



Anti HHV-1 and HHV-2 activity *in vitro* of abietic and dehydroabietic acid derivatives

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Abstract

Herpes Simplex Virus types 1 and 2 (HHV-1 and HHV-2) are human neurotropic viruses that infect most commonly the oral and genital area, respectively. Resistant viral isolates can be observed especially in immunocompromised patients. This scenario has triggered the search for new antiherpetic agents, especially those with mechanisms of action different to nucleoside analogs. The antiviral activity *in vitro* for abietic and dehydroabietic acid and controls [acyclovir (ACV), and heparin (HEP)] was evaluated against HHV-1 and HHV-2 using end-point titration technique (EPTT) with a few modifications. The compounds methyl abietate [2], abietinal [4], 8,13(15)-abietadien-18-oic acid [5], methyl abieta-8,13(15)-dien-18-oate [6], 8,13(15)-abietadien-18-ol [7] and dehydroabietinol acetate [11] showed significant anti-herpetic activity of broad-spectrum.

Keywords: abietic acid, resin acids, dehydroabietic acid, antiviral activity, HHV-1, HHV-2.

Introduction

Herpes Simplex Viruses type HHV-1 and HHV-2 are members of the family *herpesviridae*, subfamily *alphaherpesvirinae* (1, 2) responsible for a broad range of human infectious diseases persisting during the lifetime of the host, due to its latent form. Although infections are often subclinical, HHV can cause mild to severe diseases, which can be lethal, especially in neonates (3,4) and immunocompromised individuals (5,6), such as those with human immunodeficiency virus (7) and bone marrow transplants (8). Currently, there is no cure for the persistent infection, and prolonged therapy with the available antiherpetic drugs has induced the emergence of drug-resistant virus strains (9). The increasing resistance of these infections to treatment of available synthetic drugs demands the search for novel compounds. The major class of antiviral agents used for the management of herpetic-infections is acyclovir (ACV) and its derivatives. This scenario has triggered the search for new antiherpetic agents, especially those with mechanisms of action different from that of nucleoside analogs.

Abietic acid is a major component of the rosin fraction of oleoresin biosynthesized by grand fir (*Abies grandis*), lodgepole pine (*Pinus contorta*), and many other conifer species (10). The production of oleoresins (diterpene resin acids) by conifer species is an important component of the defense response against insect attack herbivores, fungal and other pathogens (11-13).

The aim of antiherpetic therapy is to block viral replication to enable shortening the duration of symptoms and to accelerate healing of the lesions associated with HHV 1 and HHV-2. The biological activity of natural abietane acids has been reviewed (14). For example, they have shown antiulcer (15), antimicrobial (16), anxiolytic (17), antiviral (18), antitumor (19), and cytotoxic activities (20). In previous reports, we have described the synthesis and biological evaluation of a number of abietic acid derivatives [compound 1] introducing an oxygenated moiety (such as methyl ester, alcohol, or alde-

hyde) into the lipophilic abietane skeleton with functional groups at C18 and isomeric double bonds [compounds 2 - 8] (21) and a number of derivatives of dehydroabietic acid from commercially available (-)-abietic acid [compounds 9 - 17] (22) (Figure 1). Among biological evaluation, the anti-HHV-1 activity in vitro on HeLa and Vero cell monolayers of abietic and dehydroabietic acid derivatives using a modified end-point titration technique (EPPT) (23) (Table 1) was also described. In this study, we compare the anti-HHV-1 and HHV-2 activities of seventeen dehydroabietic and abietic acid derivatives with the aim of finding compounds of wide antiherpetic spectrum.

The clinical manifestations most common of HHV infections are vesicular lesions affecting the mucous membranes principally of the mouth, nose, or eyes for HHV-1 and the anogenital region for HHV-2. However, an increasing proportion of genital infections are caused by HHV-1(24). The most herpetic genital infections are caused by HHV-2, although HHV-1 accounts for about half of new cases in developed countries (25). The increase in genital HHV-1 is also in part attributable to an increase in oral sex in young adults, which is viewed as safer than intercourse and as a means of averting pregnancy (25, 26). It given the changes in sexual behavior at present, several studies report the presence in young adults of HHV-1 and HHV-2 in both oral and genital mucosa (24). In addition, studies previous have also showed the herpes simplex virus (HHV) infection as a risk factor for HIV transmission (27).

