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New anti-SARS-CoV-2 aminoadamantane compounds as antiviral candidates for the treatment of COVID-19

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ABSTRACT

Here, the antiviral activity of aminoadamantane derivatives were evaluated against SARS-CoV-2. The compounds exhibited low cytotoxicity to Vero, HEK293 and CALU-3 cells up to a concentration of 1,000 μM. The inhibitory concentration (IC_{50}) of aminoadamantane was 39.71 μM in Vero CCL-81 cells and the derivatives showed significantly lower IC_{50} values, especially for compounds 3F4 (0.32 μM), 3F5 (0.44 μM) and 3E10 (1.28 μM). Additionally, derivatives 3F5 and 3E10 statistically reduced the fluorescence intensity of SARS-CoV-2 protein S from Vero cells at 10 μM. Transmission microscopy confirmed the antiviral activity of the compounds, which reduced cytopathic effects induced by the virus, such as vacuolization, cytoplasmic projections, and the presence of myelin figures derived from cellular activation in the face of infection. Additionally, it was possible to observe a reduction of viral particles adhered to the cell membrane and inside several viral factories, especially after treatment with 3F4. Moreover, although docking analysis showed favorable interactions in the catalytic site of Cathepsin L, the enzymatic activity of this enzyme was not inhibited significantly *in vitro*. The new derivatives displayed lower predicted toxicities than aminoadamantane, which was observed for either rat or mouse models. Lastly, *in vivo* antiviral assays of aminoadamantane derivatives in BALB/cJ mice after challenge with the mouse-adapted strain of SARS-CoV-2, corroborated the robust antiviral activity of 3F4 derivative, which was higher than aminoadamantane and its other derivatives. Therefore, aminoadamantane derivatives show potential

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broad-spectrum antiviral activity, which may contribute to COVID-19 treatment in the face of emerging and re-emerging SARS-CoV-2 variants of concern.

1. Introduction

The coronavirus disease 2019 (COVID-19) has been a major global concern in the past three years and it continues as an eminent threat to public health around the world. COVID-19 is caused by the severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) (CSG, 2020), which was first reported in December 2019 associated with cases of severe pneumonia (Cucinotta and Vanelli, 2020). As of September 3rd, 2023, around 770 million cases and 6.95 million deaths were reported across the globe, however, it is estimated that these numbers are two to four times higher (Adam, 2022; WHO, 2023).

The genome of SARS-CoV-2, like the genomes of other RNA viruses, is prone to random mutations that can target both structural and non-structural genes. As a result of such alterations, several SARS-CoV-2 variants have emerged around the world, including the so-called variants of concern (VOC), posing a threat to public health, and challenging the immunity granted by existing vaccines due to mutations on spike (S) protein. Since the beginning of the pandemic, five main VOCs have been identified (Alpha, Beta, Gamma, Delta, and Omicron), with genetic alterations in the viral phenotype and characteristics such as increased transmissibility, virulence, and disease severity. In this context, the emergence of new variants also raises concern about possible resistance to currently available antiviral therapies (Drożdżal et al., 2021; WHO, 2021; WHO, 2023).

Several drugs that were already used for other diseases and tested during other viral outbreaks had their antiviral activity evaluated for the treatment of COVID-19, including ACE2 inhibitors, anti-spike monoclonal antibodies, endosome maturation inhibitors, RNA-dependent RNA polymerase (RdRp) inhibitors, inhibitors of viral protein synthesis and maturation, and viral shedding (Stasi et al., 2020; Basu et al., 2022). The first efficacious antiviral approved against SARS-CoV-2 was remdesivir, an adenosine analog capable of binding to RdRp and acting as an RNA chain terminator. Initially developed for the treatment of Ebola virus (EBOV), and repurposed against other RNA viruses (*e.g.*, betacoronaviruses), such as SARS-CoV and MERS-CoV, the use of remdesivir lead to an improvement in the clinical condition of COVID-19 patients, more precisely promoting rapid recovery and higher probability of reducing mortality among patients with moderate disease (Mulangu et al., 2019; Beigel et al., 2020; McCreary and Pogue, 2020).

Moreover, in late 2021, two oral formulations were approved as antiviral drugs for treating COVID-19, molnupiravir and the ritonavir-boosted nirmatrelvir combination, named Paxlovid™. Molnupiravir is a prodrug with an antiviral activity that is metabolized to the cytidine nucleoside analog *N*-hydroxycytidine (NHC), which is taken up by cells and phosphorylated to form the active ribonucleoside triphosphate (NHC-TP). NHC-TP is incorporated into SARS-CoV-2 RNA by the viral RNA polymerase, resulting in an accumulation of errors in the viral genome, thus inhibiting viral replication. Nirmatrelvir, on the other hand, is a SARS-CoV-2 main protease inhibitor (M^{pro}), whilst ritonavir is a human immunodeficiency virus 1 (HIV-1) protease inhibitor and cytochrome P450 (CYP) 3A inhibitor, acting as a pharmacokinetic enhancer for nirmatrelvir (Fischer et al., 2021; Pfizer et al., 2021; Fischer et al., 2022; Saravolatz et al., 2023).

However, there are known limitations of Paxlovid™ clinical use, such as detrimental drug-drug interactions (*e.g.*, ritonavir interference and nirmatrelvir efficacy reduction), and resistant strains to nirmatrelvir were also reported, threatening its use. In this sense, other antivirals could be repurposed or enhanced against SARS-CoV-2, as potential COVID-19 therapeutic options (Arbel et al., 2022; Girardin et al., 2022; Heskin et al., 2022, Zhou et el., 2022, Abdelnabi et al., 2023, Iketani et al., 2023). Adamantane is a canonic antiviral drug that contains a tricyclobridged hydrocarbon structure and acts by interfering with the viroporin protein channel of RNA viruses, which plays an important role in virus maturation and release. Amantadine, or aminoadamantane (AMA), is characterized by the addition of an amine to the structure of adamantane and was the first drug based on AMA derivatives to be used as antiviral against the Influenza A virus (Wanka et al., 2013). It was described that AMA can inhibit SARS-CoV-2 *in vitro*, indicating not only a repurposing potential of this drug for the treatment of COVID-19, but also an attractive drug model for modifications and production of derivatives with enhanced potency (Fink et al., 2021; Ozunal and Sahin, 2021; Zhou et al., 2021; Rejdak et al., 2022). In this context, in the present study, five new AMA derivatives were synthesized, and their anti-SARS-CoV-2 antiviral potency was assessed as potential candidates for the treatment of COVID-19.

2. Results

2.1. Synthesis of AMA derivatives

The synthesis of AMA derivatives (Fig. 1) involved a straightforward condensation reaction between the corresponding aldehydes and aminoadamantane as described in the material and methods.

