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Inhibition of HSV-1 replication and HSV DNA polymerase by the chloroxoquinolinic ribonucleoside 6-chloro-1,4-dihydro-4-oxo-1-(β-D-ribofuranosyl) quinoline-3-carboxylic acid and its aglycone

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Abstract

We describe in this paper that the synthetic chloroxoquinolinic ribonucleoside 6-chloro-1,4-dihydro-4-oxo-1-(β-D-ribofuranosyl) quinoline-3-carboxylic acid (compound **A**) and its free aglycogene base (compound **B**) inhibit, with low cytotoxicity, the replication of herpes simplex virus type 1 and 2 (HSV-1 and HSV-2). Compound **A** inhibited HSV-1 replication in Vero cells with an EC_{50} of 1.3 and 1.4 μM for an acyclovir (ACV)-sensitive strain and an ACV-resistant strain of this virus, respectively. Additionally, it inhibited HSV-2 replication with an EC_{50} of 1.1 μM. Compound **B** also inhibited the ACV-sensitive and -resistant HSV-1 strains, and HSV-2 at EC_{50} values of 1.7, 1.9 and 1.6 μM, respectively. Time-of-addition assays, performed with compound **A**, suggested that this molecule at an early time point of the HSV replication cycle. Kinetic assays demonstrated that compounds **A** and **B** inhibit the HSV DNA polymerase activity in a noncompetitive fashion, with a K_i equal to 0.1 and 0.2 μM, respectively. Taken together, our results suggest that compounds **A** and **B** represent promising lead molecules for further anti-HSV drug design.

Keywords: Herpes simplex virus type 1; HSV-1 DNA polymerase; Chloroxoquinolinic ribonucleoside; Inhibitor

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are lipid-enveloped DNA viruses whose replication has been well characterized (Boehmer and Lehman, 1997; Roizman and Sears, 1996; Whitley, 1996). Among the most important HSV proteins, the early enzymes thymidine kinase (TK) and HSV DNA polymerase play a key role during viral replication, and thus, drugs that target these proteins can impair the viral replication cycle (Hayden, 2001; Naesens and De Clercq, 2001).

Herpesvirus and cellular DNA polymerases are classified according to their sequences and functional homologies. The viral and several eukaryotic polymerases, including human α and δ polymerases, belong to the family of type B DNA polymerases (Braithwaite and Ito, 1993), since they share six to seven highly conserved domains, labeled I–VII, in decreasing order of conservation (Hwang et al., 1992). Additionally, herpesvirus DNA polymerases also share an additional region with human δ polymerase, the conserved domain C (Phillips, 2006; Grundy and Naylor, 1999). Therefore, the blockage of HSV DNA polymerase without inhibition of the cellular type B DNA

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polymerases is an important task.Among the antiviral drugs used in the treatment of HSV-infected individuals, acyclovir (ACV) and its L-valyl ester prodrug, valacyclovir, have been considered safe because they have been shown to cause minimal side effects. However, some ACV-resistant strains of HSV-1 have emerged, mainly from immunocompromised patients (Stránská et al., 2005). These strains also present cross-resistance with other antivirals currently in use (Morfin and Thouvenot, 2003). Furthermore, these drugs can lead to renal failure when administered at high intravenous doses (Johnson et al., 1994; Lyon et al., 2002) and, in some cases, they can lead to neurotoxicity (Johnson et al., 1994; Ernst and Franey, 1998). Other antiviral molecules that are in use clinically, such as penciclovir and its diacetyl ester prodrug famciclovir, are less potent at inhibiting HSV-1 DNA polymerase (Hayden, 2001). Moreover, ganciclovir is associated with neurotoxicity (Young, 2005), bone marrow suppression (Liu et al., 2004), and carcinogenicity (Wutzler and Thust, 2001). Foscarnet and other phosphonoformate (PFA) analogs are associated with renal failure (Zanetta et al., 1999). These drugs when administered for long periods (Hwang et al., 1992) can also lead to the emergence of mutant human DNA polymerases that are less efficient in their activity. Altogether, these observations motivate the search for novel anti-herpesvirus molecules with low cytotoxicity and high selectivity.

Nucleoside analogs are a fruitful source of antiviral molecules, and these derivatives often inhibit viral polymerases such as HSV DNA polymerase competitively (Naesens and De Clercq, 2001). In parallel, it has been shown that quinolinic acid derivatives are endowed with anti-herpetic activity due to their noncompetitive mechanism of action towards the enzyme HSV-1 DNA polymerase (Wathen, 2002). As we have synthesized novel oxoquinolinic ribonucleosides (da Matta et al., 1996, 1999), including the 6-chloro-1,4-dihydro-4-oxo-1-(β-D-ribofuranosyl) quinoline-3-carboxylic acid denominated compound **A** (Fig. 1) and its free base 6-chloro-1,4-dihydro-4-oxo-1H-quinoline-3-carboxylic acid, henceforward named compound **B** (Fig. 1), we investigated their ability to inhibit HSV replication in Vero cells. We found that compounds **A** and **B** inhibited the HSV DNA polymerase activity in a non-competitive fashion and thus impaired both HSV-1 and HSV-2 replication.

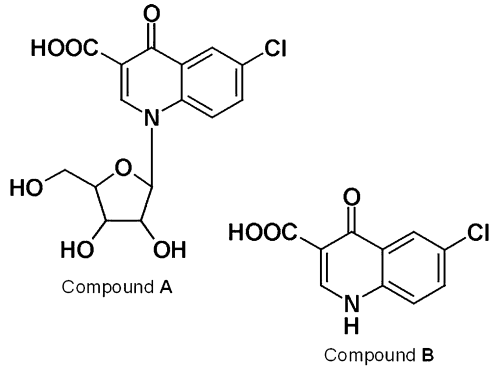

Fig. 1. The molecular structure of the chloroxoquinolinic derivative compound **A** (6-chloro-1,4-dihydro-4-oxo-1-(β-D-ribofuranosyl) quinoline-3-carboxylic acid) and compound **B** (6-chloro-1,4-dihydro-4-oxo-1H-quinoline-3-carboxylic acid).

