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Antiherpes virus activities of new 6-19 carbon-bridged steroids and some synthetic precursors

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Three synthetic 6,19-carbon bridged steroids: 3β,20β-diacetyloxy-5α-chloro-19a(R)-hydroxy-6,19-methanopregnane, 3β,20β-diacetyloxy-5α-chloro-6,19-methanopregnane, 6,19-methanopregn-4-ene-3,20-dione and four synthetic precursors: 3β,20β-diacetyloxy-19-hydroxypregn-5-ene, 3β,20β-diacetyloxy-pregn-5-en-19-al, 3β,20β-diacetyloxy-19(E)-(methoxymethylidene)-pregn-5-ene and 20β-acetyloxy-3β-hydroxy-19(E)-(methoxymethylidene)-pregn-5-ene were tested against herpes virus replication in cell cultures. Several compounds were cytotoxic for stationary cells. Antiviral studies performed with all compounds against HSV-1 indicated a dose-dependent virus susceptibility with selectivity indexes (SI) values in the range 1.7–183.2. Selected compounds were also tested against HSV-2 and the SI values obtained were in the range of 31–273. Attempts to reveal the step of virus multiplication affected by pregnanes were performed with one compound. HSV-1 virus incubation with the compound did not alter the ability of virus particles to infect cells; moreover, neither virus adsorption nor penetration appeared to be affected. The drug must be present during at least the first 7 h of the virus cycle to inhibit more than 90% of virus production. All these results suggest that these novel molecules interfere with an intracellular step of virus multiplication, thus behaving like true antivirals.

Keywords: herpes virus, antiviral activity, pregnanes, carbon-bridged steroids

Introduction

Our laboratory has been engaged for several years in search of antiviral activity in natural plant steroidal hormones (brassinosteroids) and a series of synthetic analogues of the 24(S)-ethylbrassinone (Caballero *et al*., 1997). Brassinosteroid derivatives were tested in cell cultures against pathogenic RNA and DNA viruses. Many of the compounds tested were potent inhibitors of arenaviruses (Wachsman *et al*., 2000) and some of them behaved as moderate antivirals against the measles virus (Wachsman *et al.*, 2002). A study of structure–activity relationship performed with 40 brassinosteroid derivatives tested against herpes simplex viruses type 1 and 2 (HSV-1, HSV-2) indicated that at least three of them showed valuable antiviral activity (Talarico *et al*., 2003). Furthermore, tk+ and tk- HSV-1 strains were equally susceptible (Wachsman *et al*., 2000).

Mammalian steroidal hormones, like progesterone and several synthetical analogues displaying a great variety of different biological functions, share with brassinolides a basic structural backbone (Burton *et al*., 1995; Veleiro *et al*., 2003; Vicent *et al*., 1997). Thus, as a furtherance of our studies with brassinosteroids, we tested the effect of seven modified pregnanes against HSV.

A total of seven new synthetic pregnanes were studied in this paper. Among them, four compounds (3β,20β-diacetyloxy-19-hydroxypregn-5-ene, 3β,20β-diacetyloxy-pregn-5-en-19-al, 3β,20β-diacetyloxy-19(E)-(methoxymethylidene)-pregn-5-ene and 20β-acetiloxy-3β-hydroxy-19(E)-(methoxymethylidene)-pregn-5-ene) have different polar and/or voluminous groups tethered on C-19. The other three compounds (3β-20β-diacetyloxy-5α-chloro-19a(R)-hydroxy-6,19-methanopregnane, 3β,20β-diacetyloxy-5α-chloro-6,19-methanopregnane and 6,19-methanopregn-4-ene-3,20-dione) have a bridging carbon joining C-10 and C-6 that gives rise to an additional ring on the upper face of the molecule. The latter compounds have a highly rigid conformation and, hence, a well-defined orientation of the polar groups in the molecule (Joselevich *et al*., 2003).

Materials and methods

Cells and viruses

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% inactivated bovine calf serum and 50 µg/ml of gentamycine and maintained after monolayer formation in MEM supplemented with 1.5% inactivated calf serum.

BHK21 cells were grown and maintained in Dulbecco (DMEM), supplemented with 3% inactivated bovine fetal serum, 5% inactivated bovine calf serum and 50 µg/ml of gentamycine.