2.2. Antiviral activity of AMA derivatives

The ability of AMA and derivatives to block SARS-CoV-2 replication was assessed in a concentration-response assay using a Vero (ATCC® CCL-81™) cell line. It was possible to observe a statistically significant reduction in the number of viral genomic copies of SARS-CoV-2 for the different compounds (AMA, 3F2, 3F3, 3F4, 3F5, and 3E10) among the concentrations tested (0.1 to 100 μM), as shown in Fig. 2. AMA presented an IC_{50} of 39.71 μM, whilst its synthetic derivatives 3F2, 3F3, 3F4, 3F5, and 3E10, showed a statistically significant reduction at IC_{50} values of 2.48, 2.85, 0.32, 0.44, and 1.28 μM, respectively. Worthy of mention are compounds 3F4 and 3F5, which could reduce IC_{50} values by 124 and 91 times when compared to AMA, respectively, indicating a high and robust increase in the antiviral activity of the derivatives after chemical modifications.

Selectivity indexes (SI) were calculated for each compound (table 1),

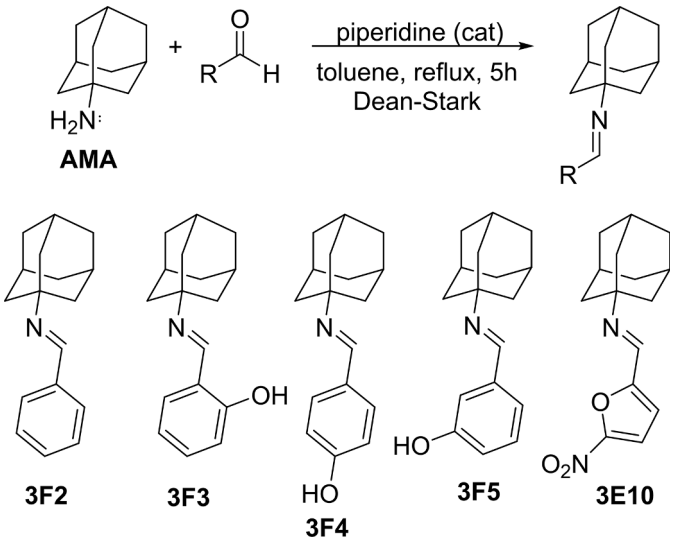


Fig. 1. Synthesis method of AMA derivatives and their structures.

as the highest viable concentration, that is, the concentration of a specific compound that reduces the viability of cultured cells by 50%, divided by the compound's IC_{50}. High SI values were obtained for the AMA derivatives (>10), as recommended for spotting a potential antiviral drug candidate (Aguiar et al., 2018). These results indicate that the cytotoxic concentration of these compounds (supplementary figure S1) is highly superior then the effective antiviral concentration that inhibits 50% of the virus in the cell culture, suggesting elevated antiviral potency of AMA derivatives, especially for 3F4, 3F5, and 3E10. For these three derivatives, the antiviral activity in the Calu-3 lung cell line was evaluated and results show that 3F4 (IC_{50} = 0.0035) has displayed robust antiviral activity, more than 10 times greater than the antiviral activity of the AMA reference compound (supplementary figure S2).

Table 1
Cytotoxicity and anti-SARS-CoV-2 activity of AMA and its derivatives (3F2, 3F3, 3F4, 3F5 and 3E10).

	Highest viable concentration[a] (μM)	IC_{50}[b] (μM)	Selectivity Index[c]
AMA	>1000	39,71	>25.18
3F2	>1000	2,48	>403.22
3F3	>1000	2,85	>350.87
3F4	>1000	0,32	>3125
3F5	>1000	0,44	>2272.72
3E10	>1000	1,28	>781.25

[a] Highest viable dose.
[b] 50 % inhibitory concentration of viral replication.
[c] Selectivity Index: the ratio between substance's highest viable concentration and IC_{50}.

2.3. Impact of treatment with AMA derivative compounds on SARS-CoV-2 spike expression by immunofluorescence microscopy (IMF)

The antiviral effect of AMA and derivative compounds was also evaluated by immunofluorescence staining (anti-Spike + Alexa Fluor 488) for protein Spike (S) of SARS-CoV-2, as well as nuclear labeling (N) by Hoechst 33,342 probe. For this, Vero CCL-81 cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.1 for 48 h at 37 °C with 5% CO_2 atmosphere. As displayed in Fig. 3A, Vero CCL-81 cells showed green staining for protein S and a reduction of cells with nuclei stained in blue, caused by cell death after viral infection. Cell control showed no green staining for protein S and abundant nuclei stained in blue. The impact of treatment was verified, demonstrating that Vero CCL-81 cells infected with SARS-CoV-2 and treated with the compounds (10 μM) showed a reduction in green staining for protein S, accompanied by an increase in blue fluorescence visualization, evidencing high viability of cells treated with new AMA derivatives.

Subsequently, S protein expression was quantified through the fluorescence intensity of the treated slides. This analysis showed that spike expression in Vero CCL-81 cells was reduced significantly after treatment with AMA derivatives at a concentration of 10 μM (Fig. 3B). Noteworthy was that the nuclei were more preserved after treatment with 3F4 as compared to all other compounds including AMA.

