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SHORT COMMUNICATION

Memantine hydrochloride: a drug to be repurposed against Chikungunya virus?

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Abstract

Background Chikungunya fever is an endemic disease caused by the Chikungunya virus (CHIKV) to which there is no vaccine or effective antiviral drug treatment so far. Our study aimed to evaluate the potential anti-CHIKV activity of memantine hydrochloride (mtnH), a drug from the class of the aminoadamantanes approved for the treatment of Alzheimer´s disease, as a possible drug to be repurposed to the treatment of Chikungunya fever.

Methods MtnH antiviral activity against CHIKV was determined by infecting BHK-21 cells with CHIKV-*nanoluc*, a virus carrying the marker *nanoluciferase* reporter, in the presence or absence of mtnH at concentrations ranging from 500 to 1.45 μM. The effective concentration of 50% inhibition (EC_{50}) was calculated. Cell viability assay (determination of CC_{50}) was also performed employing BHK-21 cells. Mutagenic assays were performed by the *Salmonella* Typhimurium/microsome assay (Ames test).

Results MtnH presented a CC_{50} of 248.4 ± 31.9 μM and an EC_{50} of 32.4 ± 4 μM against CHIKV in vitro. The calculated selectivity index (SI) was 7.67. MtnH did not induce genetic mutation in *Salmonella* strains with or without an external metabolizing system.

Conclusion With the data herein presented, it is possible to hypothesize mtnH as a viable candidate to be repurposed as an anti-CHIKV drug. Clinical assays are, therefore, encouraged due to the promising in vitro results.

Graphic abstract

The drug memantine hydrochloride is herein personified with a doubt: as a prior regulated drug against Alzheimer, could it follow the path against Chikungunya virus too?

Keywords Chikungunya virus · Drug · Repurposing · Memantine hydrochloride · Antiviral

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Extended author information available on the last page of the article

Abbreviations

CC_{50}	Cytotoxic concentration of 50%
CHIKV	Chikungunya virus
EC_{50}	Effective concentration of 50% inhibition
JEV	Japanese encephalitis virus
MI	Mutagenic index
mtnH	Memantine hydrochloride
NADP	Nicotinamide adenine dinucleotide phosphate sodium salt
PFU	Plaque-forming units
RABV	Rabies virus
SI	Selectivity index
CHIKV-*nanoluc*	Chikungunya virus-*nanoluciferase*
CMC	Carboxymethyl cellulose

Introduction

Chikungunya fever is an endemic disease in tropical and subtropical countries, and it is characterized, during its acute phase, by *dengue-like* symptoms such as fever, skin rash, backache, headache, fatigue, and arthralgia [1–3]. However, this disease can progress to a chronic condition defined by polyarthralgia, which can last from months to years [1–3]. The potential chronic character of this disease allied to its endemic feature constitutes a case of great concern to public health and economic welfare [2–4].

The etiologic agent of Chikungunya fever is the Chikungunya virus (CHIKV), an arbovirus that belongs to the *Alphavirus* genus, which is mainly transmitted to healthy individuals by the bite of infected *Aedes aegypti* and *albopictus* mosquitoes [3]. Other mosquitoes such as *Haemagogus leucocelaenus* and *Aedes terrens* have also shown to be potential vectors for this arbovirus, which aggravates the challenges related to the surveillance, prevention, and control of CHIKV [5].

The CHIKV infection primarily affects fibroblasts, keratinocytes, and epithelial cells in the dermis by permissibility that is related mostly to Mxra8 receptors as well as the presence of T cell immunoglobulin, mucin domain-1 (TIM-1), and glycosaminoglycans [6–8]. The pathophysiology is followed by a high viremia which results in infection of several tissues such as muscles, joints, liver, heart, and nervous system [9, 10]. Moreover, some CHIKV strains were associated with higher neurovirulence, suggesting a more aggressive disease in some of the infected patients [11].

Currently, there is no available vaccine for CHIKV infection nor an effective antiviral drug for the treatment of infected patients leading only to palliative therapies [1]. In this context, drug repurposing figures as a promising short to medium terms therapeutic alternative, since a drug previously approved for a specific clinical application has the potential to be capitalized more rapidly than potential drug prototypes into the treatment of another disease [12].