HSV-1 KOS strain was a gift from Dr Erik De Clercq (Rega Institute, Leuven, Belgium). HSV-2 G strain was obtained from the American Type Culture Collection (Rockville, USA).

Compounds

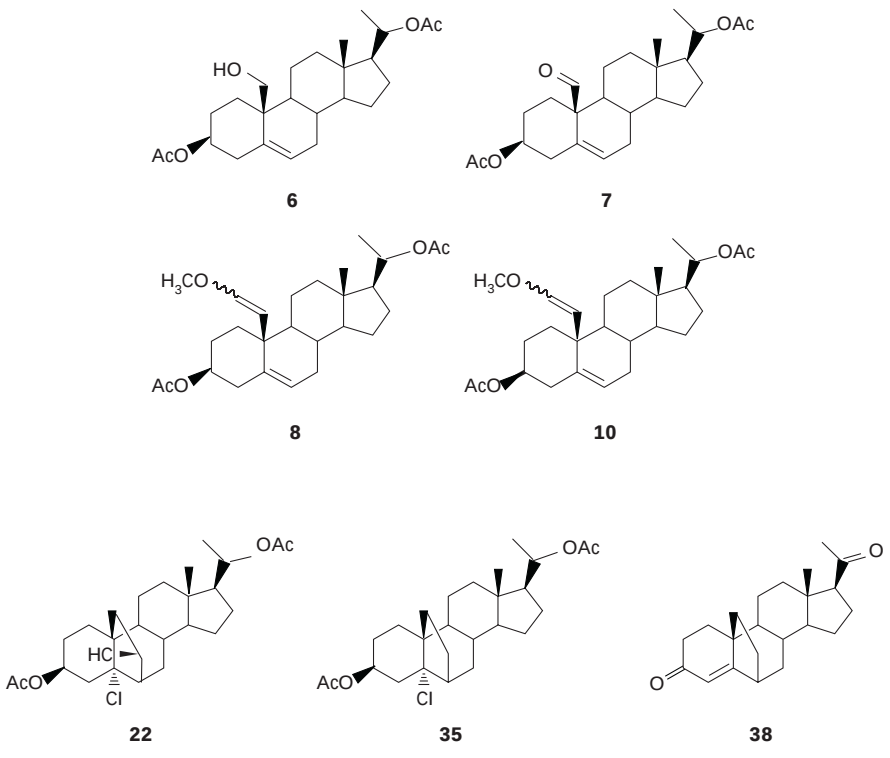
The IUPAC and working names of compounds tested in this study (Figure 1) were 3β,20β-diacetyloxy-19-hydroxypregn-5-ene (**6**), 3β,20β-diacetyloxy-pregn-5-en-19-al (**7**), 3β,20β-diacetyloxy-19(E)-(methoxymethylidene)-pregn-5-ene (**8**), 20β-acetiloxy-3β-hydroxy-19(E)-(methoxymethylidene)-pregn-5-ene (**10**), 3β,20β-diacetyloxy-5α-chloro-19a(R)-hydroxy-6,19-methanopregnane (**22**), 3β,20β-diacetyloxy-5α-chloro-6,19-methanopregnane (**35**) and 6,19-methanopregn-4-ene-3,20-dione (**38**). Compounds **6**, **7**, **8**, **22**, **35** and **38** were obtained from pregnenolone acetate as described in Joselevich *et al*. (2003). **10** was obtained as an E/Z mixture (5:1) as a byproduct in the preparation of **8** (Joselevich *et al*., 2003). The E isomer was separated by preparative t.l.c. (**R*f***=0.20, hexane-ethyl acetate 1:1); mp 103–105°C (isopropyl alcohol); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.56 (s, 3H, H-18), 1.14 (d, J=6 Hz; 3H, H-21), 2.01 (s, 3H, acetate), 3.53 (tt, J=11 Hz, 5 Hz; 1H, H-3), 3.53 (s, 3H, CH_3O), 4.83 (dq, J=17 Hz, 6 Hz; 1H, H-20), 5.52 (dd, J=3 Hz, 2 Hz; 1H, H-6), 4.48 (d, J=13 Hz; 1H, H-19), 6.09 (d, J=13 Hz; 1H, H-19a); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.3 (C-18), 19.9 (C-21), 21.2 (C-11), 21.5 (acetate), 24.3 (C-15), 25.4 (C-16), 30.9 (C-8), 31.7 (C-7), 32.0 (C-2), 36.8 (C-1), 39.1 (C-12), 41.1 (C-13), 42.1 (C-10), 42.5 (C-4), 50.4 (C-9), 54.9 (CH_3O), 55.4 (C-17), 56.0 (C-14), 71.8 (C-3), 72.9 (C-20), 106.3 (C-19), 123.9 (C-6), 137.6 (C-5), 150.4 (C-19a), 170.4 (acetate).

