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In vitro antiviral activity of dehydroepiandrosterone, 17 synthetic analogs and ERK modulators against herpes simplex virus type 1

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ABSTRACT

In the present study the in vitro antiviral activity of dehydroepiandrosterone (DHEA) and 17 synthetic derivatives against herpes simplex type 1 (HSV-1) was determined. DHEA, epiandrosterone (EA), two synthetic DHEA analogs and three synthetic EA analogs showed a selective inhibitory effect on HSV in vitro multiplication. DHEA and E2, a synthetic derivative of EA, were not found to be virucidal to cell-free HSV-1 and did not impair virus adsorption or penetration. We determined that treatment with both compounds decreased viral protein synthesis. Moreover, inhibitory effect of DHEA and E2 on extracellular viral titer was stronger than the inhibition found on total viral infectivity, suggesting that the antiherpetic activity of these compounds may also be in part due to an inhibition in virus formation and release.

Since DHEA is a known Raf/MEK/ERK signaling pathway activator, we studied the role of this pathway on HSV-1 infection. ERK1/2 phosphorylation was stimulated in HSV-1 infected cultures. UO126, a Raf/MEK/ERK signaling pathway inhibitor, impaired viral multiplication, while anisomycin, an activator of this pathway, enhanced it.

Treatment with DHEA 6 h before infection enhanced HSV-1 multiplication. On the contrary, pre-treatment with E2, which does not modulate Raf/MEK/ERK signaling pathway, did not produce an increase of viral replication. Taking together these results, the antiviral activity of DHEA seems to occur via a mechanism independent of its ability to modulate ERK phosphorylation.

1. Introduction

The incidence of diseases caused by herpes simplex virus (HSV) type 1 and 2 has increased in recent years (Azwa and Barton, 2009). HSV-1 and HSV-2 are serious human pathogens; HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections and is responsible for meningoencephalitis in neonates and meningitis in adults (Whitley and Roizman, 2001; Steiner et al., 2007). It has also been shown that HSV-2 infection represents a risk factor for the transmission of sexually transmitted diseases, such as human immunodeficiency virus (HIV) (Freeman et al., 2006; Hill et al., 2009; Mujugira et al., 2011). Nowadays, the standard therapy for the management of HSV infections includes acyclovir (ACV) and penciclovir (PCV) with their respective prodrugs valacyclovir and famciclovir. These compounds are phosphorylated by the viral thymidine kinase (tk) and then by cellular kinases. The triphosphate forms selectively inhibit the viral DNA polymerase (DNA pol) activity. HSV develops resistance to ACV, predominantly as a result of alterations in viral tks and, less frequently, from mutations in the viral DNA pol (Field, 2001; Shin et al., 2001; Morfin and Thouvenot, 2003). Drug-resistant HSV isolates are frequently recovered from immunocompromised patients but rarely found in immunocompetent subjects (Piret and Boivin, 2011). The management of ACV- or PCV-resistant HSV infections includes the use of the pyrophosphate analog foscarnet (FOS) and the nucleotide analog cidofovir. FOS directly inhibits the viral DNA pol and does not require phosphorylation by viral tk. Resistance to FOS arises via mutations in the viral DNA pol (Morfin and Thouvenot, 2003; Chilukuri and Rosen, 2003). Since most of the drugs used to target HSV infections are also nucleoside analogs, it is necessary to develop new antiherpetic compounds with different mechanisms of action.

Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, DHEA) is one of the most abundant steroids in human blood. It is a naturally occurring steroid synthesized in the adrenal cortex, gonads, brain, and gastrointestinal tract and is an intermediate product in the biosynthesis of sex steroid hormones (Dalla Valle

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et al., 1995). In addition, positive effects in the treatment of several diseases, e.g. cancer, viral infections and immune disorders, such as systemic lupus erythematosus, have been proved (Loria et al., 1988; Van Vollenhoven, 2002; Pedersen et al., 2003). The clinical utilization of this hormone presents serious limitations, mainly in women, because administration of high doses for extended time periods are necessary, resulting in an increase in circulating testosterone and dihydrotestosterone. Although it seems to be less cytotoxic than other drugs, DHEA treatment may cause masculinization (Labrie et al., 2003). Several investigations are being made in order to identify DHEA analogs that keep its beneficial effects without being able to be transformed in sexual hormones. Many analogs have been tested as antiviral compounds in vitro and in vivo (Pedersen et al., 2003; Henderson et al., 1992; Diallo et al., 2000; Mavoungou et al., 2005; Acosta et al., 2008; Romanutti et al., 2009, 2010) and previous reports have shown in vitro antiherpetic activity for other steroidal compounds, specially against HSV-1 (Wachsman et al., 2000, 2004; Talarico et al., 2002).

In order to regulate viral replication and host gene responses, many viruses are known to manipulate host-signaling machinery, including the mitogen-activated protein kinases (MAPKs) pathway. There are three main families of MAPK in mammals: ERK (extracellular signal regulated kinase), JNK (Jun-N-terminal kinase) and p38. These proteins, which are involved in diverse cellular functions such as cell cycle regulation, stress response, differentiation and cell survival, react to a diverse range of stimuli, transducing extracellular or intracellular signals, to regulate gene expression in the nucleus (Chambard et al., 2006; McCubrey et al., 2007; Sturgill, 2008; Pan et al., 2009). Numerous studies have shown that certain signaling transduction pathways, such as Raf/MEK/ERK, play an important role in viral infection (Andrade et al., 2004; Sharma-Walia et al., 2005). Moreover, it has been demonstrated that modulation of Raf/MEK/ERK pathway impairs multiplication of some viruses (Chang et al., 2005; Cai et al., 2007; Dawson et al., 2008; Lee and Lee, 2010; Zhang et al., 2010). Although previous studies showed that HSV-1 infection increases ERK phosphorylation (Qin et al., 2011), the role of this signaling pathway during HSV-1 infection is not yet fully understood. In addition, several studies have shown that DHEA modulates the Raf/MEK/ERK signaling cascade, and that this modulation may be responsible of its antiviral activity (Chang et al., 2005; Formoso et al., 2006; Liu et al., 2007).

Anisomycin is a protein synthesis inhibitor that impairs translation by binding to 60S ribosomal subunit and thus blocking peptide bond formation (Hansen et al., 2003). It is known that in mammalian cells sub-inhibitory concentrations of anisomycin activates p38, SAPK/JNK, ERK1/2 and other MAPK cascades (Hazzalin et al., 1998; Dhawan et al., 1999; Chang et al., 2000). On the other hand, UO126 is a highly selective inhibitor of both MEK1 and MEK2, two ERK kinases immediately upstream of ERK (Favata et al., 1998; Newton et al., 2000).

In this work we tested the in vitro antiviral activity of DHEA and 17 synthetic analogs against HSV-1. To further elucidate the relationship between DHEA's antiviral mechanism and its role in MAPKs signaling pathway modulation, we analyzed the action of DHEA, E2 (a synthetic analog), UO126 and anisomycin on ERK pathway; as well as the effect of these compounds on different events of viral multiplication.

2. Materials and methods

2.1. Compounds

DHEA and EA were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). The compounds D1–D6 (obtained from DHEA) and E1–E11 (obtained from EA) were synthesized in our laboratory according to previously reported methods (Romanutti et al., 2010). Formulae of tested compounds and IUPAC names are presented in Table 1. ACV, FOS and anisomycin were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO) and U0126 from Promega (Madison, WI). Drug stock solutions were prepared in dimethyl-sulfoxide (DMSO) with a final concentration of 0.1% and diluted with maintenance medium (MM) consisting of MEM (Gibco, Carlsbad, CA) with 2% inactivated fetal bovine serum (Gibco, Carlsbad, CA).

2.2. Cells and viruses

African green monkey kidney (Vero) cells were grown as monolayers in MEM supplemented with 5% inactivated fetal bovine serum and 50 μg/mL gentamycin.