2. Materials and methods

2.1. Reagents

The compounds 6-chloro-1,4-dihydro-4-oxo-1-(β-D-ribofuranosyl) quinoline-3-carboxylic acid and 6-chloro-1,4-dihydro-4-oxo-1H-quinoline-3-carboxylic acid (compound **A** and **B**, respectively; Fig. 1) were synthesized as previously described (da Matta et al., 1996, 1999). The HSV-1 inhibitor acyclovir (ACV), acyclovir triphosphate (ACV-TP) and the DNA polymerase inhibitor aphidicolin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while the reverse transcriptase inhibitor 3′-azido-3′-deoxythymidine 5′-triphosphate (AZT-TP) was from Sierra Bioresearch (Tucson, AZ, USA). All inhibitors were dissolved in 100% dimethylsulfoxide (DMSO) and they were diluted in culture medium 10^4-fold during every assay, so that the final DMSO concentrations showed no cytotoxicity. Materials for cell culture were purchased from Gibco (Grand Island, NY, USA), unless otherwise mentioned.

2.2. Cells and viruses

Vero cells (African green monkey kidney cells; ATCC, Hanassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in 5% CO_2. In order to prepare virus stocks, Vero cells were infected with HSV-1 [AR-29 (Lagrota et al., 1994), KOS (Andrighetti-Fröhner et al., 2005) strain] or HSV-2 (strain VR734; ATCC, Manassas, VA, USA) at a multiplicity of infection (MOI) equal to 0.1 for 1 h at 37 °C. Next, residual viruses were washed out with phosphate-buffered saline (PBS) and cells were cultured for an additional 48 h. After this period, cells were lysed by three cycles of freezing and thawing, centrifuged at 1500 × *g* at 4 °C for 20 min to remove cellular debris, and the supernatant was collected, titered by plaque assay and stored at −70 °C for further studies.

2.3. Cytotoxicity assay

Monolayers of 10^4 Vero cells in 96-multiwell plates were treated with various concentrations of compound **A** or **B** for 72 h and then 50 μL of a 1 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was dissolved in DMEM without serum and was added to the cell culture. MTT was removed after 3 h, 50 μL of acid–isopropanol (0.04N HCl in isopropanol) was added and the optical density (OD) was read using an automatic plate reader with a 570 nm test wavelength and a 690 nm reference wavelength (Mosmann, 1983; Denizot and Lang, 1986). The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression analysis of the dose–response curves generated from the data.

2.4. Plaque assay

Monolayers of Vero cells in six-well plates were exposed to different dilutions of the supernatant from yield-reduction assays for 1 h at 37 °C. Next, cells were washed with PBS and DMEM containing 5% FBS and 1% methylcellulose (Fluka) (overlay medium) was added to cells. After 72 h at 37 °C, the monolayers were fixed with 10% formaldehyde in PBS and stained with a 0.1% solution of crystal violet in 70% methanol, and the virus titers were calculated by scoring the plaque-forming units (PFU).

Additionally, other experiments were performed in the format of a plaque-reduction assay. In such a case various concentrations of the compounds were added in the overlay medium and, after 72 h, cells were fixed and plaques counted.

2.5. Yield-reduction assay

Monolayers of Vero cells (10^5) in 24-well plates were infected with HSV-1 (AR-29 or KOS strain) or HSV-2 (VR734 strain) at an MOI equal to 5 for 1 h at 37 °C. Cells were washed with PBS to remove residual viruses and various concentrations of the compound **A** or **B** in DMEM with 2% FBS were added. After 20 h, cells were lysed, cellular debris was cleared by centrifugation, and virus titers in the supernatant were determined by the plaque-forming assay using Vero cells, as described in the previous item. For comparison, linear regression of the dose-response curves for ACV was also performed to calculate EC_{50} values. In addition, to determine the EC_{50} values for compound **A** towards different virus inputs, we also performed experiments in which Vero cells were exposed to different MOIs of HSV-1 (KOS strain) and exposed to increasing concentrations of this compound, as described above.

2.6. Time-of-addition experiments

In order to analyze whether addition of compound **A** could be delayed without loss of its ability to block HSV-1 replication, and to get insight about which step of the HSV-1 replication cycle might be affected, we performed time-of-addition assays. Vero cells grown to confluence in six-well plates were infected with 500 PFU/well of HSV-1 (KOS strain) for 1 h, washed, and compound **A** was added at 3 μM (2× EC_{50}) at different times post-infection (0, 2, 4, 6, 8, 10, 16, 18, 20 h) (Gong et al., 2002). Whenever compound **A** was added, cells were also covered with overlay medium, and after 72 h (p.i.) viral plaques were counted. For comparison, assays using ACV at 2.5 μM (2× EC_{50}) were also performed.

2.7. Immunoblotting

To investigate whether compound **A** affects the synthesis of an important protein of the IE phase, we performed immunoblotting assays against a marker of this phase (Smith et al., 2005), the infected cell protein 27 (ICP27), as previously described (Kuo et al., 2001). In brief, Vero cells (10^6 per well, six-well plates) were infected with HSV-1 (KOS strain) at an MOI equal to 5 for 1 h at 37 °C. Cells were washed and treated for 20 h with compound **A** (3 μM) or ACV (2.5 μM). Next, cellular proteins were extracted using buffer A (bromophenol blue, 0.2%; β-mercaptoethanol 0.5%; Tris–HCl, pH 6.8, 1 M; SDS, 10%; glycerol, 1%) and 20 μg of the extracted proteins, or a series of two-fold dilutions of such a material was separated on a 9% acrylamide SDS-PAGE gel. Then, proteins were transferred to nitrocellulose filters. Filters were blocked and incubated with rabbit polyclonal anti-ICP27 and anti-Actin antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h. Specific reactive proteins were detected by the enhanced chemiluminescence method employing a goat anti-rabbit immunoglobulin antibody linked to horseradish peroxidase (Santa Cruz Biotechnology Inc.).

