 9. Interactions for the Spike_DIZE complex. Frequency of hydrophobic contacts – (blue) chain A and (green) chain B. Frequency of hydrogen bonds - (orange) chain A and (red) chain B. The frequencies correspond to the end four of molecular dynamics simulations.
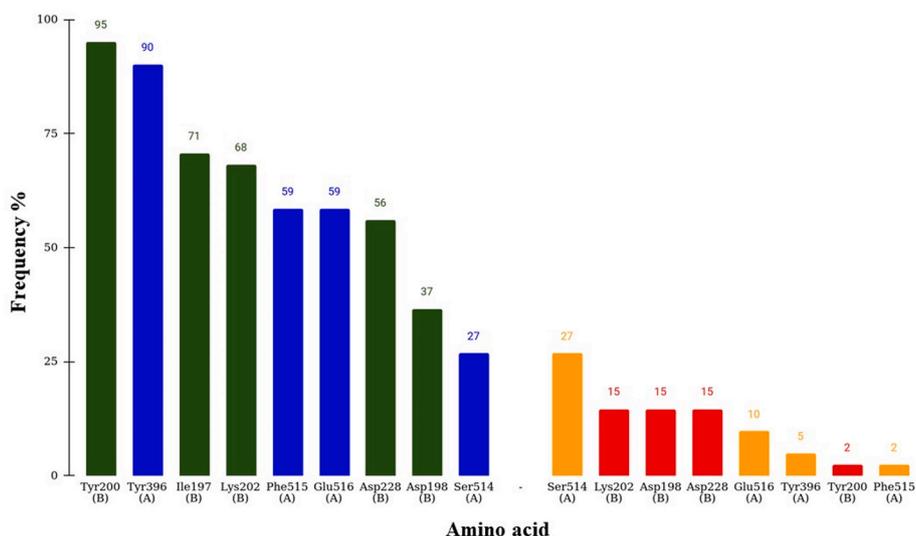
Table 1
Molecular affinity parameters of diminazene ligand with 1R42, 2DDF, 2XU3, 6LZG, 6VXX, 6VYB, 6Y2E and 2OQ5.

Complex (Protein-ligand)	ΔG_{bind}[a] (kcal. mol^{-1})	Ki[b]	Amino acids that interact through hydrogen bonds[c]	Amino acids that perform hydrophobic interaction[c]
DIM/Closed Protein S (6VXX)	- 7.87	1.71 uM	Ile973and Ala520	Phe565, Thr393, Cys391, Leu518, Asn544, Leu390, His519, Asp979, Arg983, Leu517 and Ser974
DIM/Open Protein S (6VYB)	- 5.8	55.64 uM	Pro463, Asp428, Ser514 and Thr430	Phe515, Phe429, Pro426, Lys462 and Phe464
DIM/Mpro (6Y2E)	- 6.23	26.97 uM	Phe140, Glu166, His41, His164, Cys44 and Thr25	Cys145, Leu141. His 163, Ser144, Asn142, Met49 and Met165
DIM/ TMPRSS2 (2OQ5)	- 6.08	35.17 uM	Arg41, Ser195 and Asp189	Gln192, His57, Ser214, Trp215, Glu218, Gly216, Gly226, Ala190, Val213, Cys42 and Gly193
DIM/ ADAM17 (2DDF)	- 6.0	39.76 uM	Gly362, Gly412, Glu468, Pro366, Cys365, Thr461 and Ser457	Lys465, Lys460, Gly354, Val364 and Lys367
DIM/RBD (6LZG)	- 5.92	45.54 uM	Arg393, Glu402, Glu375, His374 and Ala348	Tyr385, His401, His378, Pro346, Thr347, Asp382, Asp350 and Asn394
DIM/ Cathepsin L (2XU3)	−5.14	171.15 uM	Pro59, Gly58, Asn62, Glu63 and Glu95	Asn66
DIM/ECA2 (1R42)	- 4.06	1.05 mM	Glu402, Tyr510, Glu406 and Tyr515	Arg518, His374, His505 and Phe504

[a] Power bond in best conformation.
[b] Best conformation inhibition constant.
[c] Obtained with Ligplot program.

Table 2
RMSF values for residues that showed interactions with ligands. Root Mean Square Fluctuation values for the residues that interacted with Dize ligands and chain A and B.