HSV-1 strain F was obtained from the American Type Culture Collection (Rockville, USA). Virus stock was prepared in Vero cells.

2.3. Cytotoxicity assays

To determine cytotoxicity of the compounds, confluent monolayers of Vero cells grown in tissue culture plates for 48 h were exposed to various concentrations of the derivatives ranging from 50 to 10,000 μM in MM. After 48 h of incubation, cell viability was determined as the ability of living cells to cleave the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Sigma–Aldrich, St. Louis, MO), by the mitochondrial enzyme succinate dehydrogenase, to give a quantifiable blue product (formazan) (Denizot and Lang, 1986). The precise MTT procedure has been previously described (Wachsman et al., 2000; Talarico et al., 2006). The cytotoxicity of DHEA and the synthetic derivative E2 on growing cells was determined using the MTT assay described before for DHEA (Romanutti et al., 2010). The CC_{50} was defined as the compound concentration (μM) that reduced cell viability by 50%, calculated by regression analysis.

2.4. Virus yield reduction assay

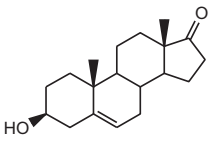
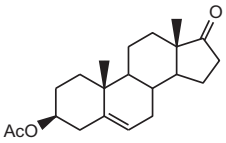
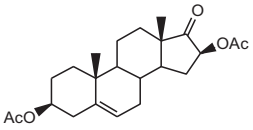
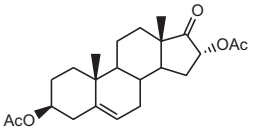
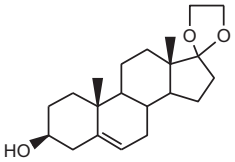
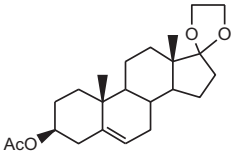
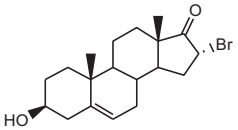
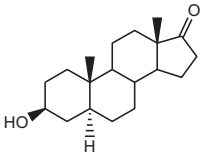
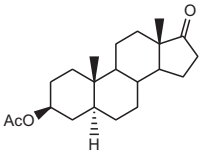
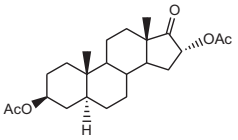
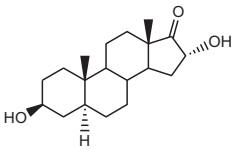
Antiviral activity was evaluated with virus yield reduction assays. For that purpose, Vero cells grown in 24-well culture plates for 48 h, were infected with HSV-1 at a multiplicity of infection (m.o.i.) of 1 PFU/cell. After 1 h of adsorption at 37 °C, cells were covered with MM containing various concentrations of the compounds. After 30 h of incubation at 37 °C, HSV-1 infected cultures were subjected to two cycles of freeze–thawing followed by centrifugation at low speed (10,000*g*). Then, the supernatants were titrated by a plaque assay to determine total infectivity. In addition, cell free virus obtained from culture supernatants were quantified when indicated.

Antiviral activity was expressed as EC_{50} (50% effective concentration) i.e. compound concentration required to reduce to 50% HSV-1 plaque formation after 72 h when compared with the untreated infected cultures. The EC_{50} values were calculated by plotting percentages of inhibition versus different concentrations of each compound.

2.5. Virucidal assay

10^7 PFU of HSV-1 were incubated either with DHEA (1.8 mM), E2 (1.8 mM) or MM for 120 min at 37 °C. Then, aliquots were taken, conveniently diluted in MM and used to infect Vero cells to test virus survival using a plaque assay.

Table 1
Cytotoxicity and antiviral activity of DHEA and their synthetic derivatives against HSV-1.

