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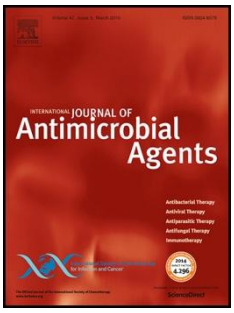

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The Thiopurine Nucleoside Analogue 6-Methylmercaptopurine Riboside (6MMPr) Effectively Blocks Zika Virus Replication

Otavio Valério de Carvalho[1#], Daniele Mendes Félix[1#], Leila Rodrigues de Mendonça[1], Catarina Maria Cataldi Sabino de Araújo[1], Rafael Freitas de Oliveira Franca[1], Marli Tenório Cordeiro[1], Abelardo Silva Júnior[2*], Lindomar José Pena[1*]

[1]Department of Virology, Oswaldo Cruz Foundation (Fiocruz), Recife, Pernambuco, Brazil;

[2]Department of Veterinary Medicine, Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil;

# These authors contributed equally to this work.

*Corresponding authors:

Lindomar Pena, PhD. Department of Virology, Oswaldo Cruz Foundation (Fiocruz). Address: Avenida Professor Moraes Rego. Recife, Pernambuco, Brazil. Email: lindomar.pena@cpqam.fiocruz.br

Abelardo Silva Júnior. Department of Veterinary Medicine, Federal University of Viçosa (UFV). Address: Av. PH Rolfs, s/n, Viçosa, MG, 36570-900, Brazil. Email: asjvet@gmail.com

Highlights

- For the first time, 6MMPr was identified as a ~~strong~~ potential antiviral drug against ZIKV;
- All assays used the epidemic ZIKV strain circulating in Brazil;
- Antiviral activity was tested in both epithelial and human neuronal cells;
- 6MMPr was much less toxic to neuronal cells compared to epithelial cells;
- 6MMPr decreased ZIKV production in both cells by more than 99%.

Abstract

Since the emergence of Zika virus (ZIKV) in Brazil in 2015, 48 countries and territories in the Americas have confirmed autochthonous cases of the disease caused by the virus. The ZIKV-associated neurological manifestations and congenital defects make the development of safe and effective antivirals against ZIKV of utmost importance. Here, we evaluated the antiviral activity of 6-methylmercaptopurine riboside (6MMPr), a thiopurine nucleoside analog derived from the prodrug azathioprine (AZA), against the epidemic ZIKV strain circulating in Brazil. In all the assays, an epithelial (Vero) and an human neuronal (SH-SY5Y) cell line were used to evaluate the cytotoxicity and the effective concentrations of 6MMPr against ZIKV. The levels of ZIKV RNA, viral infectious titer and the percentage of infected cells at the presence or absence of 6MMPr was used to determine the antiviral efficacy. We show that 6MMPr decreased ZIKV production by more than 99% in both cell lines in a dose- and time-dependent way. Interestingly, 6MMPr was 1.6 times less toxic to SH-SY5Y cells compared to Vero cells, presenting a 50% cytotoxic concentration (CC_{50}) of 460.3 µM and 291 µM, respectively. The selectivity index of 6MMPr for Vero and SH-SY5Y cells was 11.9 and 22.7 ~~µM~~, respectively, highlighting the safety profile of the drug to neuronal cells. Taken together, our results identify, for the first time, the thiopurine nucleoside analog 6MMPr as promising antiviral candidate against ZIKV that warrants further *in vivo* evaluation.

Keywords: Antiviral, Zika virus; cytotoxicity; 6MMPr; Vero; neuronal cells.

