

Effect of 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide on *in vitro* poliovirus replication

PATRICIA REGINA SANTOS¹
ANELISE S. NAZARI FORMAGIO²
BENEDITO PRADO DIAS FILHO¹
CELSE VATARU NAKAMURA¹
MARIA HELENA SARRAGIOTTO²
TANIA UEDA-NAKAMURA^{1*}

¹ Post-Graduate Program in
Pharmaceutical Sciences
State University of Maringá
87020-900, Maringá, PR, Brazil

² Post-Graduate Program in Chemistry
State University of Maringá
87020-900, Maringá, PR, Brazil

The effect of the alkaloid 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide (PMC) on the poliovirus (PV) replication cycle in Vero cells was assayed by inhibition of the cytopathic effect (CPE) and inhibition of plaque forming units (PFU). Both methodologies suggested that the mode of action was avoidance of infection progression in the host cell. The compound was able to prevent CPE and PFU formation when the cells were pre-treated with PMC for 24 h prior to PV infection. In addition, when the alkaloid was continuously maintained in the infected cultures, the spread of PV to adjacent cells was impaired. The pre-exposure and post-exposure prophylactic applications are possible situations in which an anti-PV drug might be used. Future studies are needed to elucidate the PMC mode of action and verify the feasibility of PMC use *in vivo*. No antipicornavirus agent is currently approved for clinical use.

Keywords: 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide, β -carboline, poliovirus, antiviral activity

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Members of the genus *Enterovirus* have been implicated in a large variety of human diseases, ranging from mild illnesses to severe clinical diseases, such as myocarditis, meningitis, encephalitis, and paralysis (1). PV, a member of the genus *Enterovirus* (family *Picornaviridae*), is the etiological agent for poliomyelitis, a disease that results in the destruction of neurons located in the gray matter of the anterior horn of the spinal cord (2). PV is a prototype RNA virus in general and human enterovirus in particular (3). Large-scale vaccination campaigns have been unable to eradicate PV. Endemic transmission of PV still occurs in Nigeria, Pakistan and Afghanistan (4). To achieve eradication, inactivated PV vaccine (IPV) has been suggested as replacement of oral PV vaccine (OPV), but it is still a matter of controversy whether this strategy alone would be sufficient to eradicate PV. Thus, anti-PV compounds have been considered important in supporting the IPV control program (5).

* Correspondence; e-mail: tunakamura@uem.br

The possibility of new outbreaks in the post-polio era through accidental or intentional reintroduction of the virus into the environment and lack of anti-PV drugs emphasizes the necessity of their development (6–9). Hence, evaluation of compounds with potential anti-PV activity in cell culture, particularly those which have demonstrated promising therapeutic and prophylactic effects on other picornavirus infections, has also been stimulated (8).

The antiviral activity of β -carboline alkaloids has been demonstrated against human immunodeficiency virus (HIV) (10), *Herpes simplex* virus – type 1 (HSV-1) (11) and human papillomavirus (12). We have previously reported the antiviral activity of a series of 1,3-disubstituted β -carboline derivatives that bear a substituted carbohydrazide group at C-3 against PV and HSV-1 (13). Among these, PMC exhibited low toxicity and high antiviral activity against PV infection. The aim of the present study was to investigate the mode of action of PMC on PV replication in Vero cells.

EXPERIMENTAL

Cells

All the reagents and media for cell culture were purchased from Gibco-BRL (USA). Vero cells (ATCC CCL-81), purchased from Adolfo Lutz Institute (Núcleo de Cultura de Células, São Paulo, Brazil), were propagated in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 50 $\mu\text{g mL}^{-1}$ gentamycin at 37 °C in a humidified atmosphere of 5 % CO₂.

Virus

Poliovirus vaccine was kindly provided by Dr. Rosa Elisa Linhares, State University of Londrina, Brazil. The viruses were propagated in Vero cells and titrated using a plaque assay. To this, cell monolayers were inoculated with 10-fold dilutions of the virus and after 48 h of incubation the virus titer was determined and expressed as plaque forming units (PFU) mL⁻¹. The viruses were stored at –80 °C until use.

Alkaloid

The PMC was synthesized as previously described (13). It was dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium. The final concentration of DMSO was 1 % (V/V).

Cytopathic effect inhibition assay

In this experiment, PMC was added before infection (1 or 24 h), during infection (1 h), and after infection (48 h) at concentrations of 2.4, 24 and 120 $\mu\text{mol L}^{-1}$. Vero cells were seeded into 96-well culture plates (TPP®) at a density of 2.5×10^4 cells per well and incubated at 37 °C with 5 % CO₂ for 24 h to obtain monolayers. The monolayers were then washed with DMEM and infected with 25 μL of PV in a tissue culture infectious dose of 80 % (TCID₈₀) per well (14) for 1 h at 37 °C with 5 % CO₂. The non-adsorbed viruses were

removed, and the monolayers were washed with DMEM and covered with medium with different concentrations of the compound or without it. The plates were incubated for 48 h and inhibition of cytopathic effect (CPE) was detected using the sulforhodamine B assay (15).