Compound (MW)	Structural formula	IUPAC name	CC_{50} (μM)	EC_{50} (μM)	SI
DHEA (288.42)		3β-Hydroxyandrost-5-en-17-one	3467	86.7	40
D1 (330.46)		17-Oxoandrost-5-en-3β-yl acetate	1227	176	7
D2 388.5		17-Oxoandrost-5-en-3β,16β-yl diacetate	584.3	489	1.2
D3 388,5		17-Oxoandrost-5-en-3β,16α-diyl diacetate	1730	59.2	29
D4 (332.48)		17,17-Ethylendioxyandrost-5-en-3β-ol	1955	629	3.1
D5 (374.51)		17,17-Ethylendioxyandrost-5-en-3β-yl acetate	814.4	481	1.7
D6 (367.32)		16α-Bromo-3β-hydroxyandrost-5-en-17-one	327.0	19.1	17
EA (290.44)		3β-Hydroxy-5α-androstan-17-one	4579	155	29
E1 (332.48)		17-Oxo-5α-androstan-3β-yl acetate	361.0	292	1.2
E2 (390.51)		17-Oxo-5α-androstan-3β,16α-diyl diacetate	3406	218	16
E3 (306.44)		3β,16α-Dihydroxy-5α-androstan-17-one	489.5	117	4.2

(*continued on next page*)

Table 1 (*continued*)

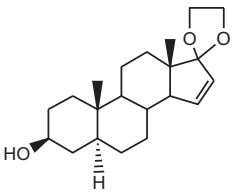
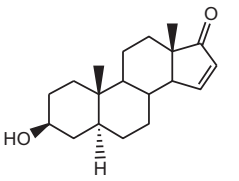
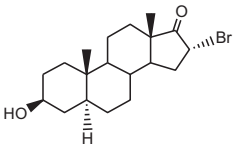
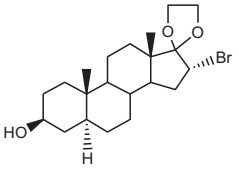
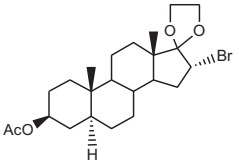
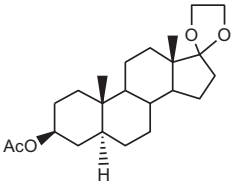
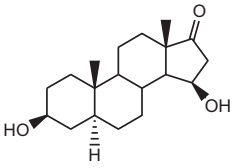
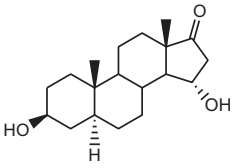
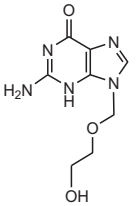
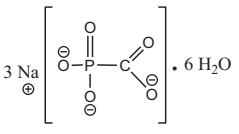
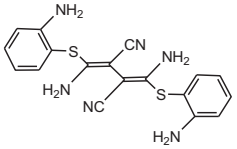
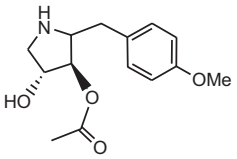
Compound (MW)	Structural formula	IUPAC name	CC_{50} (μM)	EC_{50} (μM)	SI
E4 (332.489)		17,17-Ethylendioxy-5α-androst-15-en-3β-ol	487.5	268	1.8
E5 (288.42)		3β-Hydroxy-5α-androst-15-en-17-one	220.0	15.6	14.1
E6 (369.34)		16α-Bromo -3β-hydroxy-5α-androstan-17-one	98.28	97.5	I[a]
E7 (413.39)		16α-Bromo-17,17-ethylendioxy-5α-androstan-3β-ol	2322	472	5
E8 (455.43)		16α-Bromo-17,17-ethylendioxy-5α-androstan-3β-yl acetate	64.11	42.4	1.7
E9 (376.53)		17,17-Ethylendioxy-5α-androstan-3β-yl acetate	1536	77.6	19.8
E10 (306.44)		3β,15β-Dihydroxy-5α-androstan-17-one	435.6	225	1.9
E11 (306.44)		3β,15α-Dihydroxy-5α-androstan-17-one	1108	118	9.4
ACV (225.2)		9-(2-Hydroxyetoxymetyl)Guanine	279.7	0.311	900
FOS (300.04)		Phosphonoformic acid trisodium salt hexahydrate	700	50	12