1. Introduction

Zika virus (ZIKV) is a member of the *Flavivirus* genus within the family *Flaviviridae*. This genus comprises other important arboviruses such as *Dengue virus* (DENV), *Yellow fever virus* (YFV) and *West Nile virus* (WNV) [1]. The virus was initially isolated in 1947 from a rhesus monkey in the Zika Forest in Uganda.In March 2015, Brazil reported autochthonous transmission of ZIKV and the virus has spread throughout the Americas since then. ZIKV infection has already been reported in approximately 60 countries in different continents [2, 3]. ZIKV transmission occurs through the bites of infected *Aedes* mosquitoes, although recent findings have also indicated sexual, congenital, perinatal and blood transfusion transmission. Clinically, ZIKV disease is manifested by rash, fever, arthralgia and conjunctivitis [4]. Importantly, infection of pregnant women may result in microcephaly of the fetus and others severe congenital defects such as intracranial calcifications, ventricular system dilation and neuronal migration disorders [5]. In adults, severe neurological complications such as myelitis, meningoencephalitis and Guillain-Barré syndrome (GBS) have been associated with ZIKV infection and in some patients the infection can lead to death [6-8].

Like other members of the *Flavivirus* genus, ZIKV is an enveloped, single-stranded positive-sense RNA virus whose genome is about 11 Kb. Its genome encodes three structural and seven nonstructural proteins. The structural proteins mediate the initial steps of viral host interaction including receptor binding and entry, whereas nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) play different roles in viral replication, virion assembly and evasion of immune defense mechanisms [9]. The proteins NS3 and NS5 are targets for antiviral development because of their viral role in viral replication. NS5 is the most conserved protein across the *Flavivirus* genus, and possesses N-terminal RNA methyltransferase (MTase) and C-terminal RNA-dependent RNA polymerase (RdRp) activities [10]. Several nonstructural proteins have been implicated as potential targets for drugs against flaviviruses [11].

Despite the burden of ZIKV-associated diseases, there are neither vaccines nor treatments available to block viral replication. Recent studies have suggested that the specificities of ZIKV biology compared to other flaviviruses may reveal new challenges for antiviral therapy as well as new drug targets [1]. Nevertheless, various attempts to find anti-ZIKV inhibitors are underway [12-16]

Many nucleoside and non-nucleoside analogs are inhibitors of viral polymerases, and have been described as promising molecules against RNA viruses [10]. RdRp is responsible for viral RNA synthesis and there is no similar enzyme in the host. Compounds that bind to RdRp and inhibit *de novo* initiation of viral replication have already been shown active against DENV [17] and ZIKV [14, 16]. In general, they have high selectivity and broad-spectrum activity, making them attractive candidates for *Flavivirus* antiviral development [11].

Azathioprine belongs to the class of thiopurine-modified nucleosides and has shown activity against ZIKV [12]. It is a pro-drug which is metabolized to 6-methylpurine (6MP) (inactive metabolite) by glutathione S-transferase enzyme and 6-MP, in turn, is processed to 6-thioinosine (6TI) and 6-methylmercaptopurine (6MMP) by action of thiopurine methyltransferase enzyme (TPMT), both active metabolites that can be converted to 6-methylmercaptopurine riboside (6MMPr) [18]. 6MMPr is also a nucleoside analogue thiopurine metabolite and its antiviral properties have already been demonstrated to flaviviruses such as *Hepatitis C virus* (HCV) RNA replicon, B*ovine viral diarrhea virus* (BVDV) [19, 20], YFV, DENV-2 and WNV [21].

In this work, we evaluated the antiviral activity of 6MMPr against the epidemic ZIKV strain that has recently emerged in Brazil. For the first time, we demonstrate potent *in vitro* inhibitory activity of 6MMPr against ZIKV infection in both epithelial and neuronal cell lines. Together, our results identify the thiopurine analog 6MMPr as a promising candidate for further clinical evaluation against ZIKV.

2. Material and Methods

2.1. Cells, virus, and drug preparation

Vero cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U mL^{-1} penicillin/streptomycin. Human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection (ATCC) and were grown in Minimum Essential Medium (MEM) (Inlab Diagnóstica, Sao Paulo, Brazil) supplemented with Ham's F12 Nutrient Mixture (Sigma, St. Louis, USA), 1 mM sodium piruvate (Sigma, St. Louis, USA), 1% MEM non-essential amino acids (Gibco, Carlsbad, USA), 100

U/ml penicillin, 0.1 g/ml streptomycin, 2 mM L-glutamine and 10% (vol/vol) fetal bovine serum (FBS), at 37 °C with 5% CO_2.

