Biochemical and Biophysical Research Communications 427 (2012) 107–112

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Sterol analogues with diamide side chains interfere with the intracellular localization of viral glycoproteins

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ARTICLE INFO

Article history:
Received 14 August 2012
Available online 14 September 2012

Keywords:
Azasterol
Ugi reaction
Antiviral
HSV-1
VSV
Glycoprotein transport

ABSTRACT

The need to develop novel antiviral agents encouraged us to assess the antiviral activity of synthetic sterol analogues with a diamide side chains. Cytotoxicity and antiviral activity of a family of azasterol previously synthesized was evaluated against herpes simplex virus 1 (HSV-1) (KOS and B2006) and vesicular stomatitis virus (VSV). This family of compounds was extended by the synthesis of novel analogs using an Ugi multicomponent reaction and their ability to inhibit viral multiplication was also evaluated. The results show that some of the compounds tested exert an antiviral activity. Besides, the effect of the azasterols on the intracellular localization of viral glycoproteins was examined. Strikingly, alteration on the glycoprotein D (gD) of HSV-1 fluorescence pattern was observed with both the antiherpetic compounds and the inactive azasterols.

1. Introduction

The development of antiviral agents with novel strategies of attack continues to be a challenge since viral infections remain the leading cause of worldwide death. New approaches are increasingly focused to restrain specific host-cell functions that are required for viral replication instead of the traditional approach of affecting virally-encoded molecules [1,2]. These strategies hold further advantages particularly with respect to the prevention of viral resistance and to obtaining antiviral drugs with a broad spectrum of action.

In this sense, steroids are an attractive source for antiviral drug discovery since they are known to possess several biological properties involving cellular targets. In particular, we have reported that some synthetic polyfunctionalized stigmastane have *in vitro* antiviral activity against several pathogenic viruses [3,4]. We have also found that compounds **1** and **2** (Fig. 1) exhibit anti herpes simplex virus 1 (HSV-1) activity *in vitro* and ameliorate the signs of murine herpetic stromal keratitis [5,6]. Similar studies performed in our laboratory have shown that dehydroepiandrosterone (**3**) and some synthetic derivatives display a broad spectrum of antiviral action against Junin virus, vesicular stomatitis virus (VSV) and adenovirus [7–9]. The actual knowledge about the antiviral action of these plant and animal steroids reveals that cellular targets are an important component of this activity, including modulation of cell signaling pathways [10]. On the other hand, we have also reported the isolation from *Melia azedarach* L. of the limonoid 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM) which might be affecting a cellular factor involved in the transport of viral glycoproteins [11]. The integrity of the viral glycoproteins is critical for virion entry and/or assembly, therefore, cellular factors implicated in vesicular transport, membrane trafficking and glycoprotein processing acquired much relevance as potential targets for novel antiviral drugs [12].

Otherwise, we have successfully applied a new strategy to achieve sterol analogues with a high structural diversity by a simple and fast procedure [13,14]. The synthetic approach carried out was the Ugi four-component reaction (U-4CR), in which an amino component, an acid, a carbonyl compound and an isocyanide react together to give an α-aminoacylamide. Thus, we obtained a new family of azasteroids with diamide side chains of general structure **4**, some of which showed antifungal activity [15]. Several azasteroids are known to have interesting biological properties such as anticancer, antiparasitic, antifungal and antibacterial activities [16–19], however, there are no reports on their antiviral properties.

In this paper, previously synthesized [15] and novel sterol analogues of general structure **4** (Fig. 1) were evaluated for their ability to inhibit viral multiplication and to affect the intracellular localization of viral glycoproteins.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.09.019

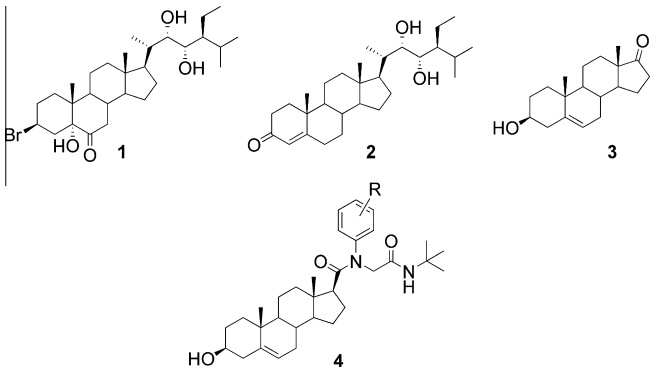


Fig. 1. Antiviral steroids.