Materials and Methods

Chemistry

The synthesis of abietanes: methyl abietate [2], abietinol [3], abietinal [4], 8,13(15)-abietadien-18-oic acid [5], methyl abieta-8,13(15)-dien-18-oate [6], 8,13(15)-abietadien-18-ol [7], 8,13(15)-abietadien-18-al [8]; and dehydroabietanes: dehydroabietinol [9], dehydroabietinal [10], dehydroabietinol acetate [11], dehydroabietinol benzoate [12], 7 α -

hydroxydehydroabietinol [13], 7 α -hydroxydehydroabietinal [14], methyl dehydroabietate [15], dehydroabietic acid [16], 7-oxodehydroabietinol, [17] were described previously (21,22) (Figure 1). Acyclovir (ACV) and heparin (HEP) were purchased from Sigma (St. Louis, MO) and used as positive controls. Stock solutions of compounds and control were prepared in dimethyl sulfoxide (DMSO, Sigma) and frozen at -70 °C until required. The concentration of DMSO (28) in biological assays was of $\leq 0.05\%$. Cell controls with DMSO at 0.05% were used.

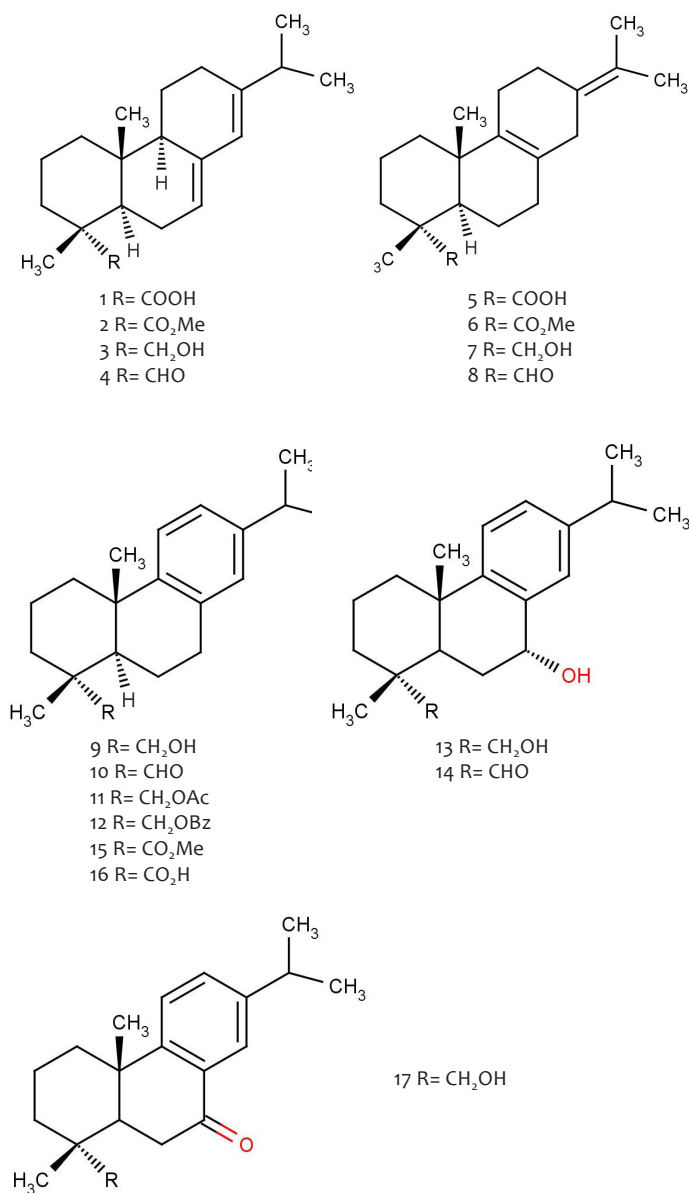


Figure 1. Chemical structures of tested abietanes and dehydroabietanes.