The Brazilian ZIKV strain, named ZIKV/H.sapiens/Brazil/PE243/2015 (abbreviated to ZIKV PE243; GenBank Acession number KX197192.1), was isolated from a patient who had the classical ZIKV exanthematous illnesses, without neurological signs [22]. The ZIKV PE243 strain was propagated and titrated on Vero cells. Viral titration was performed by the standard $TCID_{50}$ method [23] and expressed as $\log_{10}$ $TCID_{50}$ mL^{-1}.

The thiopurine nucleoside analogue 6MMPr and the control ribavirin were purchased from Sigma-Aldrich (Saint Louis, USA). Stock solution of the compound was prepared in Milli-Q H_2O, sterilized by filtering through a 0.22 µM Millipore filter, and stored at -20 °C.

2.2. Cell viability assay

The cell toxicity of 6MMPr was tested on growing cells via *in situ* mitochondrial reduction of a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, USA). Briefly, 24 h-plated Vero ($1x10^4$ cells/well) and SH-SY5Y cells ($4x10^4$ cells/well) in 96-well microplates were treated with various concentrations of the test compound. After 72 h (SH-SY5Y cells) and 120 h (Vero cells) of incubation at 37 °C, culture medium was removed and replenished with 50 µL of MTT solution (1 mg/mL) to each well and the microplate was incubated for 4 h. MTT formazan crystals were solubilized by adding DMSO and the optical densities were determined spectrophotometrically with a 96-well plate reader (BioTek, ELX800, Winooski, USA) at 540 nm. As a control for the cytotoxicity test, ribavirin was used at various concentrations (10 to 100 µM). Cell viability was calculated by subtracting the optical density fraction of treated cells from the untreated cells. Cytotoxic concentration for 50% of cell culture (CC_{50}) was defined as the concentration of the compound that caused a 50% reduction in absorbance. CC_{20} was defined as the limit point for treatment with the antiviral molecules [24].

2.3. Antiviral activity assay

Vero cells were seeded in 24-well plates a day prior to infection at the density of $5x10^4$ cells/well. The cells were infected with the ZIKV PE243 strain at a multiplicity of infection (MOI) of 0.1 and incubated for 2 h (37 °C, 5% CO_2). After virus

internalization, viral inoculum was removed, cells were washed twice with DMEM and the supernatant was replaced with fresh medium containing four concentrations of 6MMPr (7.6, 15.1, 30.3 and 60.5 µM). Controls included mock and infected non-treated cells. At 120 h post-infection (hpi), the cells supernatant was harvested and stored at -80 °C until downstream analysis by real-time quantitative RT-PCR and virus titration ($TCID_{50}$). As a control for the efficacy test, ribavirin was used at different concentrations (7.7, 15.5, 31 and 62 µM).

SH-SY5Y cells monolayers were grown in 24-well plates a day prior to infection at the density of $1x10^5$ cells/well and then infected with ZIKV at a MOI of 0.1 diluted in 100 µL of culture medium containing 2% (vol/vol) FBS for 2 h at 37 °C. Cells were washed twice and then replenished with fresh medium containing four concentrations of 6MMPr (19.7, 39.3, 78.5, 157 µM) and incubated at 37 °C with 5% CO_2. At 72 hpi, ZIKV- and mock-infected cells monolayers were processed for flow cytometry and immunofluorescence and the supernatant was titrated by $TCID_{50}$ in Vero cells.