The class of aminoadamantanes has been extensively investigated due to their diversity of pharmacological activities [13]. For instance, the drugs amantadine and rimantadine, formerly approved as anti-Influenza A virus agents, have gained attention as possible antiviral agents for other virus-driven infections [14]. In this respect, an observational study described the amantadine as a possible repurposing drug for patients with Coronavirus disease 2019 (COVID-19) [15].

Herein, we explored the potential antiviral activity of memantine hydrochloride against CHIKV. Memantine hydrochloride (mtnH; Fig. 1) is an aminoadamantane drug, employed mostly for the treatment of Alzheimer's disease (AD). This AD drug has been reported through in vitro assays as a possible antiviral agent against influenza A virus [16, 17], human coronavirus strain OC-43 (HCoV-OC43), rabies virus (RABV), Japanese encephalitis virus (JEV) [18, 19], Zika virus [20] and, most recently, SARS-CoV-2 infections [21].

Considering the impact of Chikungunya fever in tropical and subtropical countries and the severe disabilities generated by CHIKV infection, as well as the previous prospects of the mtnH for alleviating symptoms of Zika virus [20] and SARS-CoV-2 [21] infections, herein we evaluated the in vitro activity of mtnH as a promising drug to be repurposed as an anti-CHIKV drug.

Materials and methods

Reagents

Memantine hydrochloride (mtnH; purity 99%) (Fig. 1) was purchased from Acros Organics (Fisher Scientific®). The compound was dissolved in dimethylsulfoxide (DMSO) and stored at − 20 ºC. Dilutions of the compounds were made

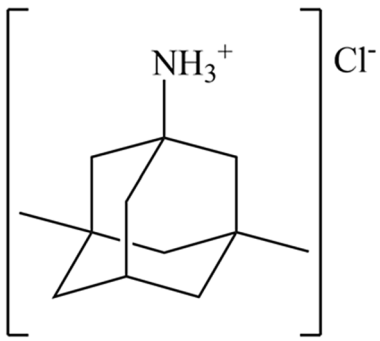

Fig. 1 Structure of memantine hydrochloride

immediately prior to the experiments. For all assays performed, DMSO was used as untreated control.

Cell culture

BHK-21 cells (ATCC® CCL-10™), derived from Syrian hamster kidney, were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich®) supplemented with 100U/mL of penicillin (Hyclone Laboratories®), 100 mg/mL of streptomycin (Hyclone Laboratories®), 1% dilution of stock of non-essential amino acids (Hyclone Laboratories®) and 1% of fetal bovine serum (FBS, Hyclonen Laboratories®) in a humidified 5% CO_2 incubator at 37 °C.

Rescue of CHIKV-*nanoluc* reporter virus

The CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 2a) used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The cDNA of CHIKV-*nanoluc* was placed under the control of a CMV promoter [22, 23]. To produce CHIKV-*nanoluc* virions, 2.3×10^7 BHK-21 cells seeded in a T175 cm^2 flask were transfected with 1.5 µg of CMV-CHIKV-*nanoluc* plasmid using lipofectamine 3000® and Opti-Mem medium. Forty-eight hours post-transfection (h.p.t.) the supernatant was collected and stored at − 80 °C. To determine viral titers, 1×10^5 BHK-21 cells were seeded in each of 24 wells plate 24 h prior to the infection. Then, the cells were infected with ten-fold serial dilutions of CHIKV-*nanoluc* for 1 h at 37 °C. The inoculums were removed, cells were washed with PBS to remove the unbound virus, and added of fresh medium supplemented with 1% penicillin, 1% streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC). Infected cells were incubated for 2 days in a humidified 5% CO_2 incubator at 37 °C, followed by fixation with 4% formaldehyde and stained with 0.5% violet crystal. The viral foci were counted to determine viral titer which was presented in plaque-forming units per milliliter (PFU/mL).

Cell viability through MTT assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich®) assay. BHK-21 cells were plated in 48 well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C. Medium containing two-fold serial dilutions of mtnH ranging from 500 to 1.45 µM was added to the cells for 16 h at 37 °C with 5% of CO_2. After treatment, the compound-containing medium was removed and MTT solution at 1 mg/mL was added to each well, incubated for 1 h, and replaced with 300 µL of DMSO (dimethyl sulfoxide) to solubilize the formazan crystals. The absorbance was measured at 490 nm on Glomax microplate reader (Promega®). Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated and untreated control groups, respectively. The cytotoxic concentration of 50% (CC_{50}) was calculated using GraphPad Prism 8.