Cells and viruses

The cells used were: Vero cells (*African green monkey kidney*, ATCC: CCL 81 line), kept in log phase of growth in MEM modified by Dulbecco (DMEM) supplemented respectively with 5% fetal bovine serum (SBF), 1 μ g/mL penicillin, streptomycin 1 μ g/mL, 1 μ g/mL of neomycin, vitamins, nonessential amino acids and 1% glutamine. The pH of 7.2 required for cell cultures was achieved with 1N sodium hydroxide, and stabilized with 0.5% aqueous solution of sodium bicarbonate 7%. Cell cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere chamber.

The virus strains used were: HHV-1, Human Herpesvirus 1 acyclovir-sensitive isolate, donated by the group of Virology at the University of Antioquia (Viral strain purchased from "The Center for Disease Control - Atlanta, GA, USA"); HHV-2, Human Herpesvirus 2 acyclovir-sensitive, ATCC:VR-734, cepa G). These virus were propagated in Vero cells and quantified by the method of 50% Cell Culture Infective Dose or Cell Culture Infectious Dose Fifty percent (CCID₅₀), corresponding to the concentration of virus that affects 50% of the cell monolayer, on Vero cell monolayer formation $\geq 80\%$, as described in protocols (29,30,31). After quantifying the virus, it was kept in aliquots at -196 °C in liquid nitrogen.

In vitro Antiviral Activity

We have determined the antiviral activity of abietane and dehydroabietane compounds against a Cell Culture Infectious Dose Fifty percent (1CCID₅₀), of HHV-1 and HHV-2, using the technique of titration end point (EPTT) (29). Vero cells grown in 96-well plates at a density of 2.0 x10⁴ cells / well, incubated at 37 °C in an atmosphere of 5% CO₂ were held to constitute 80% of the cell monolayer. Then 1CCID₅₀ viral suspensions of HHV-1 or HHV-2, with the compounds at concentrations of 100 to 6.25 μ g/mL were incubated for 30 min at room temperature. The compounds mixture / viral suspension were added later to the confluent monolayer of

Vero cells. After 48 h of incubation in 5% humidified atmosphere at 37 °C, the cytopathic effect was examined, and then the microplates were fixed with 3.5% formaldehyde and stained with 0.2% crystal violet. The cell control, compounds, and control of viral suspension were included in the test to determine both the minimal concentration of abietane or dehydroabietane derivatives, which destroys 100 percent of cells (CC_{100}) and reduces viral load. The activity was evaluated by determining the reduction factor (Rf), which corresponds to the value obtained by dividing the viral titer in the absence of compound over the viral titer obtained in the presence of compound (29,32). In other words, the reduction factor shows how many times the compound reduced viral load which it was challenged. There were two replicates of each experiment, and in quadruplicate for each concentration. It was used as positive controls Heparin and acyclovir.

Criteria for defining activity

According to the parameters established by Vlietinck *et al.* (32), the relevant or moderate antiviral activity of a natural product purified, is one whose reduction factor (RF) of viral titer is, respectively, of $\geq 1 \times 10^3$ or 1×10^2 . In this study, a reduction factor of viral titer of 1×10^1 and $\geq 1 \times 10^2$ is determined as a criterion for mild to moderately active compounds, respectively.

Results

In this study, we have determined the anti-HHV-1 activity of compounds **1-8** on and the anti-HHV-2 activity of compounds **1-17** on Vero cells, using as positive controls acyclovir (ACV) and heparin (HEP) (Table 1). The commercial abietic acid [compound **1**] did not present anti-HHV-1 activity, but showed mild anti-HHV-2 activity at a concentration of 25 µg/mL. The abietanes **5**, **6** and **7** showed a mild to moderate activity against HHV-1 and HHV-2 in a concentration range of 6.25 µg/mL to 100 µg/mL. The abietanes **2** and **4** showed a mild activity decreasing 10 times a $CCID_{50}$ of HHV-1 and HHV-2 at concentrations of

25 µg/mL and 50 µg/mL, respectively. The abietic acid derivative that showed the highest antiherpetic activity was the 8,13(15)-abietadien-18-ol [**7**] with a Rf value of 1×10^1 at 6.25 µg/mL for HHV-1 and 1×10^2 at 12.5 µg/mL for HHV-2.