2.8. Preparation of cellular and HSV-1 DNA polymerases

HSV-1 DNA polymerase was partially purified from HSV-1-infected Vero cells (Knopf, 1979). In brief, cells were infected with HSV-1 (KOS strain) at a MOI of 5 for 12 h, lysed with buffer B (0.25 M potassium phosphate (pH 7.5), 10 mM 2-mercaptoethanol (2-ME), 1 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethane sulfonylfluoride (PMSF) and 20% glycerol), sonicated and centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatant was further centrifuged at 100,000 × *g* for 90 min at 4 °C and the supernatant was dialyzed against 10 mM potassium phosphate (pH 7.5) with 10 mM 2-ME, 1 mM EDTA and 20% glycerol. Subsequently, the resulting material was eluted in two ion-exchange chromatography columns (DEAE-cellulose and phosphate-cellulose) with a linear gradient of 0.02–0.5 mM potassium phosphate. We also partially purified one of the cellular counterparts of HSV-1 DNA polymerase, the α-DNA polymerase. For this purpose, we used the same process described above with minor changes; we lysed uninfected Vero cells and used three DEAE-cellulose ion-exchange chromatography columns.

In order to analyze how pure our DNA polymerase preparations were (both HSV and α), we performed SDS-PAGE electrophoresis of our samples followed by silver-staining or immunoblotting. In the case of HSV DNA polymerase, we looked for type B DNA polymerase contamination (α and δ polymerases) using polyclonal antibodies raised against these cellular enzymes (Abcam Inc., Cambridge, MA, USA). In the case of α DNA polymerase, we looked for δ polymerase contamination, since this preparation was performed with mock-infected Vero cells. Additionally, the absence of signal, by either silver-staining or immunoblotting, was confirmed by kinetic assays. The HSV DNA polymerase kinetic assay was performed in the presence and absence of ammonium sulfate, according to Nishiyama et al. (1982) protocol and we found no DNA polymerase contamination. The confirmation of the purity of our α DNA polymerase preparations, by kinetic assays, was also analyzed according to Nishiyama's protocol. In this last case, such a preparation was incubated in reaction mixtures to detect the activity of other cellular DNA polymerases, such as δ and β (Nishiyama et al., 1982), among others. Again, we found no contamination.

2.9. DNA polymerase inhibition assay

HSV-1 DNA polymerase and α-DNA polymerase inhibition assays were carried out under conditions appropriate for first-order kinetics. The reaction mixture for HSV-1 DNA polymerase experiments was composed of 50 mM Tris–HCl (pH 8.0), 8 mM $MgCl_2$, 0.5 mM DTT, 0.5 μg/mL bovine serum albumin, 100 mM ammonium sulfate (as an inhibitor of possible contaminant cellular polymerases), 100 μM of each nucleotide (dATP, dGTP, dCTP and [^{3}H]-dTTP—0.5 μCi/nmol), 12 μg/mL activated salmon-sperm DNA and 3 U/mL of HSV-1 DNA polymerase. The reaction mixture for α-DNA polymerase experiments was composed of 40 mM potassium phosphate (pH 7.2), 8 mM $MgCl_2$, 100 μM of each nucleotide (dATP, dGTP, dCTP and [^{3}H]-dTTP—0.5 μCi/nmol), 12.5 μg/mL activated salmon-sperm DNA and 3 U/mL of α-DNA polymerase. We defined 1 U as the amount of enzyme that incorporates 1 pmol of dTTP per minute at 37 °C under standard assay conditions. The reaction mixtures were incubated with the test compound at 37 °C for 30 min, and quenched by addition of 10% trichloroacetic acid (TCA). The resulting radioactive DNAs were adsorbed onto GF/C fiberglass membranes (Whatman) and counted by liquid scintillation (Tri Carb 2600, Packard Inc.).

Dixon plots were used to determine the K_i, using 5 μM of dTTP as a substrate, values for compound **A**, **B** and other inhibitors. A Lineweaver–Burk plot was used to analyze the kinetic effects of compound **A** with respect to dTTP incorporation (K_m and V_{max}). The same assays were also performed with the reference compounds aphidicolin and ACV-TP, and AZT-TP as a negative control. Whenever we used ACV-TP, assays contained [^{3}H]-dGTP at 5 μM instead of [^{3}H]-dTTP.

3. Results

3.1. Effects of compounds *A* and *B* on cellular viability

In order to evaluate the cytotoxicity of compounds **A** and **B** towards Vero cells, they were treated with various concentrations of compounds **A** or **B** and after 72 h cell viability was measured by MTT (Table 1). The CC_{50} for compounds **A** and **B** were 1500 ± 123 μM (means ± S.E.M.) and 870 ± 150 μM, respectively, while the CC_{50} for ACV was 860 ± 58 μM, indicating that compound **A** is less toxic than ACV and compound **B** for in vitro usage. It is important to comment that by analyzing the compounds' cytotoxicity by Trypan blue dye exclusion assay, we found similar CC_{50} values (data not shown). All other assays with compounds **A** and **B** were performed using concentrations much lower than its CC_{50}.

3.2. Antiviral activity of the compounds *A* and *B*

Initially, the effect of compound **A** on HSV-1 replication in Vero cells was examined by a yield reduction assay using two viral isolates, KOS and AR-29, an ACV-sensitive strain and an ACV-resistant strain, respectively. In Table 1 we can see that compound **A** inhibited replication of both HSV-1 strains, with EC_{50} values of 1.3 ± 0.23 and 1.4 ± 0.16 μM for KOS and AR-29 strains, respectively. This finding indicates that these two strains are equally sensitive to our compound. Based on the ratio of CC_{50} to EC_{50} we calculated the selectivity index (SI) values, which were equal to 1153 and 1071 for KOS and AR-29 strains, respectively (Table 1). The anti-herpetic activity of compound **A** was not restricted to HSV-1, since HSV-2 replication was also impaired by this molecule with an EC_{50} of 1.1 ± 0.2 μM and a SI of 1363 (Table 1).