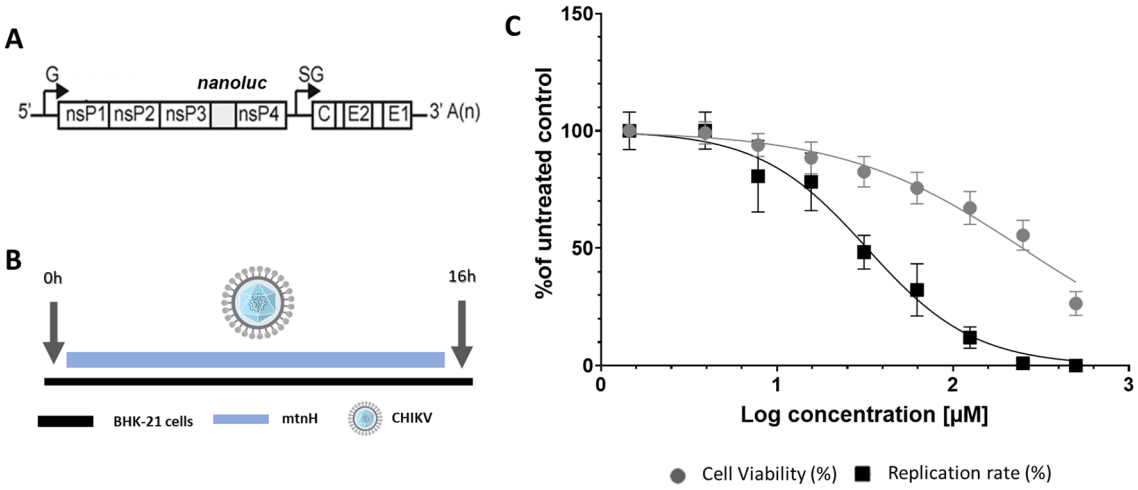

Fig. 2 Inhibition of CHIKV replicative cycle by mtnH. **a** Schematic representation of CHIKV-*nanoluc* genome. **b** Schematic representation of CHIKV infectivity assays. **c** BHK-21 cells were infected with CHIKV-*nanoluc* at MOI 0.1 in the presence or absence of mtnH ranging from 500 to 1.45 µM for 16 h.p.i.. EC_{50} and CC_{50} were determined and SI was calculated. CHIKV replication was measured by *nanoluciferase* assay (indicated by closed square) and cellular viability measured using an MTT assay (indicated by closed circle). Results are shown as a non-linear regression analysis, indicating the percentages of the cell viability or viral replication by mtnH. Mean values ± SD of three independent experiments each measured in triplicate are shown. The comparison of each curve was performed employing the F test (confidence level of 95%), resulting in a $p < 0.0001$, and $F_{2,116} = 260.5$

Antiviral activity assays

To assess the antiviral activity of mtnH, BHK-21 cells were seeded at a density of 5×10^4 cells per well into 48 well plates 24 h prior to the infection. Cells were infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 in the presence or absence of mtnH at concentrations ranging from 500 to 1.45 µM. Samples were harvested using Renilla-luciferase lysis buffer (Promega®) at 16 h.p.i. (Fig. 2b) and virus replication levels were quantified by measuring *nano-luciferase* activity using the Renilla luciferase Assay System (Promega). The effective concentration of 50% inhibition (EC_{50}) was calculated using Graph Pad Prism 8 software. The values of CC_{50} and EC_{50} were used to calculate de selective index $\left(SI = \frac{CC_{50}}{EC_{50}}\right)$.

Mutagenic assay: Ames test

The Ames test was employed to evaluate the ability of mtnH to induce gene (point) mutations measured in *Salmonella* Typhimurium strains (kindly provided by B.N. Ames, Berkeley, CA) in the presence or absence of metabolic activation. The *S.* Typhimurium tester strains TA98 and TA97a detect frameshift mutations, TA100 detects base-pair substitution mutations primarily at one of the GC pairs, and TA102 normally is used to detect mutagens that cause oxidative damage and base-pair-substitution mutations (AT) [24].