With regard to the dehydroabietanes, they showed moderate activity against HHV-2 and were not active against HHV-1. It is worth to note that the ester dehydroabietinol acetate [**11**] was found to be active both for HHV-1 and HHV-2 with a Rf value of 1×10^1 and 1×10^2 , respectively, at a concentration of 25 µg/mL. The molecules that showed optimal activity against HHV-2 were: **9**, **10**, **14**, and **15** with concentrations among 12.5 and 50 µg/mL. The dehydroabietic acid derivative that showed the highest anti-HHV-2 activity was the 7 α -hydroxydehydroabietinal [**14**] which decreased 1000 times $1CCID_{50}$ producing a dose-dependent inhibition. The control of heparin and acyclovir reduced respectively 100 and 10.000 times $1DICC_{50}$ of HHV-1 and HHV-2 of 0.1 U./mL and 6 µg/mL, respectively.

see Table 1.

Discussion

Researchers have demonstrated that resin acid derivatives can be a useful tool in the synthesis of drugs active against viruses (33, 18, 34, 35). We have previously described the antiherpetic activity of abietic acid [compounds **1**] and seven derivatives: methyl abietate [**2**], abietinol [**3**], abietinal [**4**], 8,13(15)-abietadien-18-oic acid [**5**], methyl abieta-8,13(15)-dien-18-oate [**6**], 8,13(15)-abietadien-18-ol [**7**], 8,13(15)-abietadien-18-al [**8**] on HeLa cells (21) and also dehydroabietic acid [**16**] and eight derivatives: dehydroabietinol [**9**], dehydroabietinal [**10**], dehydroabietinol acetate [**11**], dehydroabietinol benzoate [**12**], 7 α -hydroxydehydroabietinol [**13**], 7 α -hydroxydehydroabietinal [**14**], methyl dehydroabietate [**15**], and 7-oxodehydroabietinol [**17**] on Vero cells (22) against herpes simplex virus type 1 (HHV-1) using a modified end-point titration technique (EPPT), additionally, we reported their cytotoxic

and antimycotic activities. In this study, we compare the anti-HHV-1 and HHV-2 activities of seventeen abietanes and dehydroabietanes, acyclovir (ACV) and heparin (HEP), with the aim of finding compounds of wide antiherpetic range.

According to Vlietinck *et al.* (32), only the compounds with reduction factor (*Rf*) of the viral titer over 1×10^3 (*Rf*: ratio of the virus titer in the absence over virus titer in the presence of the tested compound) show relevant antiviral activity. In general, abietic acid [compound **1**] did not present anti-HHV-1 activity, but showed mild activity anti-HHV-2. To date, it has not been reported antiviral activity against HHV-2 for abietic acid. Compounds **5-7** showed a mild to moderate activity against HHV-1 and HHV-2 on Vero cells, while in our previous study on HeLa cells, methyl abieta-8,13(15)-dien-18-oate [**6**] and 8,13(15)-abietadien-18-ol [**7**] showed no activity against HHV-1 in any of the concentrations tested (21). This indicates a variation in the results possibly due to differences in non-tumor cells (Vero) and tumor type (HeLa), both cells susceptible to infection with HHV-1. However, it is noteworthy that the values “for” activity against HHV-1 for abietic acid [**1**] and 8,13(15)-abietadien-18-oic acid [**5**], did not change despite differences in cell types. On the other hand, the antiherpetic activity data in the series of abietanes indicated a significant difference of activity dependent of the isomerization of the two double bonds. Gigante and co-workers reported the effect of the catechol methyl 11,12-dihydroxyabieta- 8,11,13-trien-18-oate on HHV-1 at concentration of 15mM (35). These results are consistent with our results because the methyl abietadien-18-oate [**2**] showed anti-HHV activity being more active against HHV-2. The replacement of the ester by a hydroxyl group at C18 and the isomerization of the two double bonds enhance the antiviral activity as was showed with the 8,13(15)-abietadien-18-ol [**7**].