Moreover, the chloroxoquinolinic point of compound **A**, namely compound **B**, also inhibited HSV-1 (both KOS and AR-29 strains) and HSV-2 (Table 1) with EC_{50} values of 1.7 ± 0.2, 1.9 ± 0.1 and 1.6 ± 0.08 μM, respectively (Table 1). Since this small difference is almost negligible, the chloroxoquinolinic ring may be considered the structural part that is responsible for the observed anti-herpetic activity, as it has been proposed by others (Wathen, 2002). Interestingly, the major difference between compounds **A** and **B** rely on the SI values, which may result from the higher cytotoxicity of the latter compound when compared to the former.

For comparison, the effect of the reference compound ACV was also studied under our assay conditions. We observed (Table 1) that compound **A** and ACV gave similar results, regarding the inhibition of HSV-1 (KOS strain) and HSV-2, since the ACV EC_{50} values were 0.99 ± 0.04 μM (SI of 868) and 1.4 ± 0.1 μM (SI of 620), respectively. Remarkably, compound **A** was approximately four-fold more potent than ACV in inhibiting the AR-29 strain of HSV-1, which showed some degree of resistance to the reference compound (Table 1) (Lagrota et al., 1994).

Table 1
Cytotoxicity and anti-herpetic activity of chloroxoquinolic acid analogs

Drugs	EC_{50} (μM)[a]			
	CC_{50} (μM)[b]	HSV-1 (KOS)	HSV-1 (AR29)	HSV-2
Compound **A**	1500 ± 123	1.3 ± 0.23[c] (1153)	1.4 ± 0.16 (1071)	1.1 ± 0.2 (1363)
Compound **B**	870 ± 150	1.7 ± 0.2 (511)	1.9 ± 0.1 (457)	1.6 ± 0.08 (543)
ACV	860 ± 58	0.99 ± 0.04 (868)	5.8 ± 0.3 (149)	1.4 ± 0.1 (620)

[a] HSV-infected Vero cells were treated with different concentrations of compounds **A**, **B** or ACV for 20 h. After this period cells were lysed and virus titers were determined by plaque-forming assay.

[b] Uninfected Vero cells were treated with different concentrations of compounds **A**, **B** or ACV for 72 h and than cell viability was measured by MTT.

[c] The parentheses represent the selective index which is calculated by the ratio of CC_{50} to EC_{50}.

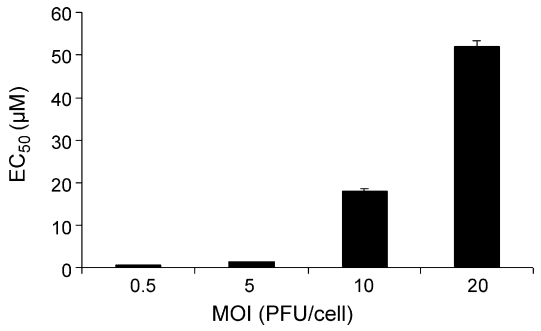

Fig. 2. Effect of compound **A** on HSV-1 replication. Vero cells were infected with HSV-1 (KOS strain) at indicated MOIs, exposed to various concentrations of compound **A**, and viral replication was measured by plaque-forming assay after 20 h of infection. Data represent means ± S.E.M. of three independent experiments.

Additionally, we compared the yield reduction assay results with those obtained with plaque-reduction assays. We observed similar findings, since in this last EC_{50} values of compounds **A**, **B** and ACV for inhibiting the KOS strain of HSV-1 were 1.5 ± 0.05, 2.2 ± 0.04 and 1.3 ± 0.01 μM, respectively. Moreover, inhibition of the AR-29 strain was achieved with EC_{50} values of 2.0 ± 0.07 μM (compound **A**), 2.6 ± 0.05 μM (compound **B**) and 6.5 ± 0.03 μM (ACV). Finally, the EC_{50} values of the above mentioned compounds for HSV-2 were 2.1 ± 0.09 μM (compound **A**), 1.9 ± 0.05 μM (compound **B**) and 1.5 ± 0.04 μM (ACV). Those EC_{50} values are the means ± S.E.M. of three independent experiments performed in duplicates.

Since compound **A** presented lower SI values than compound **B**, we next determined whether the former molecule was able to inhibit different inputs of HSV-1 (KOS strain). We found an MOI-dependent inhibition of HSV-1 replication produced by compound **A** (Fig. 2).

*3.3. Inhibition of HSV-1 replication by compound **A** in function of time of addition*

In order to analyze whether the addition of compound **A** could be delayed without losing its ability to block HSV-1 replication, we performed time-of-addition assays, adding compound **A** to HSV-1-infected Vero cells at different time points after infection (Gong et al., 2002). We found that the anti-herpetic activity of compound **A** was preserved when added to infected cells up to 6 h after infection, declining thereafter (Fig. 3). These results demonstrate that both test and reference compounds inhibit HSV-1 replication in a time-dependent manner and that their profiles of inhibition overlap. Therefore, compound **A** might inhibit HSV-1 replication, similar to ACV, at an early stage of the viral life cycle.