2. Material and methods

2.1. Synthesis

2.1.1. General

All solvents and reagents were purchased from Sigma–Aldrich Chemical Co. and were of analytical grade. ESI-HRMS were measured on a Bruker micrOTOF-Q II. Melting points were determined on a Fisher Johns apparatus and are uncorrected. All NMR spectra were recorded on a Bruker AM-500 (500 MHz for ^{1}H and 125.1 MHz for ^{13}C). Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constant (J) values are in Hz. Assignments of the NMR signals of the side chain correspond to the numbering shown in the Supplementary material. Combustion analyses indicated by the symbols of the elements were determined with an Exeter CE 440 Elemental Analyzer and were within ±0.4% of the theoretical values.

2.1.2. General synthetic procedure

The steroidal acid **5** (3β-hydroxyandrost-5-en-17β-carboxylic acid, 50 mg, 0.16 mmol [20] was suspended in 1 mL of methanol and 1.1 equivalent of the corresponding amine and 15 μL of formaldehyde (37% aq.) were added. The mixture was stirred for 15 min at room temperature and then 1.1 equivalent of the isonitrile was added. The reaction was kept under the same conditions until total disappearance of the acid (usually 48 h). The solvent was evaporated under reduced pressure and the residue was taken in EtOAc and washed with NaOH (5% aq.). The compounds were purified by silica gel column chromatography (hexane/EtOAc gradient).

2.1.3. N-((tert-butylcarbamoyl)methyl)-N-(4-fluorophenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4c**)

Yield: 75%. M.p: 114–115 °C. ^{1}H NMR (500 MHz): 0.62 (H-12α, 1H, *m*); 0.81 (H-9α and H-14α, 2H, *m*); 0.82 (C-18, 3H, *s*); 0.98 (C-19, 3H, *s*); 1.38 (H-26, 9H, *s*); 2.45 (H-17, 1H, *t*, J = 9.9); 3.49 (H-3, 1H, *m*); 3.84 (H-22a, 1H, *d*, J = 14.5); 4.38 (H-22b, 1H, *d*, J = 14.5); 5.29 (H-6, 1H, *m*); 6.32 (NH, 1H, *b.s*); 7.08 (H-3′, 2H, *m*); 7.21 (H-2′, 2H, *b.s.*). ^{13}C NMR (125 MHz): 13.7 (C-18); 19.3 (C-19); 20.8 (C-11); 24.7 (C-15); 26.0 (C-16); 28.7 (C-26); 31.5 (C-2); 31.7 (C-8); 31.8 (C-7); 36.5 (C-10); 37.2 (C-1); 38.4 (C-12); 42.2 (C-4); 45.5 (C-13); 49.8 (C-9); 51.2 (C-25); 51.7 (C-17); 55.9 (C-14); 56.2 (C-22); 71.6 (C-3); 114.6 (C-3′, *d*, J_{C-F} = 25); 121.3 (C-6); 129.7 (C-2′, *d*, J_{C-F} = 5); 139.6 (C-1′); 140.7 (C-5); 161.8 (C-4′, *d*, J_{C-F} = 250); 168.4 (C-23); 174.9 (C-20). Anal. $C_{32}H_{45}FN_2O_3$ (C, H, N).

2.1.4. N-((tert-butylcarbamoyl)methyl)-N-(2-chlorophenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4d**)

Yield: 81%. M.p: 113–115 °C. ^{1}H NMR (500 MHz): 0.60 (H-12α, 1H, *m*); 0.81 (H-9α and H-14α, 2H, *m*); 0.83 (C-18, 3H, *s*); 0.92 (C-19, 3H, *s*); 1.37 (H-26, 9H, *s*); 2.20 (H-17, 1H, *m*); 3.48 (H-3, 1H, *m*); 3.56 (H-22a, 1H, *d*, J = 14.5); 4.62 (H-22b, 1H, *d*, J = 14.5); 5.28 (H-6, 1H, *m*); 6.45 (NH, 1H, *b.s*); 7.31 (H-3′ and H-6′, 2H, *m*); 7.42 (H-4′ and H-5′, 2H, *m*). ^{13}C NMR (125 MHz): 13.9 (C-18); 19.3 (C-19); 20.8 (C-11); 24.6 (C-15); 26.3 (C-16); 28.7 (C-26); 31.5 (C-2); 31.7 (C-8); 31.8 (C-7); 36.5 (C-10); 37.2 (C-1); 38.7 (C-12); 42.2 (C-4); 45.5 (C-13); 49.8 (C-9); 51.1 (C-25); 52.6 (C-17); 55.2 (C-14); 56.2 (C-22); 71.6 (C-3); 121.3 (C-6); 127.9 (C-5′); 129.4 (C-3′); 130.2 (C-6′); 131.1 (C-2′); 132.8 (C-1′); 140.7 (C-5); 129.5 (C-4′); 168.3 (C-23); 175.2 (C-20). Anal. $C_{32}H_{45}ClN_2O_3$ (C, H, N).