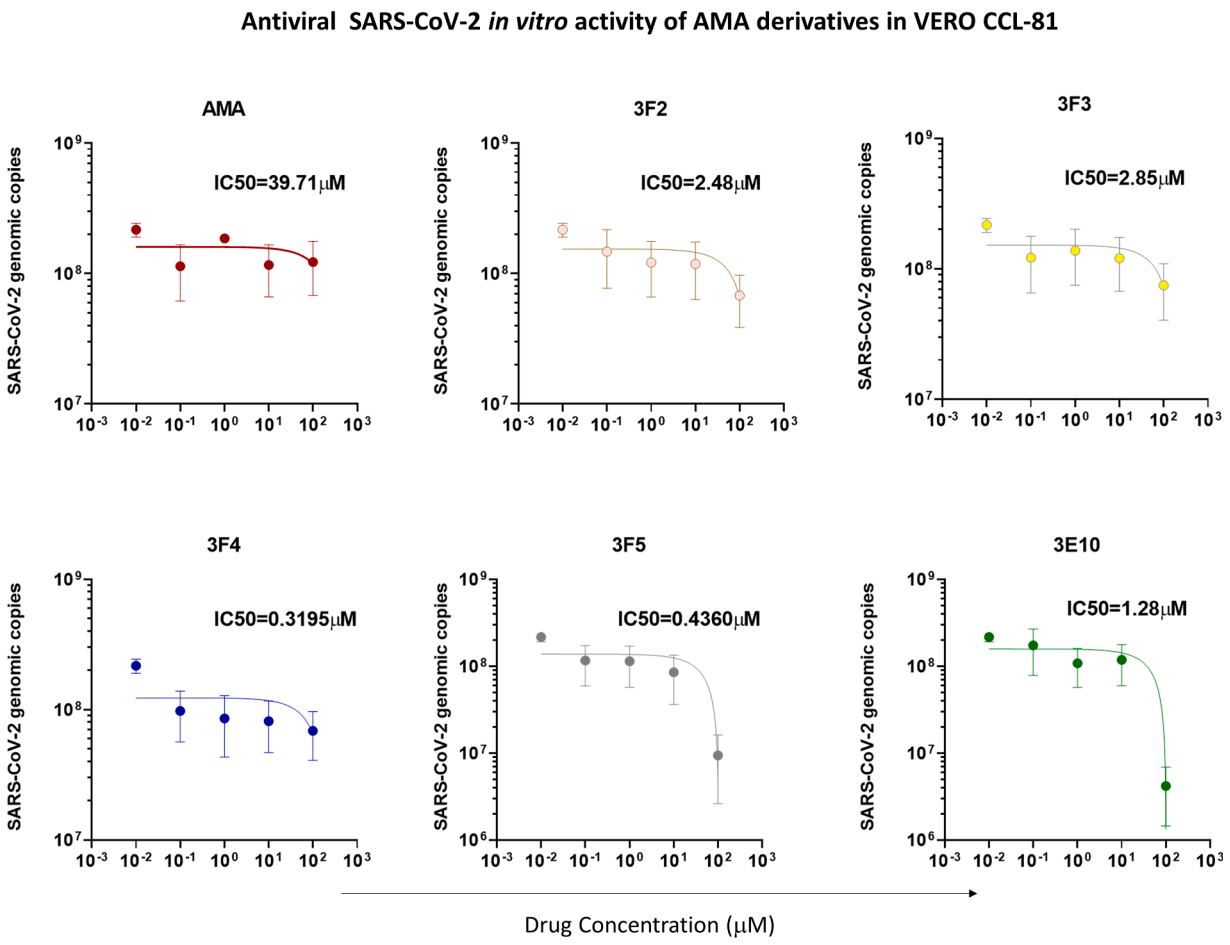


Fig. 2. Antiviral SARS-CoV-2 *in vitro* activity of AMA derivatives in Vero CCL-81 cells. A) The graphs show the median and interquartile range of the number of genomic copies of SARS-CoV-2 evaluated by RT-qPCR for the normalized E gene of the virus, in relation to the concentration of AMA and derivatives (3F2, 3F3, 3F4, 3F5 and 3E10). Mean ± Standard Deviation obtained after three replicates of two independent experiments. Progressive trend curves by non-linear logistic regression with logistic quarter are graded on the graphs. B) The graph represents the *in vitro* IC_{50} of AMA and derivatives (3F2, 3F3, 3F4, 3F5 and 3E10), indicating IC_{50} reduction values relative to the reference compound (AMA).

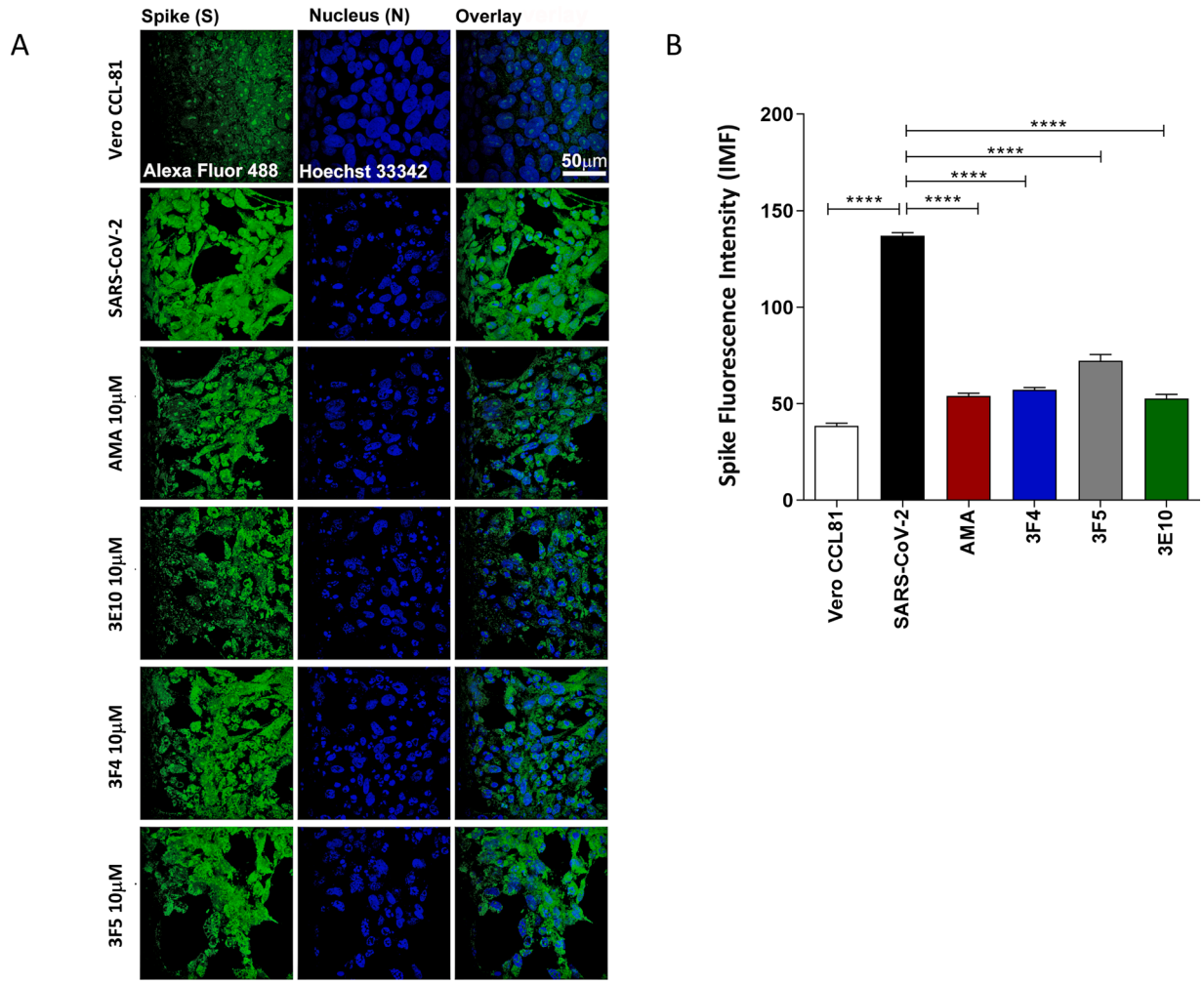


Fig. 3. Evaluation of Spike expression by Indirect Immunofluorescence of Vero CCL-81 cell infected with SARS-CoV-2 after treatment with AMA derivatives. A) Fluorescence and B) Mean Spike fluorescence intensity of Vero CCL-81 cells infected with SARS-CoV-2 and treated with 10 μM of each compound. Mean ± Standard Deviation obtained after two independent experiments. Colors indicate different treatment conditions, such as negative control (white), SARS-CoV-2 (black), AMA (red), 3F4 (blue), 3F5 (gray) and 3E10 (green). Statistical significance is evidenced by an asterisk ($p < 0.05$).

2.4. Analysis of the impact of treatment with compounds on cell and SARS-CoV-2 replication cycle by transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) was employed to take a snapshot of SARS-CoV-2 replication cycle upon infection of Vero CCL-81 cells. Changes in viral morphogenesis after 48 h after treatment with AMA derivatives were highlighted and quantified. Analysis of the compounds with the best results in the antiviral assays (3F4, 3F5 and 3E10) was performed, and the number of viral particles and viral factories within vacuoles were measured (Figs. 4B and 4C).