Table 1 (*continued*)

Compound (MW)	Structural formula	IUPAC name	CC_{50} (μM)	EC_{50} (μM)	SI
UO126 (380.49)		1,4-Diamino-2,3-diciano-1,4-bis(2-aminophenylthio)butadiene	320	15	21.3
Anisomycin (265.3)		2-*p*-Methoxyphenylmethyl-3-acetoxy-4-hydroxypyrrolidine	45	–	–

The compounds were added 1 h after infection and total infectivity was determined by a plaque assay.
CC_{50}: compound concentration required to reduce cell viability by 50%, as determined by the MTT method.
EC_{50}: compound concentration required to reduce virus yield by 50%.
SI (selectivity index): ratio CC_{50}/EC_{50}.
[a] I: inactive.

2.6. Effect of time of addition of DHEA or E2 on HSV production

DHEA or E2 at a concentration of 180 μM or 290 μM, respectively, were added to confluent monolayers of Vero cells either at 6 h before infection or at 1, 4 or 8 h post-infection (p.i.) with HSV-1 (m.o.i. = 1). Cultures were further incubated at 37 °C until 30 h p.i. and extracellular virus titer or total infectivity was quantified by plaque assay on Vero cells.

2.7. Adsorption and penetration assay

To determine the effect of DHEA (180 μM) or E2 (290 μM) on viral adsorption and penetration, about 100 PFU of HSV-1 were adsorbed for 1 h at 4 °C on confluent Vero cells grown in 24-well culture plates (2×10^5 cells), in the presence or absence of the compounds. Then, cultures were washed twice with cold phosphate-buffered saline (PBS) and overlaid with MM containing 0.7% methylcellulose to quantify virus adsorption. For the internalization assay, after virus adsorption at 4 °C for 1 h, cells were incubated at 37 °C to maximize virus penetration for various periods of time in the presence or absence of DHEA or E2. At 0, 15, 30, 45 and 60 min, the monolayers were washed twice with PBS and treated for 1 min with citrate buffer (pH 3) to inactivate any remaining attached virus. After being washed twice with PBS, cultures were overlaid with MM containing 0.7% methylcellulose, and after 72 h of incubation at 37 °C, plaques were counted.

2.8. Western-blotting

Monolayers of confluent Vero cells were infected or not with HSV-1 (m.o.i. = 1) and incubated at 37 °C for 1 h to allow virus internalization. After removing the inoculum, monolayers were covered with MM or MM containing DHEA (180 μM), UO126 (50 μM) or anisomycin (0.15 μM) and incubated at 37 °C for 30 h. Afterwards, cultures were lysed with Laemmli sample buffer (Bio-Rad, CA, USA), added with 5% β-mercaptoethanol. Samples were then heated for 2 min in boiling water before being load into 10% acrylamide gels. Following electrophoresis, proteins were transferred to a PVDF membrane (Perkin Elmer Life Sciences, Inc., MA, USA) using a dry system (LKB, Multiphor II). ERK1 and p-ERK 1/2 were revealed with rabbit anti-ERK1 and anti-p-ERK 1/2 (Santa Cruz, USA) and a peroxidase anti-rabbit immunoglobulin G (Promega, USA) as secondary antibody. For HSV-1 protein synthesis in DHEA treated, E2 treated or control cultures, glycoprotein gD was revealed with mouse anti-gD (Santa Cruz, USA) and a peroxidase anti-mouse immunoglobulin G (Promega, USA) as secondary antibody and visualized using a chemiluminiscence kit (Perkin Elmer Life Sciences, Inc., MA, USA).