With regard to the evaluated dehydroabietanes, they showed moderate activity against HHV-2 and were not active against HHV-1. In the series of dehydroabietane derivatives for anti-HHV-2 activity is not possible to establish the structure activity

relationship with hydroxyl group at C18, because the activity between ester and hydroxyl group were comparable. The ester dehydroabietinol acetate [**11**] (8, 11, 13-Abietatrien-18-yl acetate) showed active against HHV-1 and HHV-2. Fonseca and co-workers reported that dehydroabietane derivatives as methyl 13, 14-imidazolyl- deisopropyldehydroabietate, methyl 12-bromo- 13,14-imidazolyl deisopropyldehydroabietate, methyl 12,13-d-pyrrolyldeisopropyldehydroabietate, methyl 20-methyl-12-bromo- 13,14-imidazolyl-deisopropyldehydroabietate and methyl 20-trifluoromethyl- 13,14-imidazolyl-deisopropyldehydroabietate possess activity against human cytomegalovirus (CMV) and varicella-zoster virus (VZV) to reduce virus plaque formation by 50% in a concentration 5- to 10-fold lower than the cytotoxic concentration (CC_{50} : 5.1, 3.9, 12.5, 2.9, 10.8 $\mu\text{g}/\text{mL}$ respectively), when tested in human embryonic lung (HEL) cells (33).

In conclusion, our study evidenced that abietic and dehydroabietic acid derivatives can be a tool in the synthesis of antiviral drugs. The compounds methyl abietate [**2**], abietinal [**4**], 8,13 (15)-abietadien-18-oic acid [**5**], methyl abieta-8,13(15)-dien-18-oate [**6**], 8,13(15)-abietadien-18-ol [**7**] and dehydroabietinol acetate [**11**] are broad-spectrum potential candidates for antiherpetic activity. Following studies will be focused to evaluation of their anti-herpetic inhibition mechanisms and synergism with acyclovir to HHV-1 and HHV-2.

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Compound	HHV-1 (1CCID ₅₀ ^a) Vero ^b (CDC Atlanta Strain)			HHV-2 (1CCID ₅₀ ^a) Vero ^b (ATCC VR-734 Strain)	
	CC ₁₀₀ (µg/mL) ^a	Viral Reduction Factor ^b	Antiviral Activity (µg/mL) ^c	Viral Reduction Factor ^b	Antiviral Activity (µg/mL) ^c
1	>100	NA	NA	10 ¹	25
2	>25	10 ¹	25	10 ¹	25
3	>50	10 ^{0.5}	25	10 ¹	25
4	>50	10 ¹	50	10 ¹	50
5	>100	10 ¹	100	10 ^{1.5}	50
6	>100	10 ¹	100	10 ²	100
7	25	10 ¹	6,25	10 ²	12,5
8	>100	10 ^{0.5}	100	10 ^{0.5}	100
9 ^g	50	NA	NA	10 ²	12,5
10 ^g	50	NA	NA	10 ²	25
11 ^g	100	10 ¹	25	10 ²	25
12 ^{f,g}	>100	10 ^{0.5}	50	10 ^{1.5}	100
13 ^g	25	NA	NA	10 ¹	12,5
14 ^f	>50	NA	NA	10 ³	50
15 ^g	100	NA	NA	10 ²	12,5
16 ^g	>100	NA	NA	10 ^{0.5}	100
17 ^g	25	NA	NA	10 ¹	12,5
Heparin	>100 U. I./mL	10 ²	0.1 U.I./mL	10 ²	0.1 U.I./mL
Acyclovir	>600	10 ⁴	6.0	10 ⁴	6.0

Table 1: Antiviral activity: Cytotoxicity and anti-HHV-1/ HHV-2 activity of, abietic and dehydroabietic acid derivatives on Vero ** Cells determined by the end-point titration technique

^a1CCID₅₀: Cell Culture Infectious Dose Fifty; ^bVero, *Cercopithecus aethiops*, African green monkey kidney, ATCC: CCL 81 line; ^cMinimal toxic dose that detached 100% of the cell monolayer. ^dRf: ratio of the virus titer in the absence over virus titer in the presence of the tested compound. ^emaximal nontoxic dose that showed the highest viral reduction factor. NA: no activity. ^fDecreased 100 times 1CCID₅₀ of HHV-2/ dose-dependent inhibition. ^gThese activities were reported in a previous study (22).