Plaque reduction assay

The assay was performed by seeding Vero cells into 24-well culture plates (TPP®) at a density of 2.5×10^5 cells per well and incubating them at 37 °C with 5 % CO₂ until monolayer formation. The cell monolayer was infected with 200 µL of PV suspension per well (40–50 PFU per well) and incubated at 37 °C with 5 % CO₂ for 1 h. Treatments were performed according to the experimental strategies described below. The infected cell monolayer was washed with DMEM and overlaid with DMEM containing 0.5 % carboxymethylcellulose (DMEM-CMC). After 48 h of incubation, the cell monolayer was fixed with 10 % formalin and stained with 1 % crystal violet. Plaques were counted and the percentage of inhibition was calculated. All experiments were performed at least three times. The different experimental strategies are described below. In each of the experiments, the concentrations of PMC used were 0.24, 2.4, 24 and 120 µmol L⁻¹.

Cell pretreatment assay. – Confluent cell monolayers were incubated in the presence or absence of different concentrations of PMC for 24 h at 37 °C. The medium containing PMC was removed and cell monolayers were washed with DMEM and infected with PV as described above. Infected and non-infected cell monolayers were washed with DMEM and overlaid with DMEM-CMC. After incubation at 37 °C for 48 h, the monolayers were fixed and processed as described above.

Attachment assay. – To verify whether PMC was able to prevent the attachment of PV to the cells, cell monolayers were pre-chilled at 4 °C for 15 min and then simultaneously exposed to PV and different PMC concentrations. After incubation for 1 h at 4 °C, the cell monolayers were washed, overlaid with DMEM-CMC, and incubated for 48 h at 37 °C. The plaque reduction assay was then performed as described above.

Treatment during infection. – Confluent cell monolayers were infected with PV and treated with different concentrations of PMC for 1 h at 37 °C with 5 % CO₂. The supernatant was removed, and cell monolayers were washed and processed as described above to determine the reduction of PFUs.

Treatment after infection. – Vero cell monolayers infected or not with PV incubated for 1 h at 37 °C were washed with medium to remove non-attached virus and overlaid with DMEM-CMC that did or did not contain different concentrations of PMC for 48 h at 37 °C. PFUs were revealed and counted as described above.

Viral neutralization assay. – Viral suspensions were incubated with different concentrations of PMC for 1 h at 37 °C. Two-fold serial dilutions of virus-PMC suspensions were then used to infect the cell monolayers for 1 h at 37 °C. The cells were then washed with DMEM, overlaid with DMEM-CMC, and incubated for 48 h at 37 °C.

Statistical analysis

Reductions of PFUs were analyzed using the analysis of variance followed by the Tukey test. For statistical significance, $p < 0.05$ was set.

RESULTS AND DISCUSSION

The low toxicity of 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide in Vero cell cultures and its high selectivity (selectivity index = 1,322) against PV (10) led us to investigate its effect on virus-cell interactions by inhibiting PV proliferation. The viral multiplication cycle basically comprises five steps: (i) adsorption, which consists of fixing the virus to the cell surface mediated by specific receptors, (ii) penetration and stripping that allow internalization of the viral particle, (iii) biosynthesis of viral components, such as viral proteins and genetic material, (iv) viral particle assembly, and (v) the release of viral particles. Antiviral agents can exert their activity at one or more steps of viral replication. Additionally, the antiviral agent can also act by neutralizing the virus particle or exerting an immunomodulatory effect on the host cell. The present study was designed to investigate the stage of virus-cell interactions at which PMC is effective. The effect of PMC was investigated by detecting CPE caused by PV on monolayers of Vero cells, revealed by the sulforhodamine B colorimetric method at different stages of virus multiplication. Introduction of PMC simultaneously with the virus into the cell monolayer for 1 h at 37 °C and subsequent incubation for 48 h in the absence of PMC were unable to protect the cells against virus infection at any concentration tested, suggesting that PMC does not interfere with the early stages of infection (*i.e.*, adsorption and penetration steps). When the cell monolayer was treated for 24 h before inoculating PV and subsequently incubated for 48 h at 37 °C in PMC-free medium, 80 % cell protection was observed at the 24 $\mu\text{mol L}^{-1}$ concentration of PMC. The concentration that reduced 50 % of CPE (EC_{50}) was 6.4 $\mu\text{mol L}^{-1}$. This result suggests that PMC could exert a prophylactic effect when the host cell was treated 24 h prior to contact with the virus, even after removal of PMC from the culture medium. We also found that the treatment of the cell monolayer previously infected with PV for 48 h at 37 °C was still able to protect cells by 72 % against viral infection, with an EC_{50} value of 3.9 $\mu\text{mol L}^{-1}$ (Fig. 1). The same experimental strategy