2.4. Plaque-reduction assay

Antiviral efficacy of 6MMPr was also evaluated by measuring the reduction in the number of ZIKV plaque forming units (pfu) after treatment with 6MMPr. Confluent monolayers of Vero cells at a density $1x10^5$/well were seeded on 24-well plates and incubated at 37 °C in a CO_2 incubator for 24 h. Supernatants from antiviral activity assays were added to cells and after 2-h incubation, the inoculums were removed and the cells were overlaid with 2.5% carboxymethyl cellulose (CMC) with 2% FBS. The plates were incubated for 120 h at 37 °C and 5% CO_2. Monolayers were fixed using 10% (V/V) formalin diluted in PBS, stained with crystal violet solution and the plaque numbers were counted. The percentage of plaque reduction (PR%) compared to untreated infected cells was calculated using the following formula: PR (%) = (C - T) x 100/C, where, C is the mean of the number of plaques from triplicate untreated control wells and T is the mean of the number of plaques from triplicate treated wells.

2.5. Time-dependent antiviral effects

The second approach of the antiviral activity assay was the addition of 6MMPr equivalent to its CC_{20} (60.5 µM) to the infected Vero cells (MOI 0.1) and collection of supernatant at 24, 48, 72, 96 and 120 hpi to determine the effects of time-dependent treatment exposure. The RNA levels and infectious virus in cell supernatant were

quantified by quantitative real-time reverse transcription PCR (qRT-PCR) and $TCID_{50}$, respectively.

2.6. Post-treatment with 6MMPr at various time intervals

Vero cells were first infected with ZIKV at MOI of 0.1 for 2 hours. Infected cells were washed twice and then treated with 6MMPr (60.5 µM) diluted in DMEM at 6, 12, 24, 48, 72 or 96 hpi. Supernatants were collected at 120 hpi and processed for qRT-PCR and viral titration.

2.7. ZIKV qRT-PCR

Total RNA was extracted from culture supernatants using TRIzol reagent (Invitrogen Carlsbad, USA) according the manufacturer's instructions. The RNA was eluted in 20 µL and stored at -80 °C. Quantitative real-time PCR (qPCR) was conducted by using the QuantiTect Probe RT-PCR Kit (QIAGEN, Valencia, USA) with amplification in the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, USA) as per the manufacturer's protocol. Previously developed ZIKV primers (ENV 1086F 5'-CCGCTGCCCAACACAAG-3' and ENV 1162R: 5'-CCACTAACGTTCTTTTGCAGACAT-3') and probe (5'-VIC-AGCCTACCTTGACAAGCAGTCAGACACTCAA-BHQ1-3) were used [25]. Quantitative RT-PCR reactions were carried out at 50 °C for 30 min followed by denaturation at 95 °C for 15 min and 45 cycles of 95 °C for 15 sec and 60 °C for 1 min. Relative standard curve was generate with addition of RNA extracted from virus culture with known titer (PFU mL^{-1}) 10-fold serially diluted in nuclease free H_2O. All reactions were performed in triplicate. The relative quantification of ZIKV RNA was assessed using the 7500 Software v2.0.6 provided by Applied Biosystems.

2.8. Flow cytometry analysis

SH-SY5Y cells were treated as described in item 2.3 and harvested at 72 hpi. Cells were fixed using the Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences, San Jose, USA). Intracellular antigen staining was carried out following the manufacturer's instructions using mouse anti-flavivirus E protein monoclonal antibody 4G2 (ATCC: HB-112) and goat anti-mouse FITC secondary antibody (Sigma-Aldrich). Flow cytometry was performed using a FACS AriaIII (BD Biosciences) and results were analyzed using FlowJo Software (TreeStar).

2.9. Immunofluorescence microscopy

ZIKV-and mock-infected SH-SY5Y cells monolayers were grown on 13-mm-diameter coverslips and treated as described in item 2.3. Cells were fixed at 72 hpi with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature and then permeabilized with 0.2% (V/V) Triton X-100 in PBS for 15 min at room temperature. Cells were then incubated with blocking solution (0.2% [wt/vol] pork skin gelatin in PBS) for 20 min at 37 °C and incubated with mouse anti-flavivirus E monoclonal antibody 4G2 for 1 h at 37 °C. After, cells were washed in PBS and incubated with donkey anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Invitrogen Carlsbad, USA) for 30 min at 37 °C. Coverslips were mounted using ProLong® Diamond Antifade Mountant (Molecular Probes) and cells were analyzed with Leica DMI4000 B (Leica Microsystems) microscope. Images were processed using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.10. Statistical analysis