Results in Fig. 4B demonstrate that the total number of viral particles decrease significantly ($p < 0.05$) upon treatment with AMA, 3F4, 3F5, and 3E10. Overall, there was a trend towards the decreased of viral particles after treatment with the derivatives as compared to AMA alone. Vacuolization was a predominant cytopathic effect observed upon SARS-CoV-2 infection in Vero cells, therefore, the effect of AMA and derivatives in this outcome was also evaluated by quantification of vacuoles in the cell cytoplasm of SARS-CoV-2-infected cells. The results showed that AMA alone was not able to decrease this effect, whilst 3F4, 3F5 and 3E10 were able to significantly decrease ($p < 0.05$) cell vacuolization upon treatment of SARS-CoV-2-infected cells (Fig. 4C). These results confirmed the antiviral activity of the compounds. In addition to the reduced vacuolization, other cytopathic effects were also minimized, such as cytoplasmic projections, and the presence of myelin figures (supplementary figure S3) derived from cellular activation vis-à-vis SARS-CoV-2 infection. Furthermore, it was possible to observe a reduction of viral particles (ranging from 80 to 100 nm in size) adhered to the cell membrane (Fig. 5) and inside several viral factories (supplementary figure S4), especially after treatment with 3F4.

2.5. Cathepsin L docking analysis and enzymatic assays in the presence of AMA derivatives

Human Cathepsin L (CatL) is a cysteine protease that was associated with a cellular enzyme important for SARS-CoV-2 replication, and amantadine has been described as a putative CatL inhibitor. To evaluate the potential binding mode of AMA derivatives into the catalytic site of CatL, docking analysis was performed.

The binding site was defined as the amino acids within a radius of up to 6 Å from the crystallographic ligand, thus building a hypothetical box for molecular interactions within the target. In this protocol, the chemscore kinase model was used, 200 runs of genetic algorithm (GA) were performed, and the pose with the highest score was used to compare with experimental binding mode and RMSD calculation (0.76 Å), shown in Fig. 6A.

Thus, considering all ligands (Fig. 6B), except 3F3, the predicted binding mode was partially consistent with crystallographic ligand, positioning the aromatic ring in the hydrophobic cavity containing the

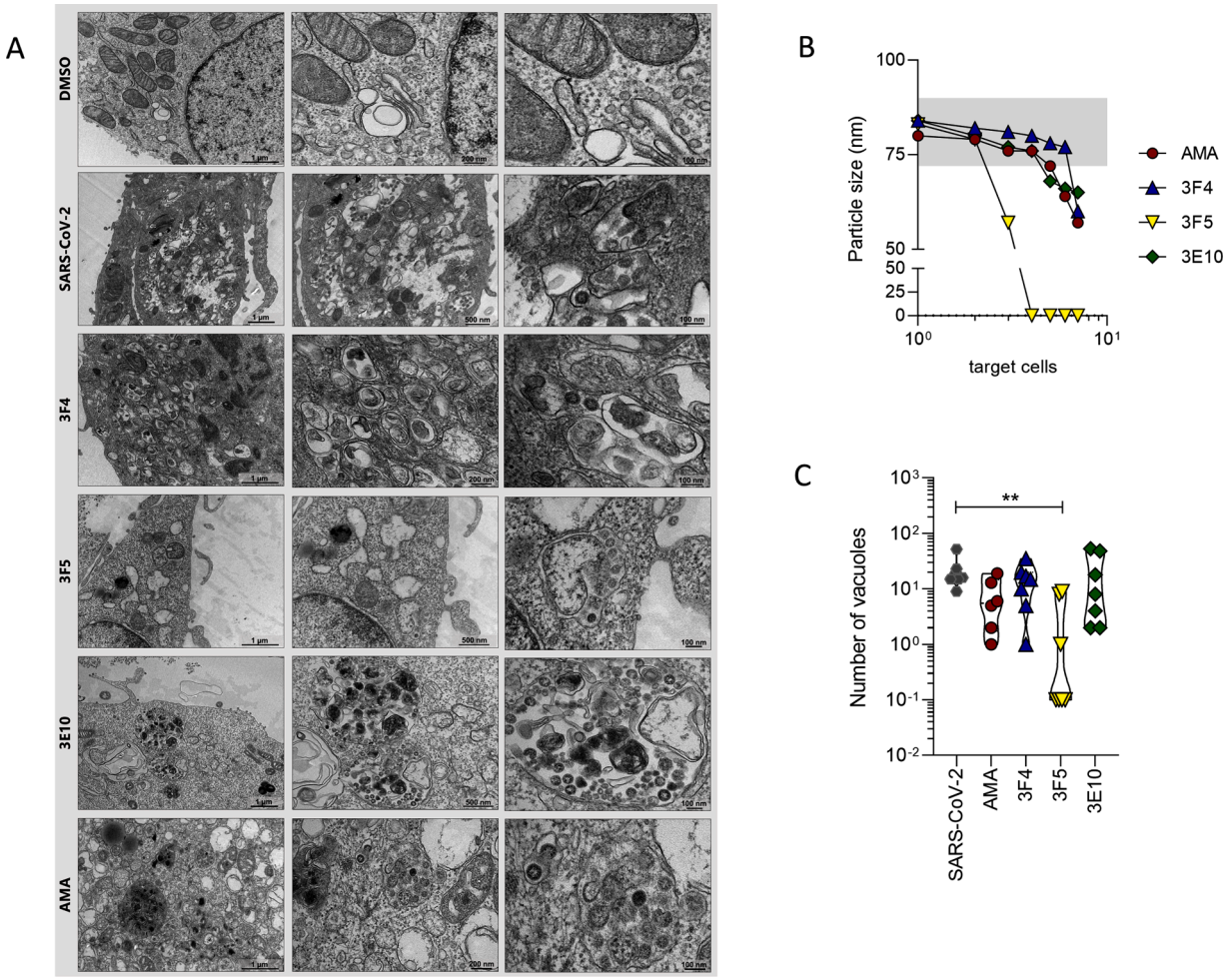


Fig. 4. Transmission Electron Microscopy of SARS-CoV-2-infected Vero CCL-81 cell upon treatment with AMA and derivatives. A) Images from Transmission Electron Microscopy of SARS-CoV-2-infected Vero CCL-81 cell upon treatment with AMA and derivatives (3F4, 3F5 and 3E10), as well as DMSO as a diluent control. On The right, violin plots demonstrating B) number of total viral particles and C) number of vacuoles for SARS-CoV-2 (non-treated), AMA, 3F4, 3F5, and 3E10. Results are representative data obtained after three replicates of two independent experiments. Statistical significance is evidenced by one ($p < 0.05$ or two asterisks ($p < 0.01$).

amino acid residues Leu69, Met70, Ala135, and Met161, in addition to the polar substituent of the aromatic ring facing the Asn66 residue. The pose of the 3F3 molecule, unlike the other analogs, may be related to the presence of a hydroxyl group in the ortho position of the aromatic ring only in this compound and, therefore, not being able to form a potential hydrogen bond with the Asn66 residue. Furthermore, as for 3F2, which does not have any polar substituents on the aromatic ring, a steric effect rather than an absence of Asn66 potential binding is suggested (Fig. 6B). Furthermore, the three most active compounds (3F4, 3F5, and 3F10) were predicted to bind in CatL binding site highly similar (Fig. 6C).