2.9. Indirect immunofluorescence (IFI) assays

Vero cells grown on glass coverslips were infected or not with HSV-1 at a m.o.i. of 1. After 1 h adsorption, cells were mock-treated or treated with DHEA (180 μM), UO126 (50 μM) or anisomycin (0.15 μM) and incubated at 37 °C for 30 h. At that time, supernatants were removed and cells were washed with PBS, fixed with methanol (10 min at −20 °C) and stained for total IFI using anti-HSV-1 purified rabbit Igs and goat-anti-rabbit IgG FITC (Sigma Aldrich, St. Louis, MO). Cells were photographed with a Zeiss microscope with epifluorescence optics.

3. Results

3.1. Antiviral activity of synthetic derivatives obtained from DHEA

In order to assess if structural changes on DHEA would translate in a modification of the antiviral properties of this steroid, we evaluated the anti HSV-1 activity of 17 synthetic derivatives: D1–D6 obtained from DHEA and E1–E11 obtained from EA, a natural non-androgenic derivative of DHEA (Table 1).

First, for compounds D5, D6, E6, E8, anisomycin, UO126, ACV and FOS, we determined the concentration that reduces cell viability to 50% of the control (CC_{50}) by using the MTT method. CC_{50} values for the other 15 derivatives have been reported previously (Romanutti et al., 2009). A virus yield inhibition assay to determine total infectivity was then performed, and the selectivity index (SI), i.e. the relationship between CC_{50} and EC_{50} values, was calculated. For comparative purposes, the inhibitory effect of ACV and FOS was also assayed.

Among the tested compounds, DHEA, EA and E2 were the less cytotoxic. The presence of other functional groups on C3, C15, C16 or C17 generally increased cytotoxicity; but additive effects were not always observed. For instance, C3 β-acetylated D1 and E1 compounds were 2- to 10-fold more cytotoxic than DHEA and EA, respectively. The presence of an additional β-acetyl group on C16 (compound D2) did not considerably change the cytotoxicity, but higher compound concentrations were needed to inhibit HSV-1. However, the replacement of the β-acetyl group by an α-acetyl group on C16 in compounds D3 and E2 rendered similar SI values to those obtained with DHEA and EA. The presence of an α-hydroxy group in C16 produced a diminishment in the CC_{50}

value with respect to EA (compound E3). D4 and D5, which are protected C17 ketone derivatives of DHEA and D1, respectively, were inactive. Compounds D6, with a 16α bromo, and E9, with a 3β acetyl group and a protected C17 ketone, presented SI values of 17 and 19.8, respectively. The presence of a hydroxyl group in C15 in α or β position (compounds E10 and E11) increased citotoxicity, thus reducing the SI values with respect to EA. Meanwhile, a double bond between C15 and C16 (compound E5) rendered a low CC_{50} but also a high reduction in EC_{50} value. Other combinations of functional groups resulted in inactive molecules (data not shown). In Table 1 the CC_{50}, EC_{50} and SI values of UO126 and anisomycin, the two ERK modulators used in this work, are also included.

We determined, under our experimental conditions, that among steroidal assayed compounds EA, DHEA, D3, D6, E2, E5 and E9 are the most active against HSV-1.

3.2. Effect of DHEA and E2 on HSV infectious particle production

In order to elucidate the mechanism of action of this family of compounds, we continued our studies with DHEA and the less cytotoxic of the synthesized compounds, the EA analog E2. To analyze the cytotoxicity of DHEA and E2 using a more rigorous test, we determined the concentration of both compounds required to inhibit cellular replication using cells in growing state. The CC_{50} values obtained were 2600 and 2400 μM for DHEA and E2, respectively. These values are slightly lower than those calculated using confluent non-growing cells (Table 1), indicating that DHEA and E2 exhibit a moderate inhibitory effect on cell growth.