For the mutation assay using a pre-incubation method, a stock solution of mtnH at 2317.4 µM was firstly prepared. Then, 0.5 mL of S9 mix or phosphate buffer (pH 7.4) was mixed with 0.1 mL of bacteria, and lastly 0.1 mL of the stock solution of mtnH, and placed in a shaking incubator at 37 ºC for 20 min. After this, 2 mL of top agar was added to the mixture and poured into a plate with an agar layer. The final concentration of mtnH was 85.83 µM, corresponding to 50 µg/plate.

The activation mixture for the S9 fraction was purchased from Moltox Molecular Toxicology Inc., Boone, USA, and freshly prepared before each test. This mixture reveals whether the substance or sample is mutagenic in its original form or if it needs to be metabolized or activated to become mutagenic. The metabolic activation system consisted of 4% S9 fraction, 1% of magnesium chloride 0.4 mol L^{-1}, 1% of potassium chloride 1.65 mol L^{-1}, 0.5% of D-glucose-6-phosphate disodium 1 mol L^{-1}, and 4% of nicotinamide adenine dinucleotide phosphate sodium salt (NADP) 0.1 mol L^{-1} in 50% of phosphate buffer 0.2 mol L^{-1} and 39.5% of sterile distilled water. The plates were incubated at 37 ºC for 48 h, and the His + revertant colonies were counted manually.

DNA damage-inducing agents were used as positive controls: 4-nitro-*o*-phenylenediamine (10 µg/plate) for TA98 and TA97a, sodium azide (1.25 µg/plate) for TA100, and mitomycin C (0.5 µg/plate) for TA102 in experiments without S9 mix. In experiments with S9 activation, 2- anthramine (1.25 µg/plate) was used as a positive control for TA98, TA97a, and TA100, and 2-aminofluorene (10 µg/plate) for TA102. DMSO was used as untreated control (100 µL/plate) and the spontaneous control (SC) corresponds to the rate of spontaneous reversion of each strain.

Statistical analysis

Individual experiments were performed in triplicate and all assays were carried out a minimum of three times to confirm reproducibility. The EC_{50} and CC_{50} were calculated using a non-linear regression considering log(inhibitor) vs. response, with variable slope (four parameters), and a comparison was also performed using the F test ($p < 0.05$). Data from the assay with *Salmonella* Typhimurium strains were expressed as mean ± SD and were submitted to a normality and lognormality test. The statistical significance was determined by one-way analysis of variance (ANOVA), complemented by the Dunnett's post hoc test. This test was performed in comparison with the control group (untreated control of the assay). The mutagenic index (MI) was also calculated as the average number of revertants *per* test plate divided by the average number of revertants *per* solvent (negative control plate). A sample is considered mutagenic if the ANOVA variation is significant, with $p < 0.05$ and the average number of revertants increase of the sample is a minimum of two folds of that found in the untreated control ($MI > 2$) [24, 25].

Results and discussion

Memantine hydrochloride impairs CHIKV infection in vitro

Considering the impact of Chikungunya fever in tropical countries and the previously described antiviral activities on mtnH [18, 19], we investigated the anti-CHIKV activity of mtnH employing BHK-21 cells infected with a recombinant CHIKV that expresses a *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 2a).

Firstly, the mtnH antiviral activity was evaluated performing a dose–response assay to determine the cytotoxicity concentration of 50% (CC_{50}) and the effective antiviral concentration of 50% (EC_{50}). For this, BHK-21 cells were infected with CHIKV-*nanoluc* and simultaneously treated with mtnH at concentrations ranging from 500 to 1.45 µM, and the viral replication was assessed 16 h post-infection (h.p.i.) (Fig. 2b). In parallel, cell viability was assessed by an MTT assay. From this range of concentrations, it was determined that mtnH has the CC_{50} of 248.4 ± 31.9 µM

and EC_{50} of 32.4 ± 4 µM ($p < 0.0001$, $F_{2,116} = 260.5$). The calculated selectivity index (SI) was 7.67 (Fig. 2c). These results suggest mntH as a potential alternative for the future treatment of CHIKV infection. Despite the relatively modest SI value compared to other drugs, mtnH has a dosing schedule of 20 mg daily for the treatment of mild to severe AD [26], with no severe adverse effects. One may suggest that the absence of adverse effects in vivo may thus outweigh the modest SI in vitro in terms of therapeutic significance. However, it is important to emphasize that the effective concentration for the treatment of infected animals and/or individuals must be further evaluated. In addition, SI values higher than 1.0 suggest a positive benefit and risk balance for the potential antiviral drug [27]. Other drugs such as Chloroquine, which is widely used to treat *Plasmodium falciparum* infections and presents substantial adverse effects to the patients, was reported as an alternative anti-CHIKV inhibitor in vitro with an ordinary selectivity index ranging from 15 to 26 depending on the type of treatment [28–30].