Statistical analysis was performed to assess differences in viral yield from infected cells treated with 6MMPr compared to untreated control, in different doses and time intervals. Data were analyzed using one-way ANOVA (analysis of variance) using the GraphPad Prism Software version 5.01 for Windows (GraphPad Software, La Jolla, California, USA). The Tukey test was used for pairwise comparisons among means. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce ZIKV titer by 50% as compared to virus control. Values of CC_{50} and IC_{50} were calculated using non-linear regression using the GraphPad Prism Software. Selectivity index (SI) was obtained by calculating the ratio of the CC_{50} and the IC_{50} values. Data were expressed as the mean ± the standard deviations from three independent experiments. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. The thiopurine drug 6MMPr has distinct toxicity profile in epithelial and neuronal cells

In order to determine the cell toxicity of the thiopurine nucleoside analogue 6MMPr (Figure 1) to Vero and SH-SY5Y cells, the MTT assay was performed. We found that CC_{20} and CC_{50} values for Vero cells were 60.5 and 291 µM, respectively

(Table 1 and Figure 2A). For the SH-5YSY cell line, the CC_{20} was 157 µM and the CC_{50} was 460.3 µM (Table 1 and Figure 2B). To determine the relative drug efficacy in inhibiting viral replication compared to cellular toxicity, the SI was established for each cell line (Table 1). Thus, 6MMPr demonstrated lower cytotoxicity for neuronal cells as compared to epithelial cells, suggesting its potential use as an antiviral against neurotropic viruses. The CC_{20} and CC_{50} values of the control ribavirin in Vero cells were 62.11 µM and 246 µM, respectively (Supplementary Table 1 and Supplementary Figure 1).

3.2. 6MMPr blocks RNA yield and infectious ZIKV production in Vero cells

The antiviral activity of 6MMPr in Vero cells was evaluated by RNA quantification, viral titration and plaque reduction assay from supernatant of treated and untreated cells infected with ZIKV. Quantification of viral RNA levels by qRT-PCR shows that the inhibition occurred in a dose-dependent way. Although the lowest concentration tested (7.6 µM) did not significantly reduce viral RNA levels, the concentrations of 15.1 µM and 30.3 µM caused a reduction of 82.73% and 91.03%, respectively. Significantly, the highest 6MMPr concentration (60 µM) decreased viral RNA levels to over 99% (Figure 3A).

Though reduction in viral RNA production in infected cells is an indicative of antiviral effects, it may not necessarily translate into reduced viral titers. To rule out this hypothesis, we quantified infectious viral yield in treated cells relative to controls. In agreement with the qRT-PCR, 6MMPr displayed a dose-dependent reduction on virus titer. We found that treatment with 6MMPr at concentrations of 30.3 µM and 60.5 µM significantly reduced ZIKV titer by 77.24% and 99.45%, respectively (Figure 3B). The IC_{50} and the SI for Vero cells was 24.5 µM and 11.9, respectively (Table 1).

To further confirm these findings, cell supernatant was also evaluated by the plaque-reduction assay. The number of viral plaques produced following 6MMPr treatment was reduced by 24% at the 7.6 µM concentration and by 47.4%, 72.7% and 96.7% at concentrations of 15.1, 30.3 and 60.5 µM, respectively (Figure 3C and D). The control ribavirin reduced viral titers by 1.3 $\log_{10}$ at the highest concentration used (62 µM). The calculated IC50 and SI of ribavirin was 31.6 µM and 4.97, respectively (Supplementary Table 1 and Supplementary Figure 2).

Taken together, these results demonstrated that 6MMPr potently blocks ZIKV RNA yield and infectious viral progeny production in epithelial cells.