2.1.5. N-((tert-butylcarbamoyl)methyl)-N-(4-methoxyphenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4e**)

Yield: 78%. M.p: 116–118 °C. ^{1}H NMR (500 MHz): 0.69 (H-12α, 1H, *m*); 0.80 (H-9α and H-14α, 2H, *m*); 0.83 (C-18, 3H, *s*); 0.92 (C-19, 3H, *s*); 1.38 (H-26, 9H, *s*); 2.48 (H-17, 1H, *t*, J = 9.9); 3.48 (H-3, 1H, *m*); 3.84 (-OMe, 3H, *s*); 3.88 (H-22a, 1H, *d*, J = 14.5); 4.39 (H-22b, 1H, *d*, J = 14.5); 5.29 (H-6, 1H, *m*); 6.44 (NH, 1H, *b.s*); 6.90 (H-3́, 2H, *d*, J = 9.3 and 1.4); 7.12 (H-2′, 2H, *b.s.*). ^{13}C NMR (125 MHz): 13.8 (C-18); 19.3 (C-19); 20.8 (C-11); 24.7 (C-15); 26.1 (C-16); 28.7 (C-26); 31.6 (C-2); 31.7 (C-8); 31.8 (C-7); 36.5 (C-10); 37.2 (C-1); 38.4 (C-12); 42.2 (C-4); 45.3 (C-13); 49.8 (C-9); 51.0 (C-25); 51.6 (C-17); 55.4 (-OMe); 56.1 (C-14); 56.2 (C-22); 71.6 (C-3); 114.6 (C-3′); 121.3 (C-6); 129.0 (C-2′); 136.3 (C-1′); 140.7 (C-5); 158.9 (C-4′); 168.8 (C-23); 175.2 (C-20). Anal. $C_{33}H_{48}N_2O_4$ (C, H, N).

2.1.6. N-((tert-butylcarbamoyl)methyl)-N-(4-methylphenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4f**)

Yield: 81%. M.p: 111–112 °C. ^{1}H NMR (500 MHz): 0.66 (H-12α, 1H, *m*); 0.80 (H-9α and H-14α, 2H, *m*); 0.83 (C-18, 3H, *s*); 0.98 (C-19, 3H, *s*); 1.36 (H-26, 9H, *s*); 2.37 (H-5′, 3H, *s*); 2.50 (H-17, 1H, *t*, J = 9.2); 3.48 (H-3, 1H, *m*); 3.88 (H-22a, 1H, *d*, J = 14.7); 4.38 (H-22b, 1H, *d*, J = 14.7); 5.29 (H-6, 1H, *m*); 6.44 (NH, 1H, *b.s*); 7.06 (H-2′, 2H, *b.s.*); 7.17 (H-3′, 2H, *d*, J = 8.5). ^{13}C NMR (125 MHz): 13.8 (C-18); 19.3 (C-19); 20.8 (C-11); 21.1 (C-5′); 24.7 (C-15); 26.0 (C-16); 28.7 (C-26); 31.6 (C-2); 31.7 (C-8); 31.7 (C-7); 36.5 (C-10); 37.2 (C-1); 38.4 (C-12); 42.2 (C-4); 45.4 (C-13); 49.8 (C-9); 51.0 (C-25); 51.7 (C-17); 56.0 (C-14); 56.2 (C-22); 71.7 (C-3); 121.3 (C-6); 127.6 (C-2′); 130.2 (C-3′); 137.8 (C-4′); 140.7 (C-5); 140.8 (C-1′); 168.7 (C-23); 175.1 (C-20). Anal. $C_{33}H_{48}N_2O_3$ (C, H, N).