In this sense, these ligand orientations at the target site also reflect on the predicted score values. Compared to the calculated ligand-target score for the crystallographic ligand in the docking simulation (69.91), the proposed ligands presented lower score values: 3F3 (50.83), 3F5 (50.37), 3F4 (48.03), 3F2 (46.16), and 3E10 (43.92). In that sense, the partial occupation of the binding sites with lower score values suggests the compounds could be considered less potent binders or non-binders of CatL. Lastly, when analyzing the 200 docking pose solutions of each compound, including a cluster analysis, or grouping of the most similar and/or most frequent poses, a compilation of docking solutions was obtained (table 2), demonstrating the consistency of the poses and interactions predicted in the analysis and, consequently, a higher reliability of presented predictions.

In parallel, the calculated similarity of 3F4, 3F5, and 3F10 with known CatL inhibitors reported in the last version of ChEMBL are very low (Fig. 6D, <40%) using three different structural fingerprints, corroborating the docking studies.

As shown in Fig. 6E, the results for the inhibition of CatL were not significant decreased in the presence of either 3F2, 3F3, 3F4, 3F5, 3E10 or AMA. These results may indicate that other mechanisms of protein downregulation of CatL or other pathways of viral inhibition were utilized by AMA derivatives for inhibiting SARS-CoV-2 replication.

2.6. *Toxicity predictions of AMA derivatives*

Toxicity predictions were conducted using two different webservers: Protox-II, which employs molecular fragments and fingerprints to calculate similarity with molecules reported in the literature (Drwal et al., 2014), and pkCSM, which employs the graph-based structural signatures method to predict process-related properties (*i.e.*, absorption, distribution, metabolism, excretion, and toxicity; ADMET) (Pires et al., 2015). In this sense, the acute oral toxicity predicted by both methods was used for comparison as a consensus prediction strategy (Pantaleão et al., 2022). Notably, both models predicted the planned analogs to be less toxic than amantadine (table 3). Although the magnitude of the predictions between the webservers was quite discrepant, the results strongly suggest a safer profile of AMA derivatives in comparison to AMA.

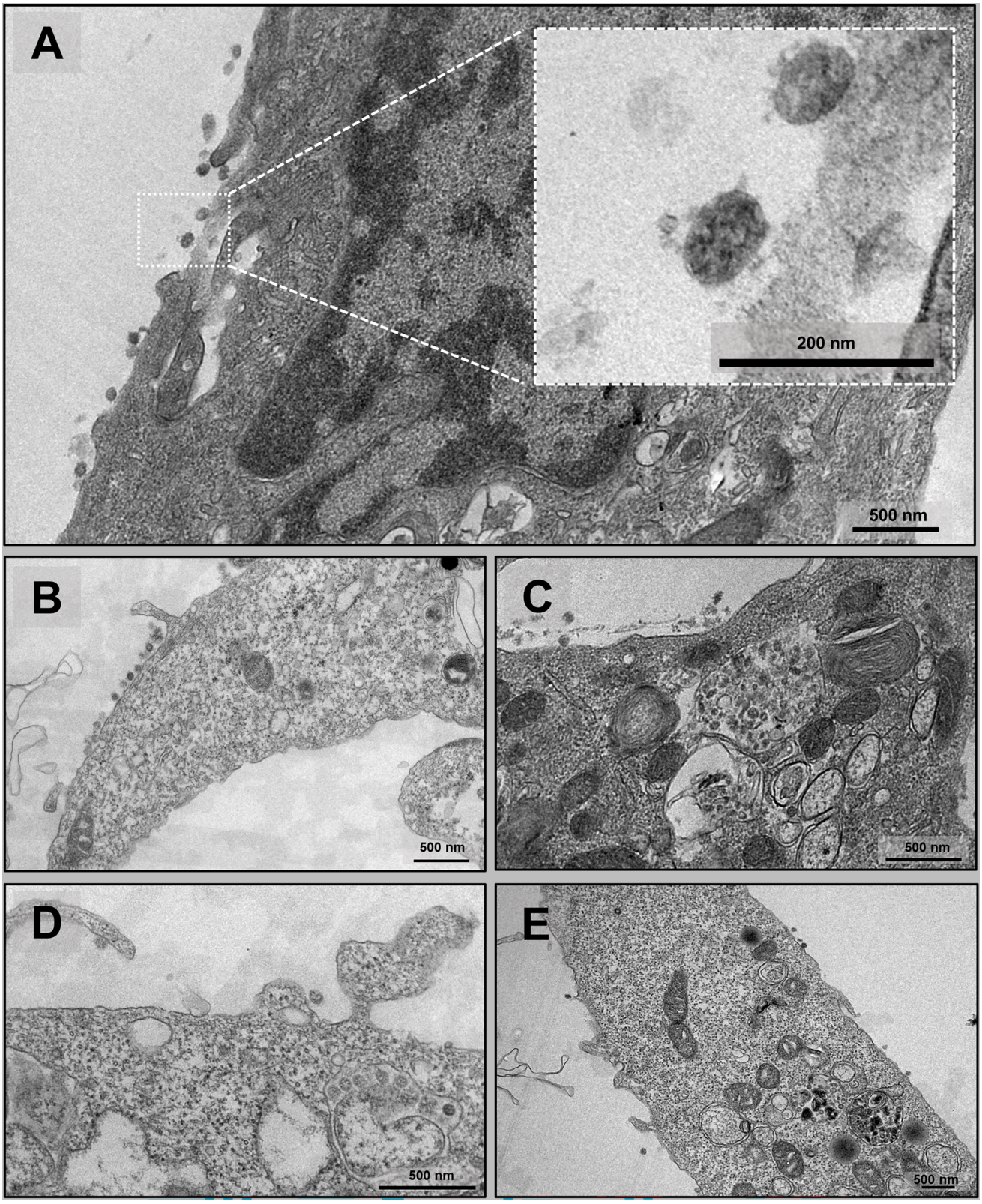
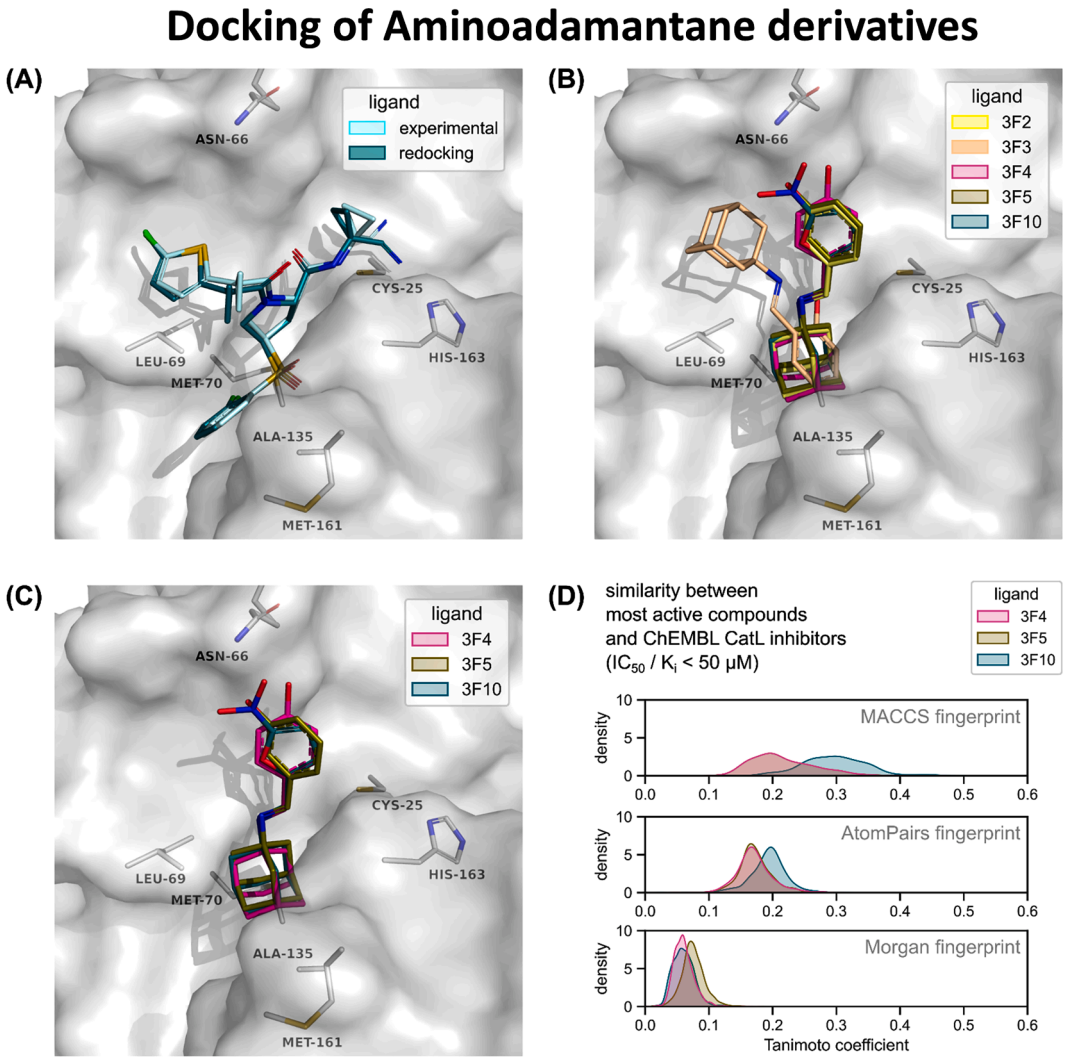


