

## New simplex Type 1 lytic infection pyridine system derivative as a novel goal against Herpes

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### \*Article History:

Received: 07/02/2017

Revised: 06/07/2017

Accepted: 08/07/2017

DOI: <https://doi.org/10.7439/ijpr.v7i7.3932>

### Abstract

The pyridine system derivatives have been studied as antiviral drug new models against HIV infection using bioisosterism as a powerful tool. In the last decades, the number of this work, thienopyridine derivatives were evaluated as an HSV-1 inhibitor to since the elevated number of acyclovir resistance in immunocompromised patients could promote a continuous viral pool increase and disease recurrence. In these substances, thienopyridine derivative with radical nitrate in para position (109) was able to inhibit 99% of HSV-1 replication in Vero cells with EC<sub>50</sub> value of 100 pM, and SI value three times higher than acyclovir. In kinetic enzymatic assay this substance was also able to inhibit about 50% of HSV-1 DNA polymerase but in a non-competitive mechanism a new synergic system to control herpes infections. Preliminary in vivo model studies revealed that substance 109 is toxic when administered in 300 µg/mL concentrations. In this work, we concluded that thienopyridine derivatives can be considered a promising new anti-HSV-1 drug and can be used for clinical testing.

**Keywords:** Prevalence, proton pump inhibitor, Acid Peptic disorder.

### 1. Introduction

HSV-1 infection represents a persistent human virus that resides in infected hosts for their lifetime mainly due to their ability to keep his genome in epigenetic form inside nuclear neurons [1]. The primary infection occurs during childhood and adolescence through direct oral exposure causing orolabial and facial lesions for symptomatic infection. Studies have shown that HSV-1 has become a major causative agent of genital herpes in some developed countries and that it could be responsible for viral transmission by asymptomatic persons [2].

Clinical manifestations severity may range from subclinical to those causing permanent damage and even death as in newborn encephalitis cases [3]. Indeed, the

severity of infection is dependent on immune status as elderly, immunocompromised and transplant patients [4]. Until today, no vaccine is effective to control latent manifestations and an effective vaccine must be multivalent and capable of inducing a multi-immune response, which means that we first have to deeply understand the immune mechanisms underlying human herpes pathogenesis.

The recently discovered model is a complex with synthetic peptide epitopes and agonist of Toll-like receptor 2 (TLR-2), which is abundantly expressed on vaginal and ocular mucosa dendritic and epithelial cells, and therefore can lead to protective immunity induction against herpes [3, 5]. Although the immunization schedule represents the best way to prevent diseases, it seems that for HSV infections

the antiviral drugs design is still strongly supported. Despite the effective action of anti-HSV-1 drugs, specially acyclovir, a prototype to viral DNA polymerase inhibition, we found various levels of toxicity and resistance in many drugs [6].

In immunocompetent patients developing recurrent keratitis, for example, HSV-1 strains revealed single guanine insertions that greatly reduce TK expression. The substitution, although distant from substrate binding sites, reduced acyclovir phosphorylation >100 fold. Indeed, patients infected with human immunodeficiency virus (HIV) showed 6% of HSV-1 resistance to acyclovir [7]. In such cases the only available alternative is foscarnet, but it has high toxicity and can only be administered intravenously.

As an alternative, a recently developed topical cream containing 5% acyclovir and 1% hydrocortisone (AHC) in a novel cream vehicle has shown to be safe and effective for the early treatment of recurrent labial herpes in immunocompetent adult and adolescent patients [8]. New helicase-primase (HPI) inhibitors research for successful drugs against HSV is promising, but there are limitations to overcome [9].