The antiviral activity of mtnH and other adamantanes against other virus strains has been suggested to be related to the inhibition of viroporins ion channels [21, 31]. Viroporins are multifunctional proteins expressed mainly by RNA viruses [32], in which the self-assembled oligomerization forms an ion conductive transmembrane pore [33, 34]. This pore is then responsible for modifying the cellular environment, unbalancing the ionic gradient as well as the charge of the membrane, which favors the viral replication [32]. The CHIKV encodes a viroporin (viral protein 6 K), which is common among alphaviruses and it is responsible for the formation of a cation-selective ion channel in the lipid bilayers [31, 35]. The lack of protein 6 K is related to a higher susceptibility to pH alterations as well as a greater sensibility to higher temperatures [36], ultimately resulting in an impairment of the viral replication. Therefore, considering the anti-CHIKV activity of amantadine, which presents a similar structure to that of mtnH, and the recent report of SARS-CoV-2 viroporin E impairment by mtnH, it is possible to suggest that mtnH also abrogates the viral replication through the interaction with CHIKV viroporin 6 K [21]. Moreover, the NMDA channel receptor blockage performed by mtnH may support this hypothesis [37], suggesting a tropism of mtnH for ion channels. However, further studies are necessary to unveil the mtnH mechanism of action against CHIKV.

Whats is more, it was observed that CHIKV can infect different cell types such as fibroblasts, dendritic cells, and macrophages [6, 10], with a possible tropism for the central nervous system (CNS) cells such as astrocytes, microglia, and neuroblastoma cells [6, 10]. This feature may induce neurological complications such as Guillan-Barré syndrome, encephalitis, and, meningitis [6, 10]. The previously described in vivo activity of mtnH controlling inflammation and commonly associated with its neuroprotective effect [38] may, thus, indicate an additional therapeutic effect of mtnH that can be exploited seeking the minimization of the potential neurological effects triggered by CHIKV infection.

Finally, it is important to emphasize that mtnH is a worldwide approved and relatively low-cost drug with a well-established therapeutic profile both in terms of its biological outcomes (dose–response activity and potential adverse effects), as well as its physicochemical and pharmacokinetic properties [39]. In this context, from the data herein presented, and considering the lack of significant adverse effects in short-term therapies for healthy individuals (without AD, e.g., Clinical Trials NCT03121820 and NCT01999894), it is possible to hypothesize that mtnH may follow a cost- and time-reduced path in clinical trials, with a considerable potential to be capitalized as a CHIKV antiviral drug.

Effect of mtnH in cell mutagenicity

The results of mutagenicity assays are presented in Table 1 encompassing the mean and standard deviation (SD) of the number of revertants per plate, and the mutagenic indexes (MI), with (+S9) and without (-S9) metabolic activation. The results were validated using the untreated and positive controls. The positive control of each cell line produced the expected mutagenic response and was used to validate the susceptibility of the system to standard mutagens.

The strain TA98 of *S. Typhimurium* when treated with the positive control induced an increase in the revertants per plate compared to the DMSO control in the absence of S9 (−S9: 654 ± 91 vs. control 20 ± 4, $p < 0.0001$, $F_{3,20} = 830.1$) and in the presence of S9 (+S9: 1221 ± 73 vs. control 21 ± 5, $p < 0.0001$, $F_{3,20} = 2634$). The same results were seen in the presence of positive control for the other strains: TA100 (−S9: 1458 ± 85 vs. control 93 ± 3, $p < 0.0001$, $F_{3,20} = 2431$; +S9: 1132 ± 85 vs. control 106 ± 5, $p < 0.0001$, $F_{3,20} = 1904$), TA102 (−S9: 1982 ± 249 vs. control 274 ± 36, $p < 0.0001$, $F_{3,20} = 358.9$; +S9: 2359 ± 114 vs. 270 ± 47, $p < 0.0001$, $F_{3,20} = 803.3$), and TA97a (−S9: 1518 ± 123 vs. control 96 ± 13, $p < 0.0001$, $F_{3,20} = 1293$; +S9: 1442 ± 152 vs. control 112 ± 18, $p < 0.0001$, $F_{3,20} = 278.8$) (Table 1). Differently, the treatment with mtnH did not induce mutagenicity in the strains TA98 (−S9: 16 ± 1, $p = 0.98$, and +S9: 27 ± 9, $p = 0.96$), TA100 (−S9: 109 ± 15, $p = 0.73$, and +S9: 113 ± 3, $p = 0.94$), TA102 (−S9: 266 ± 47, $p = 0.99$, and +S9: 231 ± 12, $p = 0.8$), and TA97a (−S9: 83 ± 10, $p = 0.94$; and +S9: 121 ± 14, $p = 0.99$) (Table 1). In addition, the DMSO, used as the parameter to determine the mutagenic potential of the mtnH, did not statistically differ of revertants number from the spontaneous control, that yielded the spontaneous His + revertants.