*3.4. Compound **A** did not impair ICP27 expression*

As compound **A** inhibits the beginning of HSV-1 replication, we investigated its ability in inhibiting the synthesis of a key regulatory protein of the IE phase, the ICP27 (Smith et al., 2005). We found that compound **A** caused no inhibition of

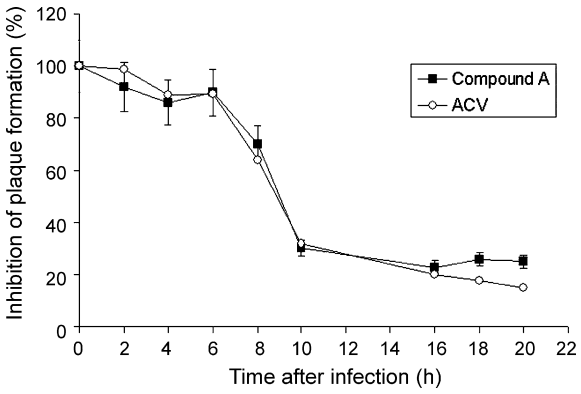

Fig. 3. Time-of-addition assay. Vero cells were infected with 500 PFU/well (MOI of ∼0.005) of HSV-1 (KOS strain) and compound **A** (3 μM) or ACV (2.5 μM) was added at different times after infection, as indicated. HSV-1 replication was measured after 72 h by plaque assay. Data represent means ± S.E.M. of three independent experiments.

the synthesis of this protein (Fig. 4A), with expression levels being similar to HSV-1-infected cells treated or not with ACV (Fig. 4B), suggesting that the antiviral activity of compound **A** could not be explained by the inhibition of this protein's synthesis.

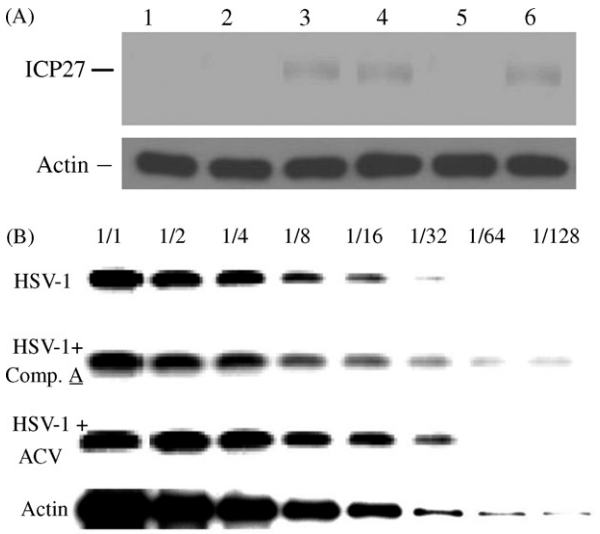

Fig. 4. Compound **A** does not inhibit ICP27 synthesis. Vero cells were infected with HSV-1 and treated with compound **A** (3 μM) or ACV (2.5 μM). After 20 h, cells were lysed and immunoblotting assays were performed against ICP27 and Actin. In panel (A): (1) mock-infected Vero cells, (2) mock-infected Vero cells treated with compound **A**, (3) HSV-1-infected Vero cells, (4) HSV-1-infected Vero cells treated with compound **A**, (5) mock-infected Vero cells treated with ACV and (6) HSV-1-infected Vero cells treated with ACV. The gel displayed in this panel is a representative of three independent experiments. In panel (B) two-fold dilutions of the protein extracts derived from HSV-1-infected Vero cells treated or not with compound **A** or ACV were also immunoblotted for ICP27 and Actin detection. The figure shown in panel (B) is also representative of three independent experiments. The difference found between the groups presented in this panel is not significant, since this variation happened between the analyzed groups from each replicate. As judged by densitometry analysis (EagleSight software 3.21; Stratagene, La Jolla, CA, USA), the band displayed in the Actin blot (dilution 1/128) is thicker than the ICP27 band (dilution 1/128) of the HSV-1 + compound **A** blot.

Table 2
Inhibitory activity of chloroxoquinolinic acid analogs and other molecules on HSV-1 and α-DNA polymerase activity

Inhibitors	K_i [μM]	
	HSV-1 polymerase	α-DNA polymerase
Compound **A**	0.1 ± 0.03	6.0 ± 0.30
Compound **B**	0.2 ± 0.02	6.0 ± 0.50
ACV-TP	0.4 ± 0.04	8.0 ± 1.00
Aphidicolin	0.3 ± 0.02	2.6 ± 0.80
AZT-TP	>21	>21

HSV-1 and α-DNA polymerase were partially purified from infected and uninfected Vero cells, respectively. Polymerase reactions were carried out for 30 min with various concentrations of the indicated compounds according to Section 2. Data represent means ± S.E.M. of five independent experiments. K_i values were calculated from Dixon plots.

3.5. Compounds **A** and **B** inhibit HSV-1 DNA polymerase activity

We performed a cell-free based assay to evaluate whether the nucleoside analog (compound **A**) and its free aglycone base (compound **B**) could inhibit the HSV-1 DNA polymerase (Boehmer and Lehman, 1997; Roizman and Sears, 1996). We observed (Table 2) that the K_i for compound **A** against HSV-1 DNA polymerase was approximately four- and three-fold lower than that observed for the clinically available derivative ACV-TP and for the classical type B polymerase inhibitor aphidicolin, respectively. On the other hand, AZT-TP, which is a clinically available derivative for the inhibition of HIV-1 replication, failed to show any anti-herpetic activity. Compound **B** was 2- and 1.5-fold more potent than ACV and aphidicolin in inhibiting HSV-1 DNA polymerase, respectively (Table 2).

Although compound **A** is a nucleoside analog, it inhibited the in vitro activity HSV-1 DNA polymerase without prior phosphorylation. Therefore, its mechanism of action may differ from that of ACV, since this reference compound needs to be phosphorylated to its triphosphate form in order to target the HSV-1 DNA polymerase (Morfin and Thouvenot, 2003).