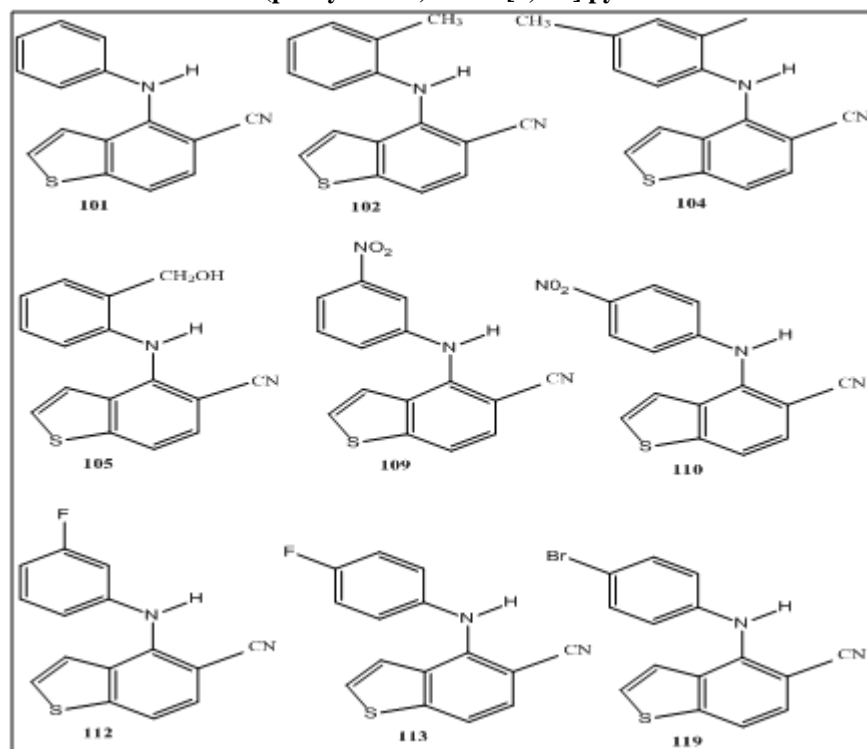
## 2. Materials and Methods

### 2.1 Virus, Cell line, Culture conditions and substances

The HSV-1 particles (AR-29, resistant to acyclovir) were diluted in Dulbecco's Minimum Essential Medium (DMEM) without serum, and propagated in Vero

cells, using a 0.1 multiplicity of infection (MOI) [10, 11]. After 24h post-infection (pi), cells will be lysed by freezing/thaw cycles. The content was centrifuged at 400x g for 20min at 4°C. The viral titer was determined by testing viral plaque reduction [12]. Vero cells from kidneys of African green monkeys (*Cercopithecusaethiops*) were grown in DMEM supplemented with 5% serum fetal bovine (SFB), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 2.5 µg/mL of amphotericin B at 37 ° C in an atmosphere of 5% CO<sub>2</sub>. The cells were treated with PBS-1X/EDTA (0,02%) and 0.25% trypsin (GIBCO) to achieve experimental conditions. All thienopyridine derivatives substances were diluted in 100% dimethyl sulfoxide (DMSO) and stored in concentrations of 50 mM at 4°C until the experimental conditions. The thienopyridine derivatives tested in this work were synthesized by nucleophilic reaction of 5-carboethoxy - 4- clathieno [2,3-b] pyridine with different anilines to produce 4-(phenylamino) thieno [2,3-b] pyridine -5- carbonitrile (**101**); 4-(2 - methyl-phenylamine) thieno [2,3-b] pyridine-5-carbonitrile (**102**); 4-(4 -methyl-phenylamine) thieno [2,3-b] pyridine-5-carbonitrile (**104**); 4-(2 -carboxy-phenylamino) thieno [2,3-b] pyridine-5carbonitrile (**105**); 4-(3 -nitro-phenylamine) thieno [2,3-b] pyridine -5carbonitrile (**109**); 4-(4 -nitro-phenylamino) thieno [2,3-b] pyridine-5carbonitrile (**110**); 4-(3 -fluoro-phenylamino) thieno[2,3-b] pyridine-5carbonitrile (**112**) and 4-(4 -fluoro-phenylamino) thieno [2,3-b] pyridine-5-carbonitrile (**113**) (Figure 1).

Figure 1: Structure formula of 4-(phenylamino) thieno[2,3-b] pyridines-5-carbonitriles derivatives



### 2.2 Viability assays -CC<sub>50</sub>

The viability assay was evaluated using mitochondrial salt reduction method (MTT;Sigma). It was performed with Vero cells cultivated in 96 multiwell plates (1x 10<sup>4</sup> cells) in the presence of 50, 100, 250, 500 and 1000 μM of the substances for 72 hours at 37 ° C in a 5% CO<sub>2</sub> atmosphere. Afterwards, 50 μL of a 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added for 4 hours at 37 ° C in a 5% CO<sub>2</sub> atmosphere, and 100 μL/well of dimethyl sulfoxide (DMSO) to further define the 50% of cytotoxic concentration (CC<sub>50</sub>) using a spectrophotometer (540 nm) [13]. The CC<sub>50</sub> values were calculated by linear regression analysis of the dose-response curves. All experiments were performed in triplicate at least three times.