Ligand_chain	Residue	RMSF (nm)
Dize_A	Tyr396	0.1163
	The515	0.1183
	Glu516	0.1499
	Ser514	0.1024
Dize_B	Tyr200	0.1198
	Ile197	0.1187
	Lys202	0.0928
	Asp228	0.1106
	Asp198	0.1637
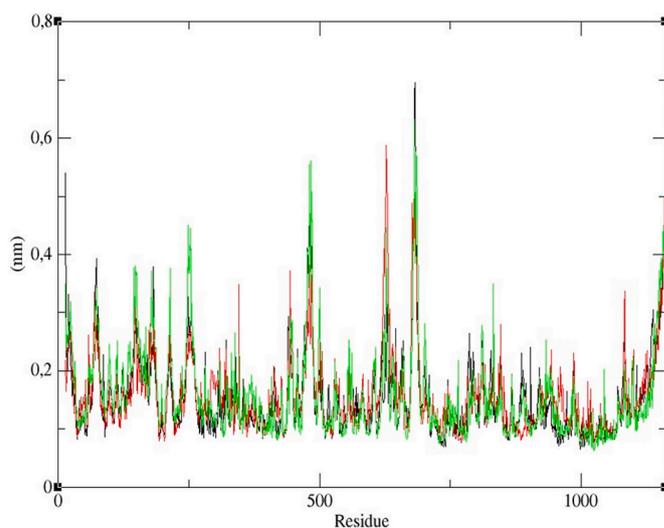
Fig. 10. RMSF plots for chains A, B and C for ligand DIZE. Root mean square fluctuation (RMSF) - Complex Spike_Dize. Chain A (black), Chain B (red), Chain C (green). Graphical images were produced by XMGRACE software (Turner, 2005).

identified that amino acid Met82 from ACE2 can bind to Leu472 from RBD and residue Thr487 in RBD interacts with Tyr41 and Lys353 from ACE2 [69], but our results demo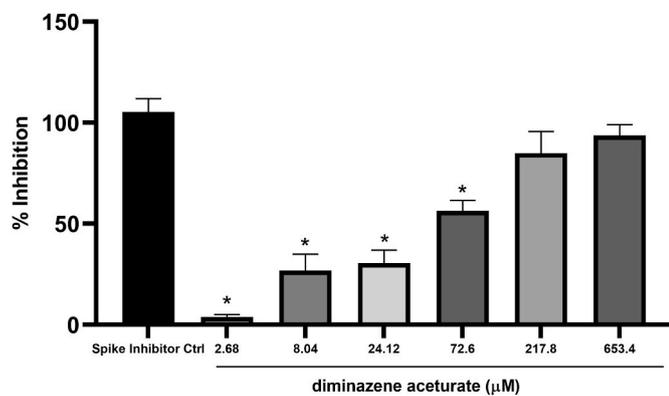nstrate that DIZE does not interact with any of these residues; however, such energy obtained suggests that our compound can prevent the virus from binding to ACE2. A recent study argue that the amphoteric nature of DIZE may allow binding to acidic

Fig. 11. ACE2 S-RBD protein inhibitor screening assay. %inhibition of SARS-Cov-2 spike protein binding to ACE2. Data represent the means of the results in triplicate and are expressed as mean ± standard error of the means. *$p < 0.05$ in relation to Spike inhibitor control (black bar), followed by ANOVA and Tukey's test.

and basic sites of SARS-CoV-2 and possibly block the entry of the virus to ACE2 in the lungs [55].

Cathepsin L (2XU3) plays an important role in SARS-CoV-2 infection, promoting cleavage of the spike glycoprotein S1 subunit [11]. Our results demonstrate that DIZE can interact with CatL (−5.14 kcal/mol). Such results are similar to those found by Vivek-Ananth and collaborators [70], showing that E64-d, an inhibitor of cathepsin proteases including L, showed lower energy (−5.0 kcal/mol) than DIZE, and the catalytic site residues are Cys25, His163, Asp162, Met161, Ala135, Met70, Leu 69, and Gly68; Hardegger and collaborators [71] demonstrate that the amino acid Gly61 from the S3 pocket is found in the active site of the enzyme; however, DIZE does not act directly on the active site amino acids, indicating that the interaction can happen by another mechanism and that the energy generated by our compound is superior to the inhibitor, thus suggesting that it may promote inhibition.

We also demonstrate the *in silico* relationship of DIZE with the ACE2 (1R42) enzyme that functions as a gateway for the coronavirus in human cells [72]; since this drug is an activator of this enzyme in the RAS [6]. The analysis showed that DIZE promoted interaction with ACE2 (−4.06 kcal/mol). According to Pal and Talukdar [73], the amino acids of the active site of ACE2 would be: Pro426, Ile439, Leu440 and Glu442, and Kulemina and Ostrov [6] demonstrated that DIZE binds outside of the active site and can modulate the catalytic activity of ACE2 *in vitro*, increasing the maximum initial velocity of the enzyme by 2-fold. The activation of ACE2 by DIZE most likely involves a binding site different from the binding site for AngII and is probably not specific [55].