2.1.7. N-((tert-butylcarbamoyl)methyl)-N-(3,4-dichlorophenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4g**)

Yield: 72%. M.p: 112–113 °C. ^{1}H NMR (500 MHz): 0.65 (H-12α, 1H, *m*); 0.80 (C-18, 3H, *s*); 0.85 (H-9α and H-14α, 2H, *m*); 0.98 (C-19, 3H, *s*); 1.36 (H-26, 9H, *s*); 2.46 (H-17, 1H, *t*, J = 9.5); 3.49 (H-3, 1H, *m*); 3.83 (H-22a, 1H, *d*, J = 14.5); 4.34 (H-22b, 1H, *d*, J = 14.5); 5.30 (H-6, 1H, *m*); 6.11 (NH, 1H, *b.s*); 7.15 (H-6′, 1H, *b.s*); 7.40 (H-2′, 1H, *b.s.*); 7.47 (H-5′, *d*, J = 8.5). ^{13}C NMR (125 MHz): 13.7 (C-18); 19.3 (C-19); 20.9 (C-11); 24.7 (C-15); 26.0 (C-16); 28.7 (C-26); 31.6 (C-2); 31.8 (C-8); 31.8 (C-7); 36.5 (C-10); 37.2 (C-1); 38.3 (C-12); 42.2 (C-4); 45.8 (C-13); 49.7 (C-9); 52.0 (C-25); 52.0 (C-17); 55.5 (C-14); 56.2 (C-22); 71.6 (C-3); 121.3 (C-6); 127.6 (C-6′); 129.0 (C-4′); 131.1 (C-5′); 130.0 (C-2′); 133.2 (C-3′); 140.7 (C-5); 143.0 (C-1′); 168.3 (C-23); 175.2 (C-20). Anal. $C_{32}H_{44}Cl_2N_2O_3$ (C, H, N).

*2.1.8. N-((tert-butylcarbamoyl)methyl)-N-(2-fluorophenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4h**)*

Yield: 72%. M.p: 113–114 °C. ^{1}H NMR (500 MHz): 0.56 (H-12α, 1H, *m*); 0.80 (C-18, 3H, *s*); 0.97 (C-19, 3H, *s*); 1.35 (H-26, 9H, *s*); 2.36 (H-17, 1H, *m*); 3.47 (H-3, 1H, *m*); 3.99 (H-22a, 1H, *d*, *J* = 15); 4.32 (H-22b, 1H, *d*, *J* = 15); 5.28 (H-6, 1H, *m*); 6.39 (NH, 1H, *b.s*); 7.15 (H-4′, 1H, *m*); 7.19 (H-5′, 1H, *m*); 7.33 (H-6′, 1H, *m*); 7.40 (H-3′, 1H, *m*). ^{13}C NMR (125 MHz): 13.9 (C-18); 19.3 (C-19); 20.8 (C-11); 24.6 (C-15); 26.3 (C-16); 28.7 (C-26); 31.5 (C-2); 31.7 (C-8); 31.8 (C-7); 36.5 (C-10); 37.2 (C-1); 38.7 (C-12); 42.2 (C-4); 45.5 (C-13); 49.8 (C-9); 51.1 (C-25); 52.6 (C-17); 55.2 (C-14); 56.2 (C-22); 71.6 (C-3); 113.6 (C-3′, *d*, J_{C-F} = 30); 121.3 (C-6); 126.0 (C-5′); 127.1 (C-6′, *d*, J_{C-F} = 7); 127.4 (C-4′, *d*, J_{C-F} = 7); 129,5 (C-1′, *d*, J_{C-F} = 28); 140.7 (C-5); 157.7 (C-2′, *d*, J_{C-F} = 255); 168.3 (C-23); 175.2 (C-20). Anal. $C_{32}H_{45}FN_2O_3$ (C, H, N).