3.3. Time-dependent efficacy in Vero cells

In the previous experiments, cells were treated with various drug concentrations after virus internalization and the antiviral readout was done at 120 hpi. Here, we sought to determine the time-dependent effects of 6MMPr at its CC_{20}. To this end, virus inoculation was done for 2 h, then the cells were treated once with 6MMPr 60.5 µM and supernatant was collected every 24 hours for five days. There was a reduction in viral RNA levels and infectious virus yield greater than 99% in all time points, (Figure 4A and B), highlighting the ~~strong~~ anti-ZIKV effects of 6MMPr.

3.4. Post-treatment with 6MMPr at various time intervals

In this assay, Vero cells were infected with ZIKV for 2 h followed by 6MMPr (60.5 µM) treatment at 6, 12, 24, 48, 72 or 96 hpi in order to determine how long the addition of the compound can be postponed before decreasing its inhibitory effects. Treatment between 6 and 24 hpi inhibited viral RNA production by more than 99%. Later treatments at 48 and 72 hpi reduced viral RNA yield by 96.67% and 47.63%, respectively. At 96 hpi, inhibition was not significant (Figure 4C). Viral titration showed that the drug reduced viral growth when added up to 24 hpi (6 h – 98.76%; 12h - 98.03%; 24 h - 86.39%); however, there was not significant reduction at later time points (Figure 4D). These results demonstrate that early treatment of 6 MMPr have the most significant reduction in ZIKV replication.

3.5. 6MMPr shows robust inhibition of ZIKV infectivity in neuronal cells

Since neural tissues constitute a target of ZIKV infection in human patients, the human neuronal cell line SH-SY5Y was chosen to evaluate the effects of 6MMPr on ZIKV infection under controlled conditions. SH-SY5Y cells monolayers were infected with ZIKV and then treated with four drug concentrations at 2 hpi (Figure 5). At the MOI of 0.1, 44% of the cells were infected by ZIKV. Treatment with 6MMPr significantly reduced the number of infected cells in a dose-dependent manner (Figure 5A, B and D). Viral quantification of cell supernatant showed that the treatment resulted in up to 99.83% in infectious ZIKV yields at the highest dose used (Figure 5C). Although the number of infected cells was lower at the concentration of 157 µM, there

was statistical difference between 78.5 µM and 157 µM regarding infectious viral titers (Figures 5 B and C). Analyses of ZIKV infectious titer showed IC_{50} values of 20.3 µM for 6MMPr treatment in infected SH-SY5Y cells.

Flow cytometry analysis showed a dose-dependent inhibition of ZIKV infection in neuronal infected cells, revealing an inhibition of almost 70% of ZIKV replication in cells treated with 157 µM of 6MMPr, compared to untreated infected cells (Figure 5D). Similar pattern of inhibition was observed by immunofluorescence microscopy (Figure 5E), confirming the results obtained by viral titration and flow cytometry.

Thus, 6MMPr display low toxic effects and efficiently inhibits ZIKV infection in neuronal cells. Overall, these results suggest that 6MMPR is a promising novel antiviral candidate against ZIKV.

4. Discussion

Since its emergence in Brazil in 2015, ZIKV has rapidly spread in the Americas resulting in an epidemic of great public health concern. Despite some serious complications caused by the virus, including neurological disorders and congenital malformations, there are currently no available vaccines or specific antiviral drugs against this feared pathogen [26]. Therefore, the discovery and development of therapeutic strategies able to effectively control ZIKV is an urgent need. We showed here that the nucleoside analog 6MMPr inhibits replication of the Brazilian ZIKV strain in different cell types. 6MMPr is a nucleoside metabolite derived from the thiopurine prodrug azathioprine (AZA) and has been shown previously to exhibit antiviral properties against diverse flaviviruses [19-21].