All compounds were dissolved in dimethylsulphoxide (DMSO) at 90 mM or higher concentrations , in order to exclude any antiviral or cytotoxic effect of DMSO, and stored at –20°C until used. Acyclovir [9-(2-hydroxyethoxymethyl)guanine] (GlaxoSmithKline, Research Triangle Park, NC, USA) was solubilized in DMSO and then diluted with maintenance medium. Heparin (Sigma Chemical Co., St Louis, Mo., USA) was solubilized in sterile water and then diluted with maintenance medium.

Cytotoxicity assay

Cell viability was determined using the cleavage of tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) (Denizot & Lang, 1986). The absorbance of each well was measured on an Eurogenetics MPR-A 4i microplate reader,

Figure 1. Chemical structures of compounds used in this study



using a test wavelength of 570 nm and a reference wavelength of 630 nm. The number of surviving cells was determined by interpolation in a standard calibration curve correlating optical density values and number of viable cells determined by counting with a haemocytometer. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration (µM) required to reduce cell viability by 50%. These values were calculated by regression analysis. The assays were performed in duplicate and the data represent the mean of at least two independent experiments.

Antiviral assays

The antiherpetic activity of the compounds was evaluated by a virus–yield reduction assay. Confluent Vero cells grown in 24 well microtitre plates were inoculated with HSV-1 KOS or HSV-2 G at an input of 100 PFU (plaque forming units) per well. After 1 h incubation, residual virus was removed and the infected cells were further incubated with MEM 1.5% in the absence or presence of various concentrations of the compounds. After 24 h incubation at 37°C, the cultures were harvested and virus yields measured by plaque assay on Vero cell monolayers incubated for 48 h at 37°C with MEM containing 0.7% of methyl cellulose.

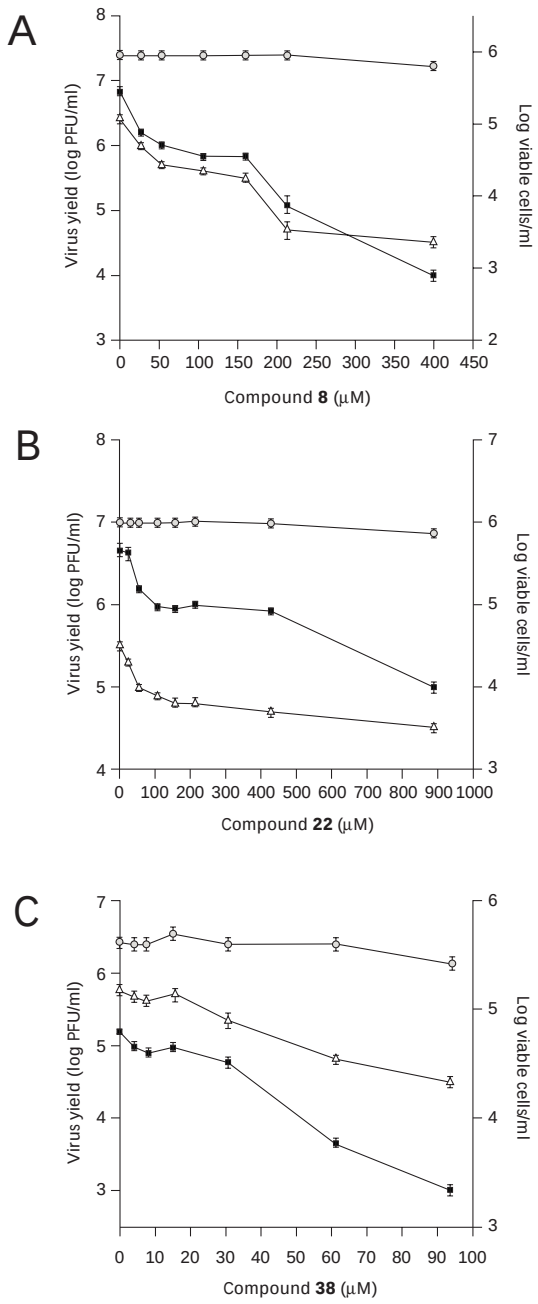
Dose-response curves were constructed from at least six different drug concentrations, starting at 900, 400 or 100 µM, and, subsequently, twofold dilutions of these. Antiviral activity was expressed as 50% effective concentration (EC_{50}) or concentration (in µM) required to reduce virus yields by 50% as compared to the untreated control. The assays were performed in duplicate and the data represent the mean of two independent experiments.