*2.1.9. N-((tert-butylcarbamoyl)methyl)-N-(2-ethyl-6-methylphenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4i**)*

Yield: 70%. M.p: 114–117 °C. ^{1}H NMR (500 MHz): 0.67 (H-12α, 1H, *m*); 0.72 and 0.78 (H-9α and H-14α, 2H, *m*); 0.93 (C-18, 3H, *s*); 0.98 (C-19, 3H, *s*); 1.21 (H-7′, 2H, *t*, *J* = 7); 1.36 (H-26, 9H, *s*); 2.27 (H-9′, 3H, *s*); 2.46 (H-8′, 2H, *q*, *J* = 7); 2.60 (H-17, 1H, *m*); 3.48 (H-3, 1H, *m*); 3.85 (H-22a, 1H, *d*, *J* = 14.7); 4.23 (H-22b, 1H, *d*, *J* = 14.7); 5.29 (H-6, 1H, *m*); 7.10 (H-3′, 1H, *m*); 7.16 (H-5′, 1H, *m*); 7.22 (H-4′, 1H, *m*); 7.46 (NH, 1H, *b.s*). ^{13}C NMR (125 MHz): 14.1 (C-18); 14.4 (C-8′); 19.3 (C-19); 19.4 (C-9′); 20.8 (C-11); 24.1 (C-7′); 24.7 (C-15); 26.0 (C-16); 28.7 (C-26); 31.6 (C-2); 31.7 (C-8); 31.7 (C-7); 36.5 (C-10); 37.2 (C-1); 38.4 (C-12); 42.2 (C-4); 45.1 (C-13); 49.7 (C-9); 50.9 (C-25); 52.1 (C-17); 56.1 (C-14); 56.8 (C-22); 71.7 (C-3); 121.4 (C-6); 126.5 (C-3′); 128.4 (C-5′); 128.9 (C-4′); 135.6 (C-6′); 135.7 (C-2′); 140.7 (C-5); 141.4 (C-1′); 168.9 (C-23); 177.5 (C-20). Anal. $C_{35}H_{52}N_2O_3$ (C, H, N).

*2.1.10. N-((tert-butylcarbamoyl)methyl)-N-(3-trifluorophenyl)-3β-hydroxyandrost-5-ene-17β-carboxamide (**4j**)*

Yield: 71%. M.p: 111–113 °C. ^{1}H NMR (500 MHz): 0.55 (H-12α, 1H, *m*); 0.80 (H-9α and H-14α, 2H, *m*); 0.82 (C-18, 3H, *s*); 0.98 (C-19, 3H, *s*); 1.37 (H-26, 9H, *s*); 2.41 (H-17, 1H, *t*, *J* = 10); 3.48 (H-3, 1H, *m*); 3.90 (H-22a, 1H, *d*, *J* = 15); 4.39 (H-22b, 1H, *d*, *J* = 15); 5.29 (H-6, 1H, *m*); 6.19 (NH, 1H, *b.s*); 7.50 (H-2′ and H-6′, 2H, *b.s.*); 7.54 (H-5′, 1H, *t*, *J* = 8); 7.61 (H-4′, 1H, *d*, *J* = 8). ^{13}C NMR (125 MHz): 13.7 (C-18); 19.3 (C-19); 20.8 (C-11); 24.7 (C-15); 26.0 (C-16); 28.7 (C-26); 31.6 (C-2); 31.8 (C-8); 31.8 (C-7); 36.5 (C-10); 37.2 (C-1); 38.4 (C-12); 42.2 (C-4); 45.7 (C-13); 49.7 (C-9); 51.3 (C-25); 52.0 (C-17); 56.1 (C-14); 56.2 (C-22); 71.7 (C-3); 120.5 (C-2′, *q*, J_{C-F} = 20); 121.3 (C-6); 124.0 (-CF$_3$, *q*, J_{C-F} = 285); 124.3 (C-6′); 124.4 (C-4′, *q*, J_{C-F} = 7); 131.2 (C-5′, *q*, J_{C-F} = 2); 128.9 (C-3′, *q*, J_{C-F} = 28); 140.7 (C-5); 145.4 (C-1′, *q*, J_{C-F} = 3); 168.0 (C-23); 174.7 (C-20). Anal. $C_{33}H_{45}F_3N_2O_3$ (C, H, N).

2.2. Cells and viruses

Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% inactivated fetal bovine serum (FBS) and 50 μg/ml gentamicin and maintained after monolayer formation in MEM supplemented with 1.5% FBS (MEM 1.5%).

The Indiana strain of VSV, HSV-1 strain KOS and the TK$^-$ mutants B2006 strain were propagated at low multiplicity of infection (moi) and plaque-assayed on Vero cells.

2.3. Antiviral compounds

Compounds **4a–j** were dissolved in dimethylsulfoxide (DMSO) and diluted with MEM 1.5% for testing. The maximum concentration of DMSO tested (1% or 2%) exhibited no toxicity under *in vitro* conditions.