DHEA and E2 inhibited the multiplication of HSV-1 in confluent Vero cells, using a virus yield reduction assay, in a dose-dependent manner. The highest inhibition of virus replication obtained was 99.9% at 180 μM for DHEA and 290 μM for E2 (Fig. 1A). To establish if these compounds produce a direct effect on the viral particle, we performed a virucidal assay. Treatment of viral suspensions with DHEA or E2 (1.8 mM) did not affect viral infectivity, when compared to untreated controls, indicating that the antiviral action of these compounds is not due to the inactivation of virus particles (data not shown). To determine if these compounds interfere with early events of the virus multiplication cycle, we analyzed the effect of DHEA and E2 on virus adsorption and penetration. No differences in the amount of adsorbed (data not shown) or internalized virus (Fig. 1B) were observed between treated and untreated infected control cells.

To determine if DHEA or E2 affect viral protein synthesis, Vero cells were infected with HSV-1 and treated with different concentrations of DHEA ranging from 45 to 180 μM or E2 ranging from 70 to 290 μM. The concentrations used for both compounds in this experiment were chosen considering their dose response curve (Fig. 1). Afterwards, a polyacrylamide gel electrophoresis followed by a Western-blot analysis was performed. As shown in Fig. 2, viral protein gD synthesis was reduced in a dose dependent manner with both compounds. To further characterize the inhibitory action of DHEA and E2, a time of addition experiment was performed. For that purpose, DHEA (180 μM) or E2 (290 μM) were added to HSV-1 infected Vero cells at different times after infection and at 30 h p.i., cell free and total infectivity were determined. Total virus yields were reduced by approximately 1 log when DHEA was present since 1 h p.i, and by 2 log when E2 was added at 1 h p.i. On the other hand, yields of released HSV-1 were reduced by approximately 3 log when the compounds were added at 1 h p.i. (Fig. 3). The higher reduction in released virus yields suggests that DHEA and E2 could be affecting mainly the release of infectious particles. However, when the compounds were added at 4 h p.i. higher viral titers were observed if compared with the results obtained when they were present since 1 h p.i. Thus, indicating that antiviral activity is not only due to impairment in virus release. Other events in viral replicative cycle could be affected, as seen with viral protein synthesis in Fig. 2. Surprisingly, when DHEA was added 6 h before infection (and not further maintained after HSV-1 adsorption) an increase of approximately 1 log in total and extracellular released virus was observed. On the contrary, the synthetic analog E2 did not produce an enhancement of viral titer when added in the same conditions.