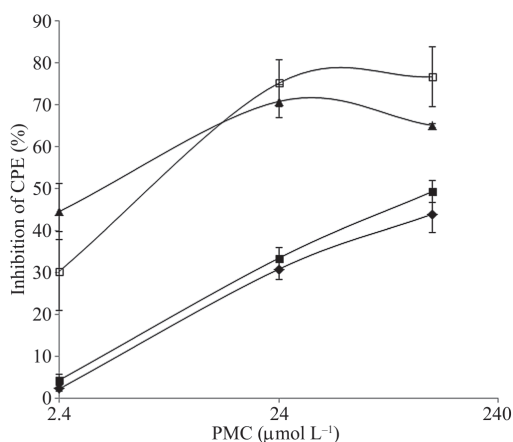


Fig. 1. CPE inhibition assay. Vero cells infected by PV were treated with the alkaloid 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide added (PMC): 1 h before infection (◆), during infection (■), after infection (▲), pretreatment for 24 h (□). Each point represents mean \pm SD of two independent experiments. Each concentration was tested in quadruplicate.

was used in the plaque reduction assay, and plaque formation was not inhibited by PMC (*i.e.*, the number of PFUs did not decrease even at higher concentration), but the reduction of plaque size was evident (Fig. 2). Results of the plaque reduction assays showed that PMC was not able to neutralize the virus particle or interfere with the adsorption and penetration phases (Fig. 3). In fact, results of the plaque reduction assay suggest that the PMC may exert a prophylactic effect against PV infection, as cell protection of approximately 70 % (Fig. 3a) was observed in accord with the results obtained in the prophylactic assay by inhibition on CPE. In Figs. 3b and c, the contact of PMC with the virus-cell system was restricted to a short time, only sufficient to exert its action during the entry process of the virus (adsorption and penetration), and no cell protection occurred. Treatment of the infected cell monolayer during 48 h (Fig. 3d) and the PV particle treatment with PMC before cell infection (in an attempt to neutralize PV) were not able to protect cells against PV (Fig. 3e). Vehicle control (final concentration of DMSO 1 %) was also carried out, and no interfer-

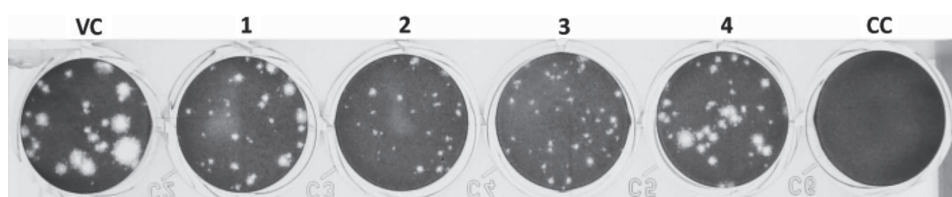


Fig. 2. The size of plaques in the cell monolayer produced in the virus control (VC) compared with cells infected with PV and treated for 48 h at 37 °C with different concentrations of the alkaloid 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide: 1 – 120, 2 – 24, 3 – 2.4, and 4 – 0.24 $\mu\text{mol L}^{-1}$. CC – cell control.

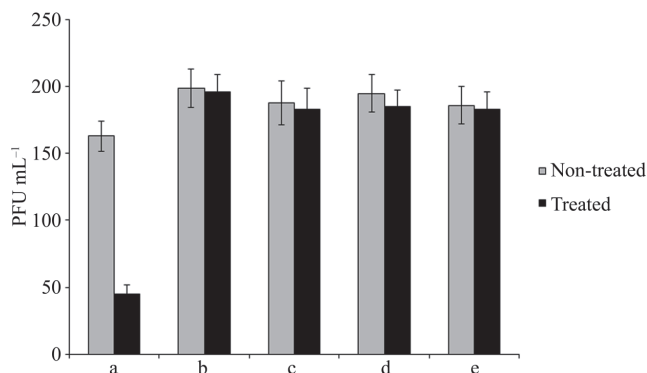


Fig. 3. Effects of the alkaloid 1-(phenyl)-*N'*-(4-methoxybenzylidene)-9*H*-pyrido[3,4-*b*]indole-3-carbohydrazide (PMC) at different stages of PV infection evaluated by the plaque reduction assay. The compound added (120 $\mu\text{mol L}^{-1}$) to Vero cells: a) in pretreatment assay (24 h at 37 °C), b) in attachment assay (1 h at 4 °C), c) during adsorption and penetration (1 h at 37 °C), and d) after infection (48 h at 37 °C), e) Neutralization assay was performed by incubating PMC with PV for 1 h at 37 °C prior to infection of the cell monolayer. The data are expressed as the mean \pm SD of three independent experiments. * $p < 0.05$; significant reduction of plaque forming units (PFU) (ANOVA followed by Tukey test).

ence in the virus activity or cell viability was observed (data not shown). Potential of the PMC to exert a prophylactic effect is particularly interesting considering that pre-exposure and post-exposure prophylactic applications are possible situations in which an anti-PV drug might be used (4).

CONCLUSIONS

We have observed that the anti-PV effect exerted by PMC appears to be attributable to an apparent ability of PMC to prevent the establishment of viral infection. However, the exact mechanism remains unknown and further studies are needed to elucidate how PMC could prevent PV proliferation at molecular level and the feasibility of its use *in vivo*. Moreover, other picornaviruses, especially human enteroviruses, could be susceptible to this compound.

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