### 2.3 Inhibition of viral production – EC<sub>50</sub>

To determine the concentration of the substances that inhibits 50% of viral production, monolayers of Vero cells in 24 multiwell plates (4 x 10<sup>4</sup> cells) were infected with HSV-1 (MOI = 1) for 1hour at 37 ° C in 5% CO<sub>2</sub> atmosphere. After incubation 50μM of 4-(phenylamino) thieno[2,3-*b*] pyridines-5-carbonitriles derivatives (101, 102, 104, 105, 109, 110, 112, 113 and 119) were incubated for 24hour at 37 ° C and 5% CO<sub>2</sub> and the supernatant was used to determine the number of plaques forming virus units (PFU/mL) compared to mock HSV-1 control.

### 2.4 Inhibition of HSV-1 DNA polymerase

To obtain viral DNA polymerase, Vero cells were first infected with HSV-1 (MOI=5) for 12 hours at 37 ° C in the presence of 5% CO<sub>2</sub>. After incubation a buffer containing 0.25 mM potassium phosphate (pH 7.2), 10mM 2-mercaptoethanol, 1mM EDTA, Triton-X-100, 20% de glycerol and 0.5mM phenylmethylsulfonyl fluoride (PMSF, Sigma) was added and the cells were sonicated in cycles of 15 seconds. The content was centrifuged at 2400g for 10 min at 4 ° C (Knopf, 1979). The enzymatic reaction was performed at 30 min at 37 ° C in a final volume of 100 μL using a buffer containing 0,1nM of 4-(phenylamino) thieno[2,3-*b*] pyridines-5-carbonitrile derivatives, 50 mM Tris-HCl (pH 8.0), 0.5mM Dithiothreitol (DTT), 8 mM MgCl<sub>2</sub>, 100 mM ammonium sulphate, 0.1 mM dNTP with [3H]-dTTP (0.5 μCi/nmol) and 25 μg of DNA from sperm salmon. After incubation, the reaction was stopped with 10% trichloroacetic (TCA) and the samples were filtered on glass filter GF/C (Whatman) previously washed with 10% TCA. The incorporation was monitored by scintillation liquid phase [14].

### 2.5 Inhibition of HSV-1 Protein Synthesis

Vero cells (2 x 10<sup>6</sup> cells) were infected with HSV-1 (MOI = 1) for 1hour at 37 ° C in a 5% of CO<sub>2</sub> atmosphere. Afterward, cells were washed and incubated for 4 hours with 50 μM of substance 109 and for 2 hours in

the presence [<sup>35</sup>S] methionine (50 μCi / mL) on the same conditions. The cells were lysed with 1M Tris-HCl buffer (pH 6.8) containing 0,02% of bromophenol blue, 5%-mercaptoethanol, 10% of SDS and 10% of glycerol. To determine the incorporation, samples were first stopped with 10% TCA and filtered in glass filters (Whatman) GF/A previously washed with 10% TCA [14].

## 3. Results

To evaluate the substances cytotoxicity, we used tetrazolium salt (MTT) to determine the concentration that inhibited 50% on the Vero cells viability (CC<sub>50</sub>). For thienopyridine derivatives, it was observed that all substances showed very similar CC<sub>50</sub> values, except for halogenated radical **101**, which shows higher value in the absence of phenylamino ring replacement with CC<sub>50</sub> > 300 μM (Table 1). The results were obtained by linear regression of measurements obtained from the average of five different concentration triplicates (50, 100, 250, 500, and 1000 μM).

**Table 1: Cytotoxicity values (CC<sub>50</sub>) of thienopyridine derivatives**

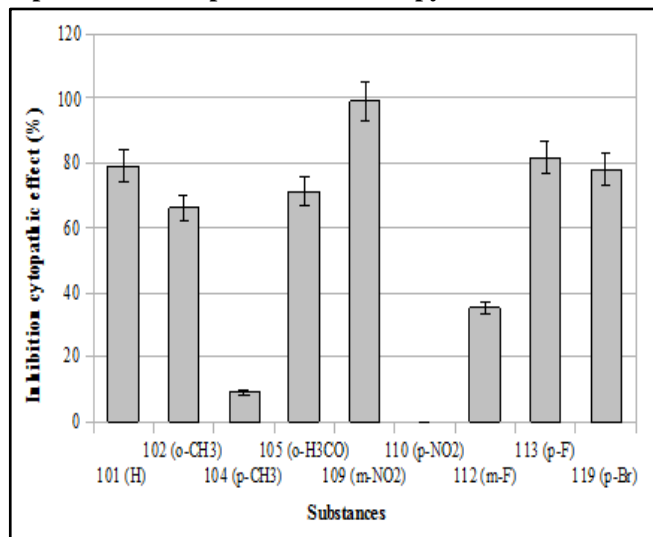
Thienopyridine derivatives	CC <sub>50</sub>
<b>101</b> (R = H)	> 300
<b>104</b> (R = p-CH <sub>3</sub> )	<300
<b>109</b> (R = m-NO <sub>2</sub> )	< 300
<b>110</b> (R = p-NO <sub>2</sub> )	<300
<b>112</b> (R = m-F)	< 300
<b>113</b> (R = p-F)	< 300
<b>119</b> (R = p-Br)	< 700