To test the potential effect of the compounds to inhibit HSV-1 cytopathic effect (CPE), confluent Vero cells were grown in 96-well microtitre plates (5×10^4 cells/well) and then inoculated with serial HSV-1 dilutions from 10^{-2} to 10^{-7}. After virus adsorption, every three rows of cells were incubated either with maintenance medium (control) or medium containing compound **8** (115 µM), **22** (478 µM) or **38** (44.5 µM). Compound concentration tested in each case was equivalent to approximately EC_{90} values derived from Figure 2. A concentration of 0.32 µM of ACV was used as a control. Cell death was quantified at 72 h post-infection by determining visually CPE under an inverted phase-contrast microscope.

For virucidal activity testing, a virus suspension containing 1×10^6 PFU of HSV-1 was exposed to 107.1 µM of compound **22** for 1.5 h at 37°C. The sample was then chilled and diluted to determine residual infectivity by plaque formation. In parallel, an untreated control virus sample was similarly processed.

Time-of-removal experiments were performed with confluent Vero cells monolayers grown in 24-well plates inoculated with 100 PFU of HSV-1. Compound **22** (107.1 µM)

Figure 2. Dose-dependent inhibition of HSV-1 and HSV-2 multiplication by pregnanes **8**, **22** and **38**



Vero cells monolayers were incubated in the presence of different concentrations of compounds **8**, **22** or **38**. After 24 h of incubation at 37ºC cell viability was determined by the MTT assay (circle). Other set of cultures were infected with HSV-1 KOS (triangle) or HSV-2 G (square). After virus adsorption, cultures were incubated in presence of non-cytotoxic concentrations of the pregnanes. (**A**) compound **8**, (**B**) compound **22** and (**C**) compound **38**. At 24 h p.i. extracellular virus yields were determined. Data are mean values from three separate experiments.

Table 1. Antiherpes virus activity and cytotoxicity for the tested compounds

	CC_{50}						
	Stationary cells		Growing cells	EC_{50}*		SI†	
Compound tested	24 h	72 h	72 h	HSV-1	HSV-2	HSV-1	HSV-2
6	143.3	33.9	ND	19.6	ND	7.3	ND
7	300	33.6	ND	3.94	ND	76.1	ND
8	874	254.2	221.5	4.77	3.2	183.2	273.1
10	74.5	62.8	ND	44	ND	1.7	ND
22	3300.6	1571	1425.9	27.4	68.9	120.5	47.9
35	138.6	35.9	ND	12.9	ND	10.7	ND
38	634.6	90.7	ND	13.9	20.6	45.6	30.8

*All concentrations are µM.
†SI was calculated using CC_{50} for 24 h.

Table 2. Inhibition of cytopathic effect of HSV-1 by pregnanes

Compound	Virus titre ($TCDI_{50}$/ml)	% Inhibition
Medium	1.3×10^7	–
8	1.7×10^6	87.0
22	1.3×10^6	90.0
38	1.3×10^6	90.0
ACV	1.3×10^3	99.9