Here, we expand the previous findings and investigated the antiviral activity of 6MMPr against the epidemic ZIKV strain (ZIKV PE243) isolated during the 2015 epidemic in Brazil. We observed that 6MMPr decreased up to 2.4 $\log_{10}$ (99.5%) of ZIKV infectious titer in a dose- and time-dependent way. The addition of the compound could be postponed up to 24 hpi before reducing its antiviral efficiency in ZIKV-infected Vero cells (Figure 4D). Since ZIKV is a neurotropic flavivirus, we evaluated the antiviral efficacy of the drug in a human neurobastoma cell line (SH-SY5Y) infected with ZIKV. Interestingly, 6MMPr was 1.6 times less toxic to SH-SY5Y cells compared to Vero cells. The antiviral agent was capable of inhibiting viral replication in SH-

SY5Y cells as well, and ZIKV infectious titer was decreased up to 2.8 $\log_{10}$ (99.8%). Although we found similar IC_{50} mean values for ZIKV infection in 6MMPr-treated Vero and SH-SY5Y cells, the therapeutic selectivity of 6MMPr to SH-SY5Y cells was nearly twice higher than that of Vero cells. The cytotoxicity and anti-ZIKV activity of the antiviral control ribavirin was in accordance with previously published studies [27, 28] and its antiviral activity was inferior to that of 6MMPr.

RNA viruses have increased genetic diversity often due to high mutation rates as result of the lack of proofreading mechanism of their RdRps [29]. Although that diversity may represent rapid evolution and adaptability, the absence of proofreading makes RNA viruses more vulnerable to antiviral effects of nucleoside analogues. During viral RNA synthesis, the incorporation of these modified nucleosides into the genome can result in low viral infectivity correlated with deleterious mutations and viral replication errors [30]. Besides incorporation into nucleic acids, thiopurine nucleosides are also known to inhibit *de novo* purine biosynthetic pathway [31]. Decreasing purine synthesis results in reduced nucleotide pools and subsequently lower rate of virus replication. 6MMPr is a strong inhibitor of *de novo* purine synthesis and displayed the higher antiviral efficacy against BVDV among AZA metabolites [19].

Some previous studies have also shown antiviral activity of other nucleoside analogues against ZIKV infection (Barrows et al., 2016; Eyer et al., 2016). Among the purine analogues, AZA and its metabolites mercaptopurine (6-MP) and thioguanine (6-TGN) were found to inhibit ZIKV *in vitro* (Barrows et al., 2016). However, these studies did not validate the antiviral activity for the ZIKV strain isolated during the 2015-2016 outbreaks in Brazil. Moreover, none of these thiopurine compounds were evaluated in any human neuronal cell line.

There are some issues that need to be addressed in order to develop effective antivirals against ZIKV. The drug should be able to cross the placenta and the blood-brain barrier (BBB), and inhibit the virus in target cells of the central nervous system (CNS), while retaining its safety profile for pregnant women and their embryos or fetuses [32]. Besides immunosuppressant effects and hepatic dysfunction risk of AZA, thiopurines are well tolerated and there is no absolute contraindication during pregnancy. AZA and its metabolites can cross the placental barrier, but their diffusion to the fetal blood is highly limited [33]. That way, according to some studies, the effects of AZA exposure during pregnancy will not represent a significant risk to the fetus [34, 35]. Among the AZA metabolites, 6-TGN may be considered the one mainly

responsible for immunosuppressive effects. On the other hand, elevated 6MMPr concentrations has been associated with thiopurine-induced hepatotoxicity [36]. However, the measurement of 6MMPr levels for detecting liver damage during AZA therapy lacks sensitivity and specificity, and other studies have not found consistent correlation of 6MMPr levels and drug-related hepatotoxicity [36, 37].

A study published by Lim et al. , 2011 [21] performed a clinical trial with WNV-infected mice treated with 6MMPr. Despite its efficacy against WNV *in vitro*, 6MMPr did not reduce mortality of inoculated mice. In that study, it was suggested a poor bioavailability of 6MMPr in the CNS tissue to explain the results, however there is no data on 6MMPr transfer across the BBB to support the author´s conclusions. If the permeability of 6MMPr across these barriers is an issue, rational chemical modification as well as novel delivery platforms based on nanotechnology may circumvent this hypothetical issue.

Given the urgency of developing therapeutic approaches against ZIKV infection, the anti-ZIKV properties of 6MMPr identified in this study makes it a promising candidate for further *in vivo* trials. In addition, further studies combining 6MMPr with other newly identified ZIKV inhibitors are warranted and may pave the way to block the severe outcomes of ZIKV infection in the population.