Fig. 5. Impact of treatment with AMA and derivatives (3F4, 3F5 and 3E10) on the reduction of viral particles adhered to the cell membrane after 48 h of infection with SARS-CoV-2. Transmission Electron Microscopy (TEM) of Vero CCL-81 cells infected with SARS-CoV-2 and treated with AMA and derivatives A) virus control, highlighted virus particles B) reduction of viral particles adhered to the cell membrane after treatment with AMA C) 3F4, D) 3F5 and E) 3E10. Results are representative data obtained after three replicates of two independent experiments.



(E) Cathepsin L enzymatic analysis

Derivatives	% of Cathepsin L inhibition (mean ± standard error)	% of Cathepsin L activity (mean ± standard error)
AMA	3±3	97±3
3F2	1±4	99±4
3F3	-14,0±8	114±8
3F4	-51,0±19	151±19
3F5	4±1	96±1
3E10	6±6	94±6
E64 inhibitor	100±0	0±0

Fig. 6. Docking analysis of AMA derivatives with GOLD and Cathepsin L enzymatic analysis. A) Comparison between the experimental binding mode of XU3 and its redocking against human cathepsin L (PEDB ID: 2XU3), resulting in an RMSD value of 0.77 Å B) Docking results for analogs 3F2, 3F3, 3F4, 3F5and 3E10, displaying their predicted poses C) Docking results for most active compounds: 3F4, 3F5 and 3E10 D) Similarity between most active compounds and ChEMBL CatL inhibitors E) Cathepsin L enzymatic analysis,% of Cathepsin L inhibition (mean ± standard error) and% of Cathepsin L activity (mean ± standard error). Results are representative data obtained after three replicates of two independent experiments.

Table 2
Cluster analysis for putative solutions of AMA derivatives by docking.

Derivative	Total clusters	Solutions(1° cluster)	Highest score (cluster)	Highest score (individual)
3F2	31	56	45.52	46.16
3F3	41	29	50.37	50.83
3F4	19	50	47.62	48.03
3F5	17	84	50.06	50.37
3E10	8	92	43.24	43.92

Table 3
Toxicological profiling of AMA and its derivatives on ProTox-II and pkCSM webservers.

	Protox II **Tox class**	PkCSM **Oral Rat Acute Toxicity (LD50)**
AMA	50–300 mg/mg	310,976 mg/kg
3F2	2000–5000 mg/kg	479,442 mg/kg
3F3	2000–5000 mg/kg	529,874 mg/kg
3F4	2000–5000 mg/kg	533,194 mg/kg
3F5	2000–5000 mg/kg	537,280 mg/kg
3E10	2000–5000 mg/kg	647,944 mg/kg

2.7. In vivo antiviral activity of AMA derivatives in mice after SARS-CoV-2 challenge

Aiming at evaluating the antiviral activity of AMA and its derivatives *in vivo*, 10–12 weeks old BALB/cJ mice were treated with the AMA derivatives by intraperitoneal injection at a maximum concentration of 15 mg/kg of animal weight twice, at 12 and 24 h before SARS-CoV-2 MA10 intranasal challenge (Fig. 7A). The MA10 strain can simulate acute lung damage in aged BALB/cJ mice as previously described (Leist et al., 2020). Results indicate that the weight of mice after the challenge was recovered by the treatments with AMA and 3F4 alone (Fig. 7B). No significance was detected in mice monitored 3 days after the challenge and following treatment with 3F5 and 3E10. Viral titers in lungs, as determined by $TCID_{50}$ assay, confirmed these results, showing a 4-fold decrease in viral titers upon treatment with AMA and its derivatives, highlighting an expressive 16-fold decrease in SARS-CoV-2 titers in lungs of treated mice with 3F4 (Fig. 7C). These observations corroborate previous *in vitro* evaluations, pointing to the robust antiviral activity of the 3F4 derivative in comparison to the other derivatives compounds and AMA alone.

3. Discussion

The COVID-19 pandemic highlighted the importance of developing new antiviral drugs, not only to face the current global crisis, but also to prepare for future outbreaks and pandemic scenarios (Pushpakom et al., 2019, Beigel et al., 2020; Morens and Fauci, 2020, Adamson et al., 2021). The modification of chemical structures, potentially providing improvements in affinity, pharmacology, toxicity, and even drug resistance profiles are desirable, considering the high costs of currently in-use antivirals against SARS-CoV-2 (Ashburn and Thor, 2004; Adamson et al., 2021; Sahoo et al., 2021).