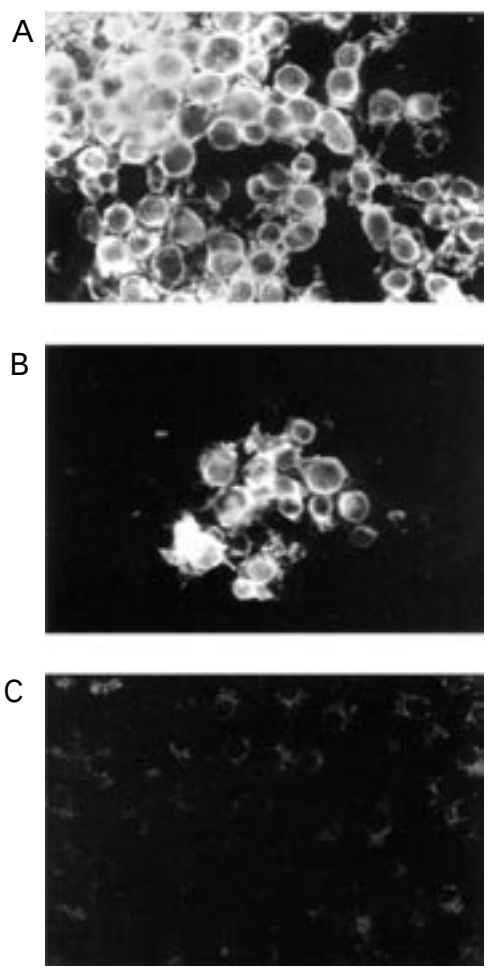
The $TCDI_{50}$ values were obtained using Reed and Müench calculations. CPE <40% was designated '–' and ≥40% '+'.

was present during the adsorption period and then removed along with virus. Cells were incubated further with medium for 24 h and supernatant harvested at 24 h p.i. The same concentration of compound **22** was added after virus infection to another set of culture cells, removed at 1, 3, 5, 7 and 24 h p.i. and replaced by maintainance medium. At 24 h post-infection all supernatants were harvested and the released virus was quantified by PFU. Cells were similarly treated with ACV (0.32 µM) used as a control. To block virus adsorption, a concentration of 10 µg/ml of heparin was used.

Indirect immunofluorescence assay

Vero cells grown in cover slips were inoculated with 100 PFU of HSV-1 and compound **22** (214 µM) was added to the culture medium after virus adsorption. At 24 h post-infection, supernatants were removed. Cells were washed with PBS and fixed with methanol (10 min at –20°C) for total, cytoplasmic and membrane IF. Indirect staining of HSV-1 antigens was carried out by using anti-HSV-1 immunoglobulins purified from hyperimmune rabbit serum reactive against all HSV-1 proteins and fluorescein-labelled goat anti-rabbit IgG (Sigma).

Figure 3. Inhibitory effect of compound **22** on HSV-1 protein expression in Vero cells



Cytoplasmic IF staining of HSV-1 infected Vero cells untreated (**A**) or treated with 214.1 µM of compound **22** (**B**). Mock-infected cells (**C**). Magnification×400.

Table 3. Step of HSV-1 virus multiplication inhibited by compound **22**

	% Inhibition		
	22*	ACV†	Hep‡
Cell treatment			
Cell free contact§	0	ND	ND
2 h before virus adsorption	0	ND	ND
1 h during virus adsorption	0	ND	96
Time of compound removal after virus adsorption			
1 h	2.25	0	ND
3 h	69.7	37.0	ND
5 h	74.2	45.6	ND
7 h	91.9	56.6	ND
24 h	92.9	98.0	ND

*Compound **22** concentration 107.1 µM.
†ACV concentration 0.11 µM.
‡Heparin concentration 10 µg/ml.
§Cell free contact: see virucidal assay in *Materials and methods*.
¶Compound **22** and ACV were added to HSV-1-infected Vero cells immediately after virus adsorption and, at different times, the compounds were removed and virus yields were determined at 24 h p.i.

Results

Cytotoxicity evaluation

A comparison of the CC_{50} values for confluent cells indicated that compounds **6**, **7**, **10** and **35** are the most cytotoxic after 72 h of incubation (Table 1). Interestingly, the CC_{50} values of compounds **8** and **22** did not change significantly for stationary or growing cells after 72 h of incubation, suggesting that cell macromolecular synthesis is not affected by these compounds.

Antiviral properties

Dose-response studies of compounds **8**, **22** and **38** were performed by means of reduction yield assays. Figure 2 showed that viral inhibition is concentration dependent in conditions not deleterious for host cells, and followed similar kinetics for both HSV-1 and HSV-2 viruses. The EC_{50} values calculated from the curves were 4.77 µM (**8**), 27.4 µM (**22**) and 13.9 µM (**38**) for HSV-1 and 3.2 µM, 68.9 µM and 20.6 µM for HSV-2, respectively (Table 1). The effect of compounds **6**, **7**, **10** and **35** was only tested against HSV-1 and the corresponding EC_{50} values are shown in Table 1. As seen when the SI values are considered, the most active compound against HSV-1 was **8** (183.2), followed by compound **22** (120.5), compound **7** (76.1) and compound **38** (45.6). SI values obtained in BHK-21 cells were comparable to those obtained in Vero cells (data not shown). Results for HSV-2 were similar; the most active compound was **8** (273.1), followed by **22** (47.9) and **38** (30.8).