Our results are consistent with the literature, and we also demonstrate that DIZE does not act directly on the active site of the enzyme; we therefore suggest that its activation occurs by another mechanism and that the interaction with ACE2 may block SARS-CoV-2 binding and prevent its internalization, in addition to avoiding the downregulation of ACE2 and its deleterious effects on the RAS, because studies demonstrate that the activation and recruitment of ACE2 has therapeutic effects, preventing fibrosis and lung injury [53,74]. In addition, DIZE has reduced several pro-inflammatory cytokines, including IL-6, one of those responsible for the seriousness of COVID-19 [22,75]; therefore, we assume that this compound can improve the prognosis of patients with the disease.

Considering that one of the best binding energies obtained from molecular docking was the DIZE/S Protein complex (6VXX) in the closed conformation, molecular dynamics was performed to observe its behavior in the system. Our results demonstrate that DIZE presented an increase in the variation (0.2–0.7 nm) of the RMSF having greater flexibility between residues 682, 685, and 1161, thus suggesting that DIZE can change the stability and flexibility of the Spike protein, causing

a change in the protein's structure. The Root Mean Square Deviation (RMSD) measures the distance between the atoms of the structure, in addition to evaluating the stability and displacement of the system [76]. The results found demonstrate a variation of the stability and displacement of the structure, stabilizing at 0.5 nm, indicating that the structure undergoes some conformational change. The RMSD graph for the Spike_dize complex has been added to the Support Information.

Ou and collaborators [77] demonstrate that SARS-CoV-2 has a furin cleavage site between S1 and S2 at residues 682–685 that are important for virus tropism and transmissibility; Egieyeh and collaborators [78] show that high RMSF values indicate greater mobility and less stability in the protein structure, indicating that the coupled drug can prevent the stable interaction between the S protein and the ACE2 enzyme. It is important to highlight that this change in the stability and conformation of the protein between S1 and S2 can prevent protein S cleavage and the externalization of S2 so that fusion with the cell membrane occurs, thus reducing the infection. Therefore, we hypothesize that DIZE can promote the reduction of infection by SARS-CoV-2.

In addition, an *in vitro* experiment was also carried out to confirm our *in silico* results. The results obtained demonstrate that DIZE at the concentration of 217.8 and 653.4 μM was able to inhibit the binding of thespike protein RBD to ACE2 in about 80%, and we believe that these results are because the binding of DIZE to S-RBD protein residues reduces the interaction with ACE2, corroborating with the results of the molecular docking. Recently, it was demonstrated using the same experimental approach that the sulfated polysaccharides, polyunsaturated fatty acids, natural and semi-synthetic bile acids, inorganic polyphosphate and stapled peptides [79–83] showed an inhibitory effect similar to those found in this study, preventing the interaction of the spike with ACE2.

Furthermore, the IC50 value obtained (20.62 μM) is similar to those found in the literature evaluating the antiviral effect against SARS-CoV-2 of lopinavir with 26 μM [84]. Recently it was demonstrated that DIZE was able to inhibit proteases, furin and TMPRSS2, in cell cultures, with IC50 of 1.35 and 13.2 μM, respectively [85]. Still, when the concentrations of our compound are increased, its effect on inhibition becomes greater; thus, we suggest that DIZE may have promising effects on the interaction between spike-ACE2 and other proteases, reducing SARS-CoV-2 infection and may generate encouraging clinical effects.

Furthermore, the use of DIZE for humans is very tolerable, one study showed that patients used DIZE for the treatment of human trypanosomiasis [86,87], being regarded as a next-generation drug discovery for the treatment of cardiovascular disease, hypertension and capable of restoring ACE2 functions and promoting cardiovascular and pulmonary protection against SARS-CoV-2, improving the prognosis of patients with COVID-19 [88,89], which can accelerate its use as a therapeutic option in patients with COVID-19.

5. Conclusion

The results found in this article demonstrate that Diminazene Aceturate can interact with all targets tested in molecular docking, acting mainly on the active sites of proteases, and may promote blocking during the viral infection process. Furthermore, molecular dynamics results showed that DIZE can promote a change in amino acid stability at the S protein cleavage site, preventing binding with ACE2. Such results were confirmed through the *in vitro* test where there was a reduction in the binding rate between the S-RBD protein and ACE2. Therefore, considering the results found and the pharmacological activities described in the literature, we hypothesized that DIZE could interact with SARS-CoV-2 molecular targets related to viral entry and replication, preventing viral infection, reducing the inflammatory process, however, other experimental models with in vivo approaches are needed to confirm this hypothesis.

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Author statement

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2022.110161.

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