3.6. Compound **A** is a noncompetitive inhibitor of HSV-1 DNA polymerase

We next investigated the kinetic effects of compound **A** on the HSV-1 DNA polymerase with respect to dTTP incorporation, and found that the test molecule was a noncompetitive inhibitor (Fig. 5A and Table 3), since it reduced values of V_{max} without affecting the K_m. This kinetic profile of inhibition is consistent with the mode of action of a compound that does not need a prior phosphorylation step to be active. For comparison, we analyzed the effect of aphidicolin on HSV-1 DNA polymerase activity (Fig. 5B) and found, as expected, that this reference compound is a competitive inhibitor of this enzyme. Therefore, the results displayed in Fig. 5A suggest that compound **A** inhibits the enzyme HSV-1 DNA polymerase with a peculiar mode of inhibition (Wathen, 2002) – different from that observed for classical anti-herpetic agents, such as ACV-TP – and probably independently of interactions with the dNTP binding site.

Table 3
Effect of compound **A** in kinetic parameters of HSV-1 DNA polymerase

Additions	K_m (μM)	V_m (fmol/min)
None	1.10 ± 0.07	105.09 ± 1.80
Compound **A**, 0.1 μM	1.20 ± 0.09	73.04 ± 1.32

The kinetic parameters were calculated from the Lineweaver–Burk plots in Fig. 5, in which we used curves (●) and (◯) to obtain the K_m and V_{max} values without and with compound **A**, respectively.

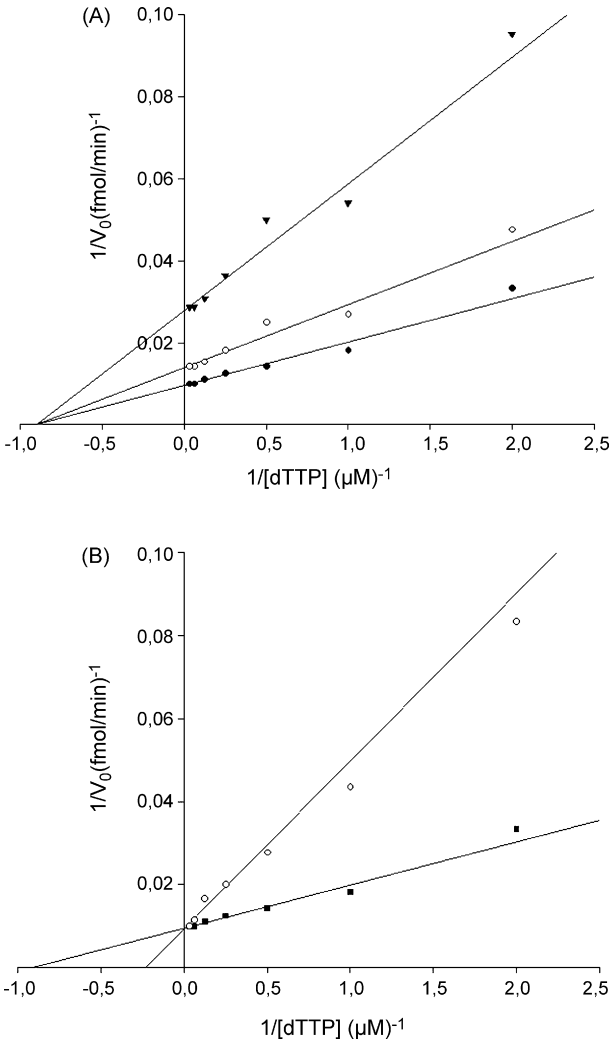

Fig. 5. Effect of compound **A** on HSV-1 DNA polymerase kinetics. Partially purified HSV-1 DNA polymerase was incubated with zero (●), 0.1 μM (◯) or 0.5 μM (▼) of compound **A** (A) or 0.3 μM (■) of aphidicolin (B), a known competitive inhibitor of the HSV-1 DNA polymerase activity with respect to dTTP incorporation. These reactions were assayed for 30 min according to Section 2. Data represent means of five independent experiments and the Lineweaver–Burk plot was generated using SigmaPlot 8.0 (Jandel Scientific).

4. Discussion

Several authors have reported the properties of synthetic compounds as anti-herpetic agents (Albin et al., 1997), including quinolinic molecules (Oien et al., 2002). Importantly, it has been shown that the presence of a chloride radical in the chem-

ical structure of quinolinic derivatives generally improves their antiviral activity (Savarino et al., 2003). We have thus investigated the anti-HSV properties of compound **A**, which is an oxoquinolinic ribonucleoside possessing a chloride radical at carbon 6 and a carboxylic acid group at carbon 3 of the quinolinic moiety and compound **B**, which represents the free base of compound **A**. Here, we demonstrate that chloroxoquinolic **A** and **B** can inhibit HSV-1 and HSV-2 replication, with low cytotoxic effects on the host cell, by impairing the viral DNA polymerase activity in a noncompetitive fashion, which raises the hypothesis of the usage of compound **A** with competitive inhibitors of HSV replication, such as ACV, in combinatory treatments. In addition, another advantage of compound **A** was its ability to inhibit the replication of an ACV-resistance strain of HSV-1.

Time-of-addition assays, immunoblotting and the protein profile of HSV-1-infected Vero cells treated with compound **A** (data not shown) suggest that this molecule acts at an early stage of HSV-1 replication, after viral entry. This stage is probably the E-phase, since the time-course of inhibition was similar to that observed for other E-phase inhibitors, such as ACV and PFA (Snoeck, 2000). Accordingly, we found that the E-protein inhibited by compounds **A** and **B** was the HSV DNA polymerase, which is targeted by almost 90% of the anti-herpetic agents currently in use (Naesens and De Clercq, 2001), and in our model viral replication and enzyme activity was inhibited with a good correlation between EC_{50} and K_i values. Since our nucleoside (compound **A**) and its free base (compound **B**) inhibited HSV-1 DNA polymerase activity, we conclude that the principal component responsible for the observed inhibition is the chloroxoquinolinic acid ring, which is in agreement with studies by Wathen (2002). Additionally, differences in the SI values of these compounds might indicate that chemical modifications may modulate their pharmacological behavior (Barbosa et al., 2004).