To determine if pregnanes were also inhibitors of the development of HSV-1 cytopathicity effect, compounds **8**, **22** and **38** were tested at their EC_{90} over a period of 72 h post-infection. Table 2 showed that all compounds reduced viral infectivity by 90%, in accordance with the selected dose.

Susceptibility of HSV-1 to compound **22** in different experimental conditions

In order to determine whether these pregnanes acted as true antivirals a series of experiments were performed with compound **22** (107.1 µM). The results obtained are shown in Table 3. The infectious capability of HSV-1 viral particles was not affected by direct contact with the compound, since virions' infectivity remained intact after 1.5 h of incubation at 37°C. Host cells treated with compound **22** remaind fully susceptible to HSV-1 infection, even after 2 h pretreatment. Virus adsorption-penetration was not impeded by compound **22**, whereas there was an inhibition of 96% in the presence of heparin. Time of drug-removal experiments showed that the presence of the compound was not necessary during the complete 24 h period to exert an inhibitory effect. The same degree of virus inhibition was achieved even when the compound was added after adsorption and remained up to 7 h p.i. However, the inhibitory effect was observed as early as 3 h post-infection, showing a pattern of inhibition similar to ACV.

Immunefluorescent studies (Figure 3) revealed a considerable inhibitory effect of compound **22** on viral antigen synthesis, confirming the data in Figure 2B.

Discussion

The pregnane steroid derivatives were investigated for their cytotoxicity effect in stationary or growing Vero cells and their inhibitory effect on HSV-1 and HSV-2 replication. A significant antiherpetic activity was found associated to compounds **7** (EC_{50}: 3.94 µM), **8** (EC_{50}: 4.77 µM), **22** (EC_{50} 27.4 µM) and **38** (EC_{50} 13.9 µM) against HSV-1 and compounds **8** (EC_{50}: 3.2 µM), **22** (EC_{50}: 68.9 µM) and **38** (EC_{50}: 20.6 µM) against HSV-2. We also demonstrated that compounds **8**, **22** and **38** inhibited the cytopathic effect of HSV-1 although none of them reached the activity level of a reference drug such as acyclovir (Table 2). The most effective compound against HSV-1 and HSV-2 was **8** (SI values 183.2 and 273.1), while **22** was the least toxic.

The comparison of compound **8** with its close analogue **10** would indicate that the presence of a free hydroxyl group at C-3 confers high cytotoxicity and should be avoided. However, comparison between **22** and **35** indicates that the hydroxyl group on the bridge (C-19a) appears to be beneficial. Compound **38**, which also lacks this moiety, showed the same tendency.

On the other hand, pregnanes with an oxygen atom bonded to the carbon directly attached to the steroidal backbone (C-19, as in **6** and **7**) are much more toxic than their analogues, where the heteroatom was placed farthest (i.e. C-19a, as in **8** and **22**). At this time, we cannot explain how pregnane analogues **8**, **22** and **38** interfere with virus replication. The results obtained with compound **22** (Table 3) indicated that, at non-cytotoxic concentration, one or more intracellular events occurring after virus penetration are inhibited.

Of particular interest is the susceptibility of HSV-2 to 6,19-methano-progesterone (**38**), since this virus is a sexually transmitted agent highly disseminated in human population (Kuklin *et al*., 1998). A concern on the potential use of this compound *in vivo* may come from the fact that progesterone administration increased susceptibility to genital herpes infection in a mouse model (Khaustic *et al*., 2003). However, it has been shown that related 6,19-bridged steroids are devoid of progestational activity (Vicent *et al*., 1997).

In summary, steroidal compounds should be considered as moderately new promissory antiviral agents. Furthermore, compared to the normal steroids, carbon-bridge moieties are expected to confer better metabolic stability and hence longer half-lives *in vivo* to these compounds, besides lowering progestional activity.

Acknowledgements

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