The mean values ± standard deviations are representative of three independent experiments. <sup>a</sup>Concentration that reduced 50% cytotoxic concentration when compared to untreated controls.

The presence of nitrate radical in meta position gives to substance **109** the most inhibition effect on the replication of HSV-1. This molecule showed a CC<sub>50</sub> value bellow 300 μM, while acyclovir, the most useful drug acting against HSV-1, is almost three times lower cytopathic (860 μM). On the other hand, the EC<sub>50</sub> of acyclovir (MOI = 1) is 1.2 μM and we found 100 pM at substance **109**. Calculating the selective index (SI), which

means how safe could be a substance for therapeutic utilization, we found  $3 \times 10^6$  for substance **109** and  $1 \times 10^3$  for acyclovir (Figure 2). These results ensure that thienopyridine derivative with nitrate radical is almost three logs more effective to inhibit HSV-1 than acyclovir, besides the  $CC_{50}$  value.

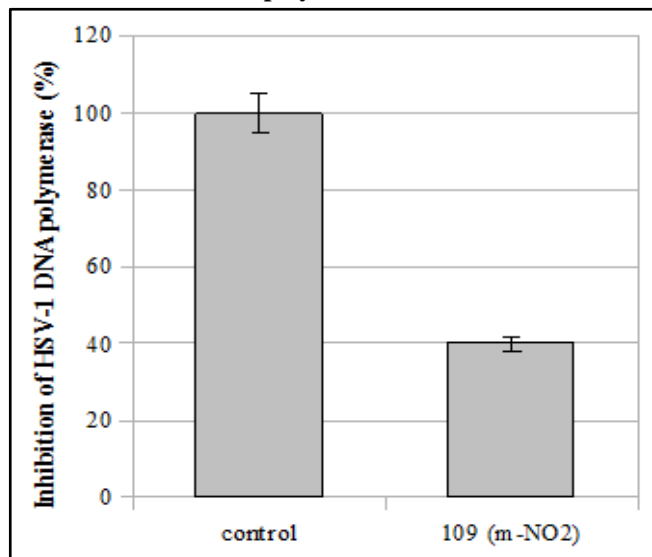
**Figure 2: Cytopathic effect inhibition to HSV-1 replication in the presence of thienopyridine derivatives.**



Error bars indicate the standard deviation and experiments were performed in triplicate.

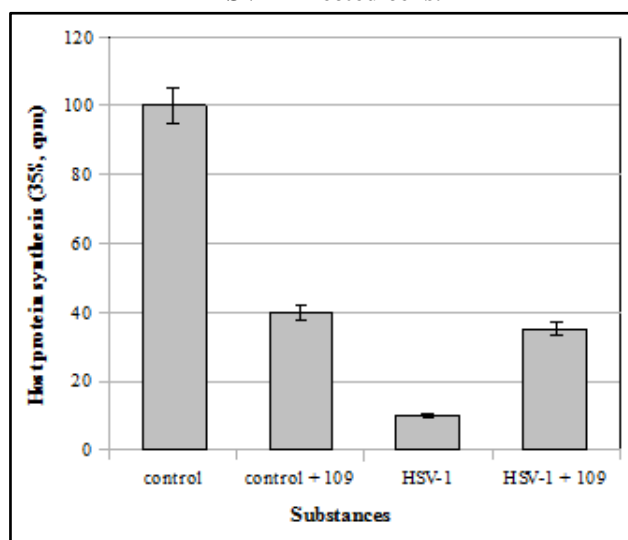
To avoid possible unspecified reactions, 100mM of ammonium sulfate was added. We could verify that the inhibition of the enzymatic activity was more than 40% in presence of the substance 109 (Figure 3).