Therapeutic agents used during treatment of HSV-1-infected individuals include nucleoside analogs, which are generally prodrugs that must be converted to their triphosphate forms to become active and target viral polymerases, such as ACV that has to be monophosphorylated by HSV TK, and then converted to its di- and triphosphate forms by cellular kinases (Morfin and Thouvenot, 2003). However, mutations in either the HSV DNA polymerase, in the TK or the absence of this kinase may lead to viral resistance (Morfin and Thouvenot, 2003). As compound **A** is a noncompetitive inhibitor of HSV DNA polymerase that does not require a prior conversion to its triphosphate form, and taking into account that this molecule inhibits the replication of the AR-29 strain of HSV-1, we might conclude that our compound inhibits ACV-resistant strains of HSV-1.

Importantly, the kinetic nature of inhibition promoted by compound **A** differs from that observed for ACV-TP, since our molecule inhibited HSV DNA polymerase activity in a noncompetitive fashion. This suggests that the effect of compound **A** on the HSV DNA polymerase is independent of prior binding of the substrate (dTTP) to this enzyme, as judged by K_m values (Auwerx et al., 2004). Thus, only the remaining enzymes, which are unaffected by the inhibitor, can catalyze the reaction, lowering the V_{max} (Segel, 1993).

Differently from ACV-TP, PFA inhibits the HSV DNA polymerase in a noncompetitive manner (Naesens and De Clercq, 2001); however, its side effects, such as renal failure are dramatic (Zanetta et al., 1999). Compounds **A**, **B** and PFA probably bind to different parts of the HSV DNA polymerase because mutations in the enzyme UL30 that lead to the emergence of PFA-resistant strains of HSV-1 occur on residues that are not required for oxoquinolinic docking (Saijo et al., 2005; Liu et al., 2006). Nevertheless, to test this hypothesis, we are performing assays with a panel of PFA-resistant strains of HSV-1 and making several passages of this virus in the presence of compound **A**.

Finally, based on the present findings, we believe that compound **A** represents a promising molecule for future anti-HSV-1 drug design. The unique chemical structure of compound **A** encourages additional studies, such as the mapping of UL30 gene of compound **A**-resistant strains of HSV-1, as well as chemical modifications of the compounds (Barbosa et al., 2004).

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References

Albin, R., Chase, R., Risano, C., Lieberman, M., Ferrari, E., Skelton, A., Buontempo, P., Cox, S., DeMartino, J., Wright-Minogue, J., Jirau-Lucca, G., Kelly, J., Afonso, A., Kwong, A.D., Rozhon, E.J., O'Connell, J.F., 1997. SCH 43478 and analogs: in vitro activity and in vivo efficacy of novel agents for herpesvirus type 2. Antiviral Res. 35, 139–146.

Andrighetti-Fröhner, C.R., Sincero, T.C., da Silva, A.C., Savi, L.A., Gaido, C.M., Bettega, J.M., Mancini, M., de Almeida, M.T., Barbosa, R.A., Farias, M.R., Barardi, C.R., Simões, C.M., 2005. Antiviral evaluation of plants from Brazilian Atlantic Tropical Forest. Fitoterapia 76, 374–378.

Auwerx, J., Stevens, M., Van Rompay, A.R., Bird, L., Ren, E., De Clercq, J., Öberg, E., Stammers, B., Karlsson, D.K., Balzarini, A.J., 2004. The phenylmethylthiazolylthiourea nonnucleoside reverse transcriptase (RT) inhibitor MSK-076 selects for a resistance mutation in the active site of human immunodeficiency virus type 2 RT. J. Virol. 78, 7427–7437.

Barbosa, J.P., Pereira, R.C., Abrantes, J.L., Cirne dos Santos, C.C., Rebello, M.A., Frugulhetti, I.C., Texeira, V.L., 2004. In vitro antiviral diterpenes from the Brazilian brown alga *Dictyota pfaffii*. Planta Med. 70, 856–860.

Boehmer, P.E., Lehman, I.R., 1997. Herpes simplex virus DNA replication. Annu. Rev. Biochem. 66, 347–384.

Braithwaite, D.K., Ito, J., 1993. Compilation, alignment, and phylogenetic relationships of DNA polymerases. Nucleic Acid Res. 21, 787–802.

da Matta, A.D., Bernardino, A.M.R., Romeiro, G.A., de Oliveira, M.R.P., De Souza, M.C.B.V., Ferreira, V.F., 1996. Nucleosides having quinolone derivatives as nitrogenated base: regiospecific and stereospecific ribosylation of 3-carbethoxy-1,4-dihydro-4-oxoquinolines. Nucleosides Nucleotides 15, 889–898.

da Matta, A.D., dos Santos, C.V.B., de Pereira, S.H., de Frugulhetti, P.P.I.C., de Oliveira, M.R.P., De Souza, M.C.B.V., Moussatché, N., Ferreira, V.F., 1999. Synthesis of novel nucleosides of 4-oxoquinoline-3-carboxylic acid analogues. Heteroatom Chem. 10, 197–202.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271–277.

Ernst, M.E., Franey, R.J., 1998. Acyclovir and ganciclovir-induced neurotoxicity. Ann. Pharmacother. 32, 111–113.

Gong, Y., Matthews, B., Cheung, D., Tam, T., Gadawski, I., Leung, D., Holan, G., Raff, J., Sacks, S., 2002. Evidence of dual sites of action of dendrimers: SPL-2999 inhibits both virus entry and late stages of herpes simplex virus replication. Antiviral Res. 55, 319–329.

Grundy, W.N., Naylor, G.J., 1999. Phylogenetic inference from conserved sites alignments. J. Exp. Zool. 285, 128–139.

Hayden, F.G., 2001. Antimicrobial agents: antiviral agents (nonretroviral). In: Hardman, J.G., Limbird, L.E., et al. (Eds.), The Pharmacological Basis of Therapeutics, 10th ed. McGraw-Hill, New York, pp. 1295–1312.