Table 1 Mutagenic activity expressed as the revertants/ plate, standard deviation, and mutagenicity index (in brackets) in the strains TA98, TA100, TA102, and TA97a of *S.* Typhimurium after treatment with memantine

	Number of revertants (M ± SD)/plate and MI							
	TA98		TA100		TA102		TA97a	
	− S9	+ S9	− S9	+ S9	− S9	+ S9	− S9	+ S9
SC	19 ± 2	24 ± 8	92 ± 10	96 ± 12	231 ± 27	270 ± 38	107 ± 7	117 ± 13
DMSO	20 ± 4	21 ± 5	93 ± 3	106 ± 5	274 ± 36	270 ± 47	96 ± 13	112 ± 18
C +	654 ± 91***,[a]	1221 ± 73***,[d]	1458 ± 85***,[b]	1132 ± 68***,[d]	1982 ± 249***,[c]	2359 ± 114***,[e]	1518 ± 123***,[a]	1442 ± 152***,[d]
mtnH	16 ± 1 (0.79)	27 ± 9 (1.29)	109 ± 15 (1.17)	113 ± 3 (1.07)	266 ± 47 (0.97)	231 ± 12 (0.86)	83 ± 10 (0.87)	121 ± 14 (1.08)

Values shown as mean (M) ± standard deviation (SD); *MI* mutagenicity index (in brackets); *SC* spontaneous control: phosphate buffer (pH 7.4) or S9 mixture (100 µL/plate), rate of spontaneous reversion; Solvent control: dimethylsulfoxide (DMSO—100 µL/plate); Memantine (mtnH—50 µg/ plate; 85.83 µM); C + = positive control

[a]4-nitro-*o*-phenylenediamine (TA98 and TA97a, 10.0 µg/plate)

[b]sodium azide (TA100, 1.25 µg/ plate); [c]mitomycin (TA102, 0.5 µg/plate), in the absence of S9 and

[d]2-anthramine (TA98, TA100, TA 97a, 1.25 µg/plate)

[e]2-aminofluorene (TA102, 10.0 µg/ plate), in the presence of S9. The assay was performed in triplicate

***$p < 0.0001$ with a confidence level of 95% compared to the DMSO control was considered significant through one-way ANOVA test followed by the Dunnett's post hoc test with $p < 0.05$, which yielded statistical difference of $p < 0.0001$ only for positive controls. The values of F, DFn, DFd, exact *p* value are described in the results

These results confirm the non-mutagenicity of this compound (Table 1), and agrees with previous studies, in which the evaluation of the genotoxic (alkaline comet assay) and mutagenic (bone marrow micronucleus test) effects of memantine on CF-1 mice showed the absence of such undesirable effects. Memantine did not cause DNA damage in the blood and brain tissues and showed antigenotoxic effects in brain tissue [40].

Final remarks and perspectives

Memantine hydrochloride has shown to be effective impairing CHIKV infection in vitro with an EC_{50} of 32.4 ± 31.9 µM, CC_{50} of 248.4 ± 4 µM, and SI of 7.67. Besides, mtnH was not mutagenic by the Ames test. With such promising data, we expect to encourage further preclinical and clinical assays of mtnH as a repurposing drug candidate for the treatment of Chikungunya fever.

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Author contributions Acquisition of data and results analysis: AKSP, IAS, and WWS. Drafting of the manuscript AKSP and IAS. Study design, supervision, and critical revision FARN, FRGB, ACGJ and PPC. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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