2.4. Cytotoxicity assay

The compounds were evaluated for cytotoxicity by a modification of the crystal violet assay [21]. Vero cells were seeded at a concentration of 10^4 cells/well in 96-well plates and grown at 37 °C for 24 h. The culture medium was replaced by fresh medium containing compounds **4a–j** at various concentrations and cells were further incubated for 48 h. Then, monolayers were fixed in 10% formaldehyde and stained with 0.05% crystal violet solution. After that, the plates were washed with de-ionized water, and dried prior to the solubilization of the bound dye with 100 μL of a 50% ethanol 0.1% glacial acetic acid solution. The absorbance at 590 nm was measured on an Eurogenetics MPR-A 4i microplate reader.

2.5. Antiviral activity

Vero cells grown in 96-well tissue culture plates were infected with HSV-1 (strains KOS or B2006) or VSV at moi of 0.07 PFU/cell. After 1 h adsorption at 37 °C the inoculum was removed and medium containing different concentrations of compounds **4a–j** was added, by triplicate. The plates were incubated at 37 °C in a 5% CO_2 atmosphere until 100% cell death was observed microscopically in untreated infected control cells. Supernatants corresponding to those triplicates were harvested after cell disruption by three cycles of freezing and thawing and pooled. Virus yields were titrated by plaque assay. For comparative purposes, acyclovir (ACV) was tested as positive control against HSV-1.

2.6. Indirect immunofluorescence assay

For total glycoprotein staining, subconfluent cells grown on glass coverslips in 24-well plates were fixed with methanol for 10 min at −20 °C. After washes with phosphate buffered saline (PBS), the coverslips were incubated with primary antibody for 30 min at 37 °C, and then returned to culture dishes and subjected to additional washes with PBS. Afterwards, cells were incubated with secondary antibody for 30 min at 37 °C. Finally, coverslips were rinsed, mounted and photographed with an Olympus BX51 microscope with epifluorescence optics. The mouse monoclonal antibody anti-gD of HSV-1 was obtained from Santa Cruz Biotechnology, USA. The rabbit polyclonal anti-gG of VSV was kindly provided by Dr. Pablo Grigera (CEVAN Buenos Aires, Argentina). Secondary goat anti-rabbit FluoroLinkTM CyTM2 and antimouse FluoroLinkTM CyTM3 antibodies were purchased from GE Healthcare Bio-Sciences, Argentina.

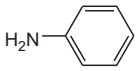
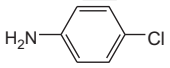
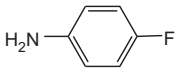
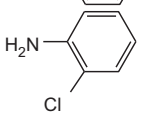
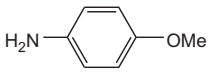
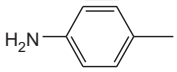
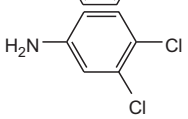
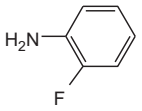
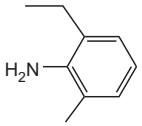
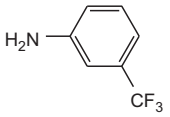
3. Results

Firstly, we performed a screening of the antiviral activity of five known compounds of general structure **4** [15]. To cover a broader spectrum of action, the assays were targeted against two mammalian viruses, HSV-1 and VSV, which belong to DNA and RNA viruses, respectively. The antiviral activity was expressed as the compound concentration required to reduce the virus yield by 50% (EC_{50}). The cytotoxicity concentration required to reduce the Vero cell viability by 50% (CC_{50}) was also calculated. The results are summarized in Table 1.

This screening showed that only two of the compounds tested (**4a** and **4b**) have an interesting antiviral effect against HSV-1 (KOS strain) and VSV although at concentrations closely related to their cytotoxicity (Table 1).

These compounds share an androstane skeleton with an α-aminoacylamide side chain involving a 17β-carboxyl group. Both are

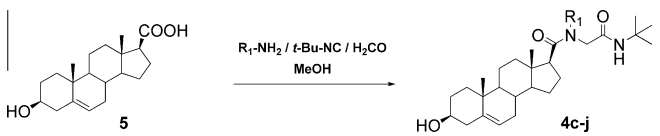
Table 1
Cytotoxicity and antiviral activity of compounds in Vero cell cultures.