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Declarations

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Figure 1. Chemical structure of 6-methylmercaptopurine riboside (6MMPr).

Figure 2. The thiopurine drug 6MMPr has distinct toxicity profile in epithelial and neuronal cells. Cell viability was determined by the MTT method. Briefly, 96-well microplates were seeded with either Vero cells ($1x10^4$ cells/well) or SH-SY5Y cells and then treated with various concentrations of the 6MMPr. After 72 h (SH-SY5Y cells) and 120 h (Vero cells) of incubation at 37°C, culture medium was removed and replenished with 50 µL of MTT working solution (1 mg/mL) and further incubated for 4 h. The optical densities were determined by spectrophotometry at 540 nm. Values are the mean ± SD of three independent experiments.

Figure 3. 6MMPr blocks RNA yield and infectious ZIKV production in Vero cells. ZIKV RNA levels following 6MMPr treatment (A). Infectious progeny titer by the $TCID_{50}$ method (B). Reduction in virus plaque forming units upon 6 MMPr treatment (C, D). Vero cells were plated in 24-well tissue culture plates and infected with the ZIKV at MOI of 0.1 for 2 h. After infection, cells were washed twice and treated with four concentrations of 6MMPr. Controls included mock and infected non-treated cells. At 120 hpi, the cell supernatant was harvested and the antiviral activity of determined using qRT-PCR, $TCID_{50}$, and plaque assay as the readout. Values are the mean±SD of three independent experiments.

Figure 4. Time-dependent antiviral effects (A, B) and post-treatment with 6MMPr at various time intervals (C, D). Infected Vero cells (MOI 0.1) were treated with 60.5 µM of 6MMPr and cell supernatant was collected at 24, 48, 72, 96 and 120 hpi to determine the effects of time-dependent treatment exposure (A,B). For time of addition studies (C,D), Vero cells were infected with ZIKV at MOI of 0.1 and then treated with 6MMPr (60. 5 µM) at different time after the infection (6, 12, 24, 48, 72 or 96 h). The RNA levels and infectious virus in cell supernatant were quantified by quantitative real-time RT-PCR (qRT-PCR) and $TCID_{50}$, respectively. Values are the mean ± SD of three independent experiments.

Figure 5. 6MMPr shows robust inhibition of ZIKV infectivity in neuronal cells. SH-SY5Y was infected with ZIKV at a MOI of 0.1 and treated with 6MMPr at different concentrations. Cells were processed for flow cytometry (A,B,D), viral titration (C) and

546 immunofluorescence analysis (E) to determine the antiviral effects of 6MMPr in
547 neuronal cells. Values are the mean ± SD of three independent experiments.
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552 **Table 1.** Cytotoxicity, antiviral activity, and selectivity index of 6MMPr in epithelial
553 and neuronal cells.

Cell line	Cytotoxicity CC_{50} (µM)[a]	Antiviral activity			
				Log_{10} reduction value[e]	
		IC_{50} (µM)[b]	SI[c]	CC_{20} (µM)[d]	$TCID_{50}$
Vero	291	24.5	11.9	60.5	2.4
SH-SY5Y	460.3	20.3	22.7	157	2.8

554 [a]CC_{50} (50% cytotoxic concentration) refers to compound concentration that caused a
555 50% reduction in viability.

556 [b]IC_{50} (50% cytotoxic concentration) refers to compound concentration required to
557 reduce viral titers by 50% as compared to untreated controls.

558 [c]Selectivity index (SI) is obtained by calculating the ratio of the CC_{50} and the IC_{50}
559 values (CC_{50}/IC_{50}).

560 [d]CC_{20} (20% cytotoxic concentration): maximum nontoxic concentration employed in
561 the antiviral assays.

562 [e] Log_{10} reduction was calculated by subtracting the log_{10} means of the ZIKV infectivity
563 in the presence of 6MMPr at CC_{20} relative to untreated cells.

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