In this sense, this work aimed at the chemical modifications of AMA, obtaining five new derivatives (3F2, 3F3, 3F4, 3F5, 3E10) that were evaluated for their ability to neutralize or reduce the SARS-CoV-2 replication cycle. The synthesis of AMA derivatives involved a condensation reaction between aminoadamantane and the corresponding aldehyde. The resulting products were isolated through precipitation using a suitable solvent, resulting in good yields ranging from 55% to 78%. These data, along with the spectroscopic analyses, confirmed the successful synthesis of the AMA derivatives, ensuring their purity and structural identity. Overall, the combination of analytical techniques used in this study allowed for a comprehensive characterization of the AMA derivatives, providing essential information for evaluating their potential as antiviral agents against SARS-CoV-2.

Intending to verify if the novel compounds were cytotoxic to the cells, the cell viability and antiviral activity were evaluated at different concentrations of these compounds in Vero CCL-81 and CALU-3 cell cultures, which demonstrated that AMA derivatives have low cytotoxicity, similarly to AMA in a variety of cell types (Fink et al., 2021; Zhang et al., 2022).

Following cytotoxicity evaluation, the *in vitro* antiviral activity of AMA was compared to its derivates in Vero CCL-81 infected with SARS-CoV-2. AMA was previously demonstrated to possess a broad range of antiviral properties against different viruses (Grieb and Redjak, 2021) as well as against SARS-CoV-2 (Fink et al., 2021). In the present work, IC_{50} values were lower than the IC_{50} of currently in-use compounds described before (Ko et al., 2021). The five derivatives were also able to decrease viral genomic copies, highlighting 3F4 and 3F5 with a reduction of 124 and 91 times in comparison to AMA. In addition, derivatives presented higher SI values, mainly 3F4, 3F5 and 3E10 (>3125, >2272.72, >781.25, respectively) in comparison to AMA (SI of 25). Compounds with SI values greater than 10 are considered an ideal prototype or a potential drug candidate and may present minimal toxicity due to the significant window between pharmacological and toxic concentrations (Aguiaar et al., 2018).

Furthermore, the expression of the S protein can give a clue to the reduction of copies observed by a given mechanism of the compounds (Hoffmann et al., 2020), as the interaction of the S protein with the ACE-2 membrane receptor of the host cell triggers viral entry and consequent replication in the host cell. In this context, AMA and its derivatives promoted a reduction in S protein expression in infected Vero CCL-81 cells, however, no difference was observed as compared to AMA, probably due to the low sensitivity of this method to detect antiviral potency. To check in more detail, the antiviral potency of the new derivatives, and changes in viral morphogenesis were investigated using transmission electron microscopy (TEM) in Vero CCL-81 cells infected with SARS-CoV-2. Classic cytopathic effects, such as detachment and rounding of cells, as well as morphological reduction/alteration of organelles and emission of cell extensions, were observed similarly to effects that were previously described (Araujo et al., 2020; Sarkale et al., 2020; Barreto-Vieira et al., 2022). Vacuolization was frequently observed in infected cells, as the virus takes the host factors and machinery such as autophagy for remodeling the endomembrane during various steps of the replication cycle, including the formation of double vesicles and viral factories (Caldas et al., 2020; Chen and Zhang, 2022). Herein, derivatives 3F4, 3F5 and 3E10 showed the highest and most significant reduction in vacuolation, which corroborates the reduction of viral load upon treatment with all three compounds.

Regarding the mechanisms involved in this process, human Cathepsin L (CatL) is a cysteine protease that is important during the SARS-CoV-2 replication cycle. Therefore, to understand the mechanisms underlying the antiviral activity of AMA derivatives, the potential inhibition of human CatL activity by the compounds were evaluated *in silico* and *in vitro*. Although molecular docking analysis predicted putative consistent results towards the inhibition of CatL, the enzymatic assays did not reveal an impact of AMA derivatives or AMA itself on the CatL activity. Several studies have already demonstrated that this protease is involved in the fusion of SARS-CoV-2 in the cell during endosome maturation, leading to the release of genomic RNA (gRNA) in its cytoplasm (Gomes et Al., 2020; Hoffmann et al., 2020; Jackson et al., 2022), and predictions of the interactions of AMA derivatives were consistent to the redocking of cathepsin L co-crystallized ligand. CatL was reported to cleave the S protein, promoting particle–cell membrane fusion. AMA was characterized as a cathepsin modulator in the context of SARS-CoV-2 infection, preventing infection both *in vitro* and *in vivo* (Zhao et al., 2021), possibly by inhibiting CatL expression rather than enzymatic activity. AMA was able to inhibit the gene expression of this protease (Smieszek et al., 2020), which may explain the inhibitory