Compd.	H_2N-R_1	CC_{50} (μM)[a]	EC_{50} (μM)[a]		
			VSV	HSV-1 (KOS)	HSV-1(B2006)
4a		144	12.4	20	20
4b		228	11.2	16	NA
4c		176	6	12	NA
4d		136.8	10	12.8	12.8
4e		154.4	NA	10.4	NA
4f		140	NA	NA	nd
4g		244	NA	NA	nd
4h		144	NA	NA	nd
4i		124	NA	NA	nd
4j		196	NA	NA	nd
ACV		>500	nd	0.25	7.5

NA = not active at the highest concentration tested.
nd = Not determined.
[a] Data represent mean values for three independent determinations.

substituted with a phenyl moiety at the nitrogen which is bound to the C-17 and a *t*-butyl group at the second amidic nitrogen.

In order to expand these findings, we decided to synthesize novel compounds structurally related to **4a** and **4b**, having a more diverse substitution pattern at the phenyl moiety. These derivatives were prepared using the previously reported synthetic strategy shown in Scheme 1 [15]. The steroidal acid **5** was treated with formaldehyde, *t*-butylisocyanide and a set of anilines to lead to the desired compounds **4c–j**. As expected, the U-4CR generally took place smoothly and in good yields (>75%). The structure of all new compounds was assigned by 1D and 2D NMR experiments. In some cases (for example compound **4i**), the NMR spectra showed that the compounds were present in the solution as a mixture of two conformers. These conformers are expected to originate from the *cis–trans* rotation around the N-substituted side chain



Scheme 1. Synthesis of compounds **4c–j**.

amide bond [22]. Because the interchange between the *cis* and *trans* isomers of these bonds is generally slow in the NMR time scale, the NMR spectrum is the composite of the NMR spectra of the two configurational isomers. For clarity, only the chemical shifts and coupling constants for the most populated conformer in each case was described in the Experimental section. In order to obtain an unambiguous characterization of the compounds, a direct analysis via high resolution electrospray ionization mass spectrometry (ESI-HRMS) was performed, and is shown in the Supplementary material.

The new azasterols were also evaluated for their cytotoxicity and activity towards HSV-1 and VSV in Vero cells. The antiviral assay showed that compounds **4c** and **4d** were active against both HSV-1 (KOS strain) and VSV, whereas the derivative **4e** displayed activity only against HSV-1 (KOS strain) and compounds **4f–j** were found to be inactive (Table 1). In addition, the five anti-HSV-1 active compounds (**4a–e**) were tested against the thymidine kinase-deficient (TK^-) B2006 strain of HSV-1, which is resistant to acyclovir. Unexpectedly, the results showed that only two of them (**4a** and **4d**) were able to inhibit the growth of the ACV-resistant strain.

Then, we examined the effect of all active compounds on viral glycoprotein expression by immunofluorescence (IF) assay. Initially, Vero cells were infected with HSV-1 (KOS strain) and treated