Hwang, C.B.C., Ruffner, K.L., Coen, D.M., 1992. A point mutation within a distinct conserved region of the herpes simplex virus DNA polymerase gene confers drug resistance. J. Virol. 66, 1774–1776.

Johnson, G.L., Limon, L., Trikha, G., Wall, H., 1994. Acute renal failure and neurotoxicity following oral acyclovir. Ann. Pharmacother. 28, 460–463.

Knopf, K.W., 1979. Properties of herpes simplex virus DNA polymerase and characterization of its associated exonuclease activity. Eur. J. Biochem. 98, 231–244.

Kuo, Y.C., Chen, C.C., Tsai, W.J., Ho, Y.H., 2001. Regulation of herpes simplex virus type 1 replication in Vero cells by *Psychotria serpens*: relationship to gene expression, DNA replication, and protein synthesis. Antiviral Res. 51, 95–109.

Lagrota, M.H.C., Wigg, M.D., Santos, M.M.G., Miranda, M.M.F.S., Câmara, F.P., Couceiro, J.N.S.S., Costa, S.S., 1994. Inhibitory activity of extracts of *Althernantera brasiliana* (Amaranthaceae) against the herpes simplex virus. Phytother. Res. 8, 358–361.

Liu, W.J., Liu, B., Guo, Q.L., Zhang, Y.C., Yuan, Y.J., Fu, X.D., Deng, Z.H., Lin, J., 2004. Influence of ganciclovir and astragalus membranaceus on proliferation of hematopoietic progenitor cells of cord blood after cytomegalovirus infection in vitro. Zhonghua. Er. Ke. Za. Zhi. 42, 490–494.

Liu, S., Knafels, J.D., Chang, J.S., Waszak, G.A., Baldwin, E.T., Deibel Jr., M.R., Thomsen, D.R., Homa, F.L., Wells, P.A., Tory, M.C., Poorman, R.A., Gao, H., Qiu, X., Seddon, A.P., 2006. Crystal structure of the herpes simplex virus 1 DNA polymerase. J. Biol. Chem. 281, 18193–18200.

Lyon, A.W., Mansoor, A., Trotter, M.J., 2002. Urinary gems: acyclovir crystalluria. Arch. Pathol. Lab. Med. 126, 753–754.

Morfin, F., Thouvenot, D., 2003. Herpes simplex virus resistance to antiviral drugs. J. Clin. Virol. 26, 29–37.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Naesens, L., De Clercq, E., 2001. Recent developments in herpesvirus therapy. Herpes 8, 12–16.

Nishiyama, Y., Maeno, K., Yoshida, S., 1982. Correlaction of increased nuclease activity with enhanced virus reactivation. Exp. Cell Res. 138, 485–489.

Oien, N.L., Brideau, R.J., Hopkins, T.A., Wieber, J.L., Knechtel, M.L., Shelly, J.A., Anstadt, R.A., Wells, P.A., Poorman, R.A., Huang, A., Vaillancourt, V.A., Clayton, T.L., Tucker, J.A., Wathen, M.W., 2002. Broad-spectrum antiherpes activities of 4-hydroxyquinoline carboxamides, a novel class of herpesvirus polymerase inhibitors. Antimicrob. Agents Chemother. 46, 724–730.

Phillips, A.J., 2006. Homology assessment and molecular sequence alignment. J. Biomed. Inform. 39, 18–33.

Roizman, B., Sears, A.E., 1996. Herpes simplex viruses and their replication. In: Knipe, D., Howley, P., et al. (Eds.), Fields Virology, third ed. Lippincott-Raven, Philadelphia, pp. 2231–2295.

Saijo, M., Suzutani, T., Morikawa, S., Kurane, I., 2005. Genotypic characterization of the DNA polymerase and sensitivity to antiviral compounds of foscarnet-resistant herpes simplex virus type 1 (HSV-1) derived from a foscarnet-sensitive HSV-1 strain. Antimicrob. Agents Chemother. 49, 606–611.

Savarino, A., Boelaert, J.R., Cassone, A., Majori, G., Cauda, R., 2003. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect. Dis. 3, 722–727.

Segel, I.H., 1993. Simple Inhibition systems. B. Non-competiive inhibition. In: Segel, I.H. (Ed.), Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, second ed. Wiley-Interscience, pp. 125–135.

Smith, R.W., Malik, P., Clements, J.B., 2005. The herpes simplex virus ICP27 protein: a multifunctional post-transcriptional regulator of gene expression. Biochem. Soc. Trans. 33, 499–501.

Snoeck, R., 2000. Antiviral therapy of herpes simplex. Int. J. Antimicrob. Agents 16, 157–159.

Stránská, R., Schuurman, R., Nienhuis, E., Goedegebuure, I.W., Polman, M., Weel, J.F., Wertheim-Van Dillen, P.M., Berkhout, R.J., van Loon, A.M., 2005. Survey of acyclovir-resistant herpes simplex virus in The Netherlands: prevalence and characterization. J. Clin. Virol. 32, 7–18.

Wathen, M.W., 2002. Non-nucleoside inhibitors of herpesviruses. Rev. Med. Virol. 12, 167–178.

Whitley, R.J., 1996. Herpes simplex viruses. In: Knipe, D., Howley, P., et al. (Eds.), Fields Virology, third ed. Lippincott-Raven, Philadelphia, pp. 2297–2342.

Wutzler, P., Thust, R., 2001. Genetic risks of antiviral nucleoside analogues—a survey. Antiviral Res. 49, 55–74.

Young, B., 2005. Review: mixing new cocktails: drug interactions in antiretroviral regimens. AIDS Patient Care STDS 19, 286–297.

Zanetta, G., Maurice-Estepa, L., Mousson, C., Justrabo, E., Daudon, M., Rifle, G., Tanter, Y., 1999. Foscarnet-induced crystalline glomerulonephritis with nephrotic syndrome and acute renal failure after kidney transplantation. Transplantation 67, 1376–1378.