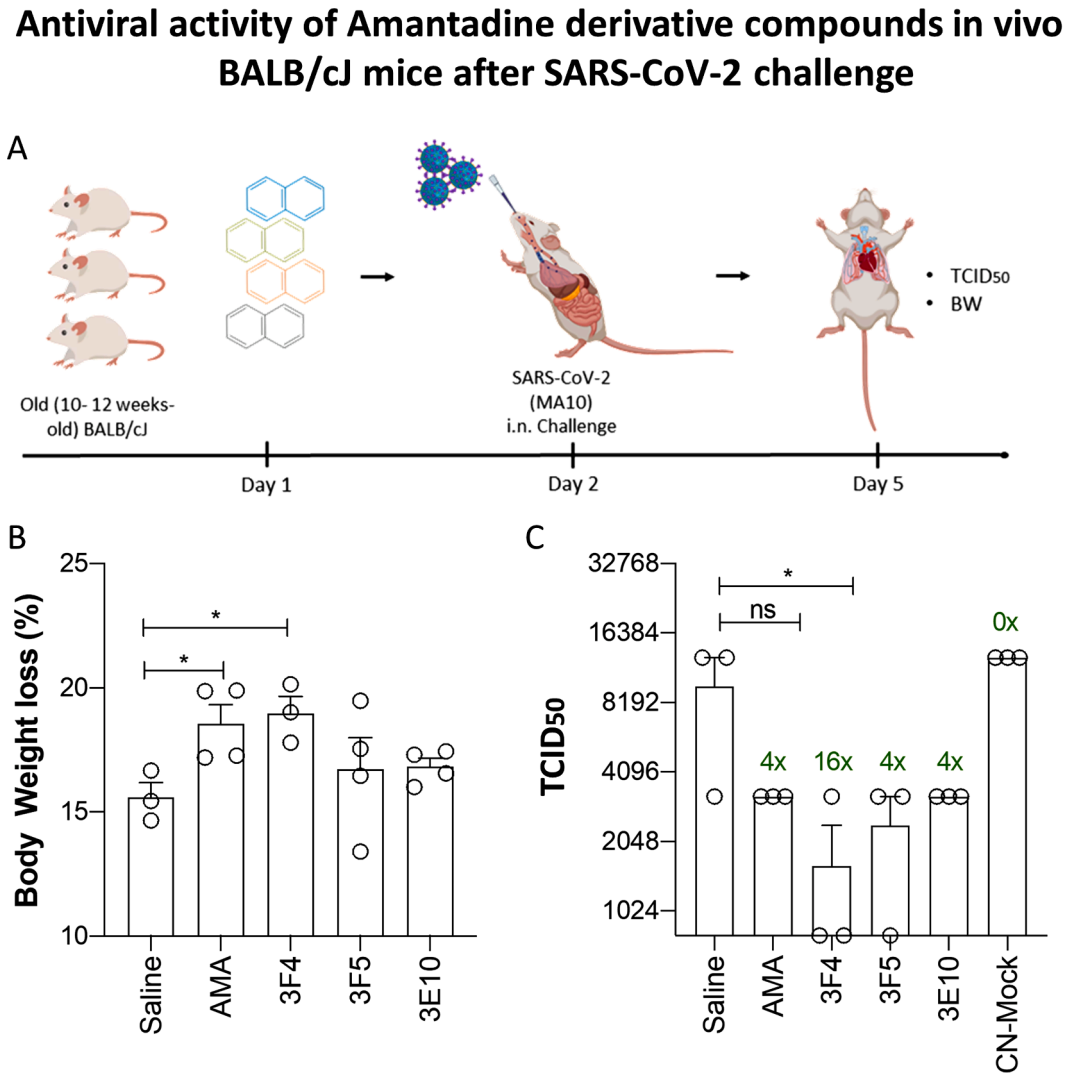


Fig. 7. Antiviral activity of AMA and its derivatives *in vivo* on BALB/cJ mice after SARS-CoV-2 challenge. A) Evaluation strategy of antiviral activity *in vivo* after treatment with AMA and derivatives (3F4, 3F5, 3E10) against the SARS-CoV-2 challenge in BALB/cJ mice. B) Percentage of body weight loss after treatment with AMA and its derivatives and SARS-CoV-2 challenge in comparison to a saline solution control. C) Lung viral titers (by $TCID_{50}$ assay) after treatment with AMA and derivatives against the SARS-CoV-2 challenge in comparison to a saline solution control. The compounds were administered by intraperitoneal injection. Results are expressed as bars with symbols representing mean and standard error. Results represent data obtained from five replicates. Statistical significance is evidenced by an asterisk ($p < 0.05$). ns: non-significant. Figure 9A was generated with BioRender.com.

activity against CatL observed previously (Zhao et al., 2021). Therefore, more scrutiny into the mechanisms underlying CatL involvement in the antiviral activity of AMA derivatives are still required.

We believe that the derivatives of amantadine could block protein E, ORF10, and ORF3a, which are viroporins of SARS-CoV-2 inhibited by the original compound, amantadine (Toft-Bertelsen et al., 2021; Fam et al., 2023). Viroporins are known to participate in different replication cycle steps, such as virus morphogenesis and release from host cells. In the case of coronaviruses, they create membrane vesicles, termed viroplasms, from intracellular membrane remodeling. Those viroplasms can act as replication sites that could enhance virus yield (Nieva et al., 2012; Breitinger et al., 2022).

The potential beneficial effects of adamantanes against SARS-CoV-2 are under examination, and clinical improvement has already been reported in SARS-CoV-2-infected patients undergoing treatment with AMA (Rejdak and Grieb, 2020; Aranda-Abreu et al., 2021; Butterworth, 2023). Finally, the present study showed that the *in vivo* antiviral activity corroborated *in vitro* results, demonstrating the robust antiviral activity of the 3F4 derivative when compared to other derivatives and AMA. 3F4 showed the highest potency as an antiviral *in vivo* as compared to other compounds, probably due to better biodistribution in the lungs and mouse tissues. The biodistribution of these compounds is now under investigation. The compounds 3F5 and 3E10 which exhibited significant antiviral activity *in vitro* did not show the same antiviral effect as observed for 3F4 *in vivo*. This discrepancy between *in vitro* and *in vivo* results has been observed and discussed previously. In this regard, Tummino et al. (2021) propose that phospholipidosis, a possible side effect induced by drugs, could be associated with antiviral activity in specific cell line systems. The phospholipidosis phenomenon relies on the physicochemical properties of compounds and does not reflect virus-targeted effects. Therefore, this process could be a toxic confounder during *in vitro* drug discovery. However, drugs that induce phospholipidosis *in vitro* are not effective in halting SARS-CoV-2 replication in mouse models. In the present study, we show that one of the Amantadine derivatives, 3F4 shows antiviral activity *in vivo*, which demonstrates that the antiviral effect of this compound could not be due to phospholipidosis, but in fact, by a confirmed anti-SARS-CoV-2 antiviral activity. However, it could be the case that 3F5 and 3E10 may have acted by this mechanism to inhibit the virus exclusively *in vitro*. New investigations on the matter are yet required for a better understanding

of the differential mechanistic details of anti-SARS-CoV-2 activity of AMA derivatives.

Altogether, structural modifications of the AMA derivatives presented in this work were successful, with proven biological and antiviral activity. Research targeting new adamantane molecules, synthesized with novel functional groups, has been presented as potential antiviral candidates for SARS-CoV-2. Even though there are antivirals available for SARS-CoV-2 with distinct properties and targets, none have proven to be cost-effective, highlighting the importance of the present findings and the quest for new, affordable, broad-spectrum strategies, considering the current scenario of eminent future pandemics with new SARS-CoV-2 variants of concern.

4. Conclusion

The results obtained in this work support the potential use of AMA derivatives as broad-spectrum antivirals, proposed as candidates that will bring benefit to COVID-19 therapeutics against SARS-CoV-2 in the future and its emerging variants of concern.

CRediT authorship contribution statement

Daisymara Priscila de Almeida Marques: Formal analysis, Methodology, Writing – review & editing. **Luis Adan Flores Andrade:** Formal analysis, Methodology, Writing – review & editing. **Erik Vinicius Sousa Reis:** Formal analysis, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Felipe Alves Clarindo:** Data curation, Methodology. **Thaís de Fátima Silva Moraes:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Karine Lima Lourenço:** Methodology, Writing – review & editing. **Wellington Alves De Barros:** Investigation, Writing – review & editing. **Nathália Evelyn Morais Costa:** Investigation, Writing – review & editing. **Lídia Maria de Andrade:** Investigation, Methodology, Writing – review & editing. **Ágata Lopes-Ribeiro:** Methodology, Writing – review & editing. **Mariella Sousa Coêlho Maciel:** Writing – original draft, Writing – review & editing. **Laura Cardoso Corrêa-Dias:** Writing – original draft. **Isabela Neves de Almeida:** Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Thalita Souza Arantes:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. **Vivian Costa Vasconcelos Litwinski:** Data curation, Investigation, Resources, Writing – review & editing. **Leonardo Camilo de Oliveira:** Investigation, Resources, Writing – review & editing. **Mateus Sá Magalhães Serafim:** Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Vinicius Gonçalves Maltarollo:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Silvia Carolina Guatimosim:** Investigation, Supervision, Writing – original draft, Writing – review & editing. **Mário Morais Silva:** Data curation, Investigation, Methodology, Writing – review & editing. **Moriya Tsuji:** Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Rafaela Salgado Ferreira:** Data curation, Formal analysis, Supervision, Validation, Writing – original draft, Writing – review & editing. **Luiza Valença Barreto:** Methodology, Writing – review & editing. **Edel Figueiredo Barbosa-Stancioli:** Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **Flávio Guimarães da Fonseca:** Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Ângelo De Fátima:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. **Jordana Grazziela Alves Coelho-dos-Reis:** .

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 available upon request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2023.199291.

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