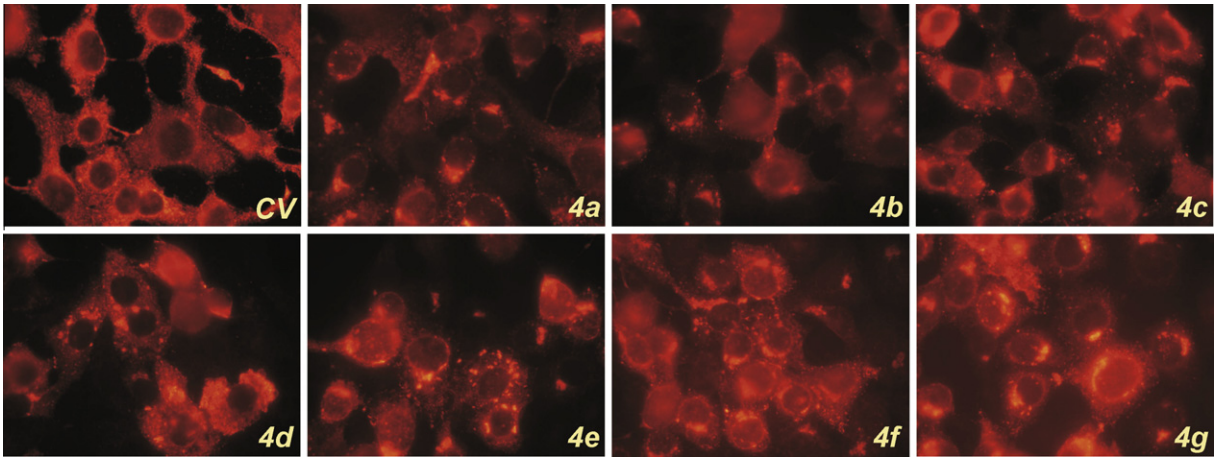


Fig. 2. Effect of compounds **4a–g** on HSV-1 viral glycoprotein D localization, Vero cells infected with HSV-1 (KOS strain) at moi = 1 were treated or not (CV) with compounds **4a–g**. IF staining was performed by adding anti-gD antibodies to cells fixed with methanol at 24 h pos-infection.

with compounds **4a–e** and an IF staining using a mouse monoclonal anti-glycoprotein D (gD) antibody was performed. By visual inspection of the images, we observed that the gD protein was distributed throughout the cytoplasm and the plasma membrane of untreated infected cells, whereas it appeared to be associated with the perinuclear region in most of the treated cells (Fig. 2). Similarly, when cells were infected with VSV and treated with compounds **4a–d**, the glycoprotein G (gG) fluorescence was localized at the juxtanuclear area of both the infected and treated cells (data not shown). Strikingly, the alteration on the gD fluorescence pattern was also observed for compounds **4f** and **4g**, which have no activity against HSV-1 (Fig. 2).

4. Discussion

In the present paper we show that several synthetic azasteroids are able to reduce the viral multiplication of HSV-1 and/or VSV, a property that has not been previously described for this class of molecules. Further, it is interesting to note that small changes in the chemical structure of the compounds considerably modify their biological properties. Results in Table 1 show that the introduction of different groups at the *para* position in the phenyl moiety alters both the cytotoxicity and the spectrum of the antiviral action of the compounds. For example, addition of a chloro or a fluoro group (**4b** and **4c**, respectively) resulted in cytotoxicity lower than that of compound **4a**, but did not affect their antiviral activity against HSV-1 and VSV. Instead, compounds **4e** and **4f** in which the substituent is a methoxy and a methyl group, respectively, displayed CC_{50} values similar to that of compound **4a** although compound **4e** was active only against HSV-1 and compound **4f** was devoid of antiviral activity. Moreover, the influence of the substituent position was also evident with compound **4h**, which has a fluoro group at *ortho* position instead of at *para* position and shows no antiviral activity against any of the viruses tested, in contrast to compound **4c**. This feature allows us to design new compounds with the aim of improving the antiviral properties and reduce the cytotoxic effects of these analogues.

Another remarkable aspect of the sterol analogues is the differential susceptibility of the TK^- strain to the five anti-HSV-1 active compounds. How some compounds can inhibit the wild type strain of HSV but not TK^- strain is a question that still remains open. It is hard to think that the thymidine kinase has a role in the mechanism of action of these derivatives since the chemical structure of azasterols is very different from that of nucleoside analogues.

Finally, we have also demonstrated that sterol analogues with or without antiviral activity interfere with the intracellular transport of viral glycoproteins. It is important to point out that there are multiple potential targets on the glycoprotein traffic that might be hampering by azasteroids. For example, alterations in Golgi and ER cholesterol content would immediately impact on protein transport and organelle structure. The sterol analogues could be affecting the activity of the Oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) which constitute a large gene family that participate in sterol signaling and/or transport. In particular, ORP9L maintains cholesterol flux from the endosomal pathway to the trans-Golgi/TGN by transferring cholesterol to the ER or other organelles [23].

In conclusion, the azasteroids evaluated in this study show interesting biological properties not previously reported, such as antiviral drugs and compounds interfering with the glycoprotein transport. Besides, our synthetic procedure allows a rapid synthesis of derivatives with novel modifications to improve their activities. Further studies are under way to explore the potential cellular targets of these sterol analogues that might explain both, the effect on virus replication and the alteration on glycoprotein traffic.

Acknowledgments

This work was supported by grants from the Universidad de Buenos Aires (UBACyT X-029), the Agencia Nacional de Promoción Científica y Técnica (ANPCyT PICT2008-1394 y PME 2007-354) and the Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 0315, 100516, 1007). We are grateful to UMYMFOR (UBA-CONICET) for the analytical and spectroscopic determinations.

Drs. A.A. Barquero, G.M. Cabrera and J.A. Ramírez are Research Members of the CONICET.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.019.

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