**Figure 3: Effect of substance 109 on HSV-1 DNA polymerase**



To investigate if the promising molecule 109 could reverse the inhibition of Vero cell proteins, we used  $^{35}S$  methionine at several conditions: uninfected, uninfected with substance 109, infected, and infected at the presence of  $50\mu M$  of the substance 109. As we expected there was an inhibition of protein synthesis during the infection (Figure 4) but also in the in control groups.

**Figure 4: Effect of substance 109 on protein synthesis in HSV-1 infected cells.**



#### 4. Discussion

In contrast to antibacterial and antifungal drugs, antiviral drugs collection is extremely low and most those are used to treat HIV infections [6]. Bioisosterism consists of a rational way to obtain new molecules that may have biological activity, using a substance with a well-known structure and biological activity as model, and afterwards synthesizing and evaluating new structures analogues to already known drugs. Thus, the amino derivatives were synthesized using basic ribavirin, which is a broad spectrum antiviral nucleoside synthetic. The derivatives have an amino triazole, which is also present in ribavirin. Ribavirin has a triazole linked to ribose, mimicking a ribonucleoside. It is well documented that treatment of cells with ribavirin results in inhibition of Dehydrogenase Inosine Monophosphate (IMPDH), whereas RMP competes with IMP (the natural substrate the IMPDH), decreasing the level of intracellular GTP. Decreased amount of these nucleotides certainly interfere with viral replication, since viruses, as well as host cells, are dependent on GTP and dGTP for the replication of genetic material. So, it is likely that the inhibition of IMP dehydrogenase is responsible for ribavirin toxicity in human cells, and also for its broad-spectrum antiviral activity.

This nucleotide pool imbalance may cause viral polymerase to replace GTP by alternative nucleotides (ATP, UTP or CTP), but for the effectiveness inhibition of IMPDH as antiviral target, viral replication would have to be more sensitive to levels of intracellular GTP declines in cells hosts [15], as thienopyridine derivatives were also synthesized exploiting bioisosterism concept.

Structural characteristics were observed on two derivatives of the system pyrazolopyrimidine that exhibit antiviral activity [16] where one of them presented antiviral activity against the enzyme transcriptase reverse the HIV-1 and the other against the vaccinia virus [17]. Phenylamino group present in these molecules at the C-4 was maintained, and the pyrazole and thiophene rings were proposed by bioisosterism synthesis in some thienopyridine derivatives of 4 - (phenylamino) brominated thieno [2,3-b] pyridine-5-carbonitriles, which includes all the thienopyridine derivatives presented in this work. The thienopyridine system is also within substances that present antiviral activity like HSV-1 [18, 19] and HIV-1 [20]. Finally, use of bioisosterism as a tool to design new molecules is extremely promising and requires biological assays to verify if they could act as a potential antiviral. The essential conditions for any antiviral drug are efficacy and minimum toxicity in host cells to ensure his function as therapeutic agent [21, 22]. So we first evaluated the cytotoxicity effect of those substances in Vero cells. The cytotoxicity defined by Nardone [23] in 1977 represents several modifications in cells when submitted to different substances that alters their metabolic pathways and replication. The lesions intensity depends on the substance concentration, exposition time and cell type [24].

To evaluate if the presence of methyl, nitric oxide, fluorine and bromine radicals replacement in para and meta positions within tienopyridine structure could inhibit the replication of HSV-1, Vero cells were infected in the presence of 50  $\mu$ M of each of tienopyridine derivatives. We observed that viral inhibition effects varied greatly according to the radicals and their position in the molecules (Figure 1). For example, comparing the antiviral effect of substance 101 with nitro radical in para position with substance 109, with the same radical in meta position, we observed the inhibition of cytopathic effect ranging from zero to almost 100%, respectively. Nevertheless, this association was not shown with substances 112 and 113, which received fluorine radicals in meta and para positions, respectively. The presence of fluor radical in meta position reduced almost 60% the anti-HSV-1 effect showed when the same radical was in para position. We then concluded that the meta and para positions of radicals are not strictly dependent of these molecules biological activities.

## 5. Conclusions

The HSV-1 genome is able to produce most of the proteins necessary for virus replication and maturation, including DNA polymerase (UL30/UL42 complex) [25], which is also the main target in almost 90% of the commercial antiherpetic drugs [26]. For this reason and regarding the possible site of action of substance 109 in the viral DNA polymerase as acyclovir, we performed enzyme kinetics with DNA from salmon sperm. To avoid possible unspecified reactions, 100mM of ammonium sulfate was added. Our results robustly suggest that HSV-1 polymerase is the target of thienopyridine derivative with nitrate in meta position.

## Acknowledgments

This work was supported by Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ).

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