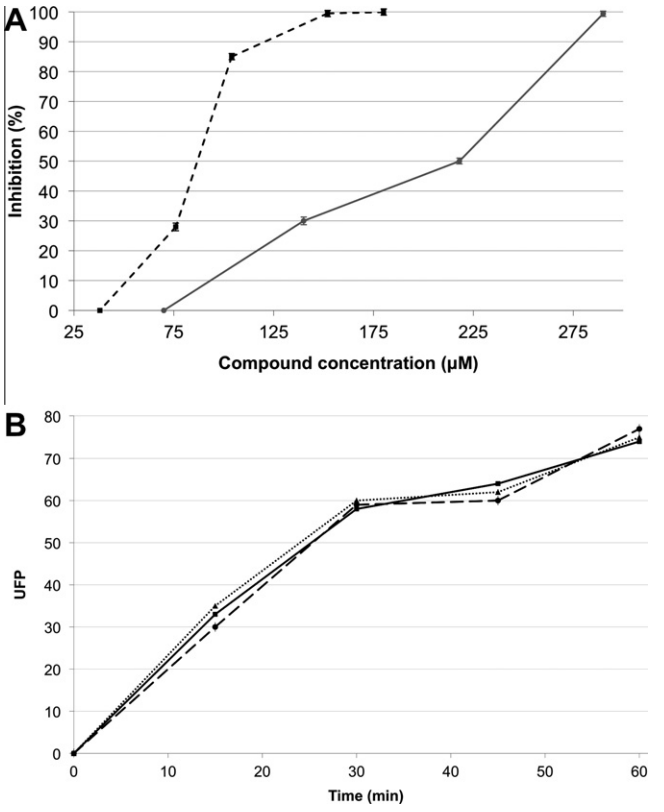


Fig. 1. Effect of DHEA or E2 on HSV-1 multiplication. (A) Vero cells infected with HSV-1 (m.o.i. = 1) were incubated with different concentrations of DHEA or E2 for 30 h. Then, supernatants were harvested and cell free virus yields were determined by a plaque assay. Black dashed line is for DHEA treatment and gray full line for E2 treatment. (B) 100 PFU of HSV-1 were adsorbed for 1 h at 4 °C on Vero cells in the presence of DHEA 180 μM (full line), E2 290 μM (dashed line) or without compounds (dotted line). Then, cells were incubated at 37 °C and at 0, 15, 30, 45 and 60 min any remaining attached virus was inactivated with acid pH. Finally, cultures were overlaid with MM containing 0.7% methylcellulose and 72 h later plaques were counted. Values are means ± SD from three independent experiments, where each titration was carried out in duplicate.

3.3. HSV-1 leads to activation of ERK1/2

As previous studies demonstrated that DHEA is a potent activator of the Raf/MEK/ERK signaling cascade and that some viruses make use of this pathway, we thought that the increase in viral titers when DHEA was present 6 h previous infection could be related with DHEA's activation of this pathway. To shed light on this, we analyzed the kinetics of Raf/MEK/ERK pathway activation in HSV-1 infected cells. To this purpose, confluent Vero cells were infected with HSV-1 at a m.o.i. of 1, and cells were harvested at 0, 20 and 40 min and at 1, 2, 3, 5, 7, 9 and 30 h p.i. Following polyacrylamide gel electrophoresis, p-ERK1/2 and ERK1 expression was analyzed by Western-blot. To determine whether other proteins present in the media influenced ERK1/2 phosphorylation or if it fluctuates naturally, we also analyzed uninfected cell lysates. We did not observe any increase of ERK1/2 phosphorylation during the time course experiment (Fig. 4B). On the contrary, as shown in

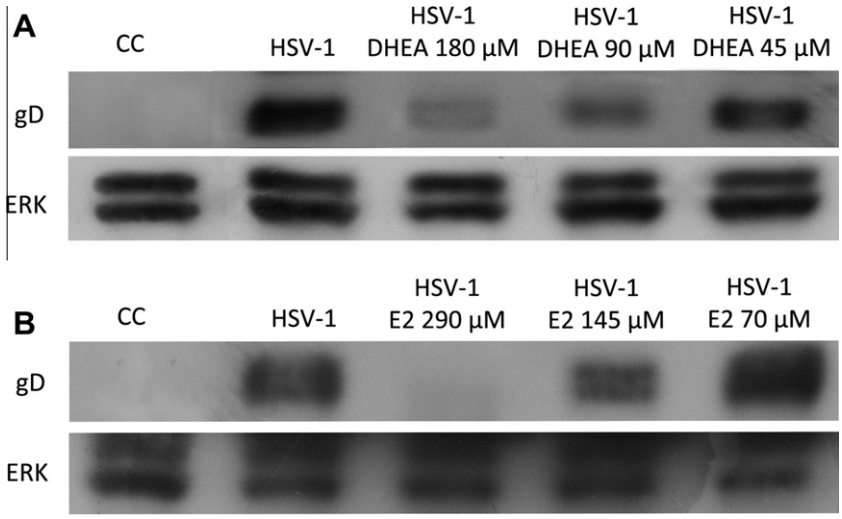


Fig. 2. Analysis of HSV-1 protein synthesis in the presence of DHEA or E2.Vero cells infected or not (CC) with HSV-1 at a m.o.i. of 1 were incubated at 37 °C for 1 h to allow internalization. Afterwards, the inoculum was discarded and the monolayers were covered with MM or MM containing DHEA (A) or E2 (B) at different concentrations and incubated at 37 °C. At 30 h p.i. cell lysates were collected and subjected to Western blotting and glycoprotein gD was analyzed with an antibody against gD. To verify equal loading, Western blotting was performed with an antibody to ERK1. Data are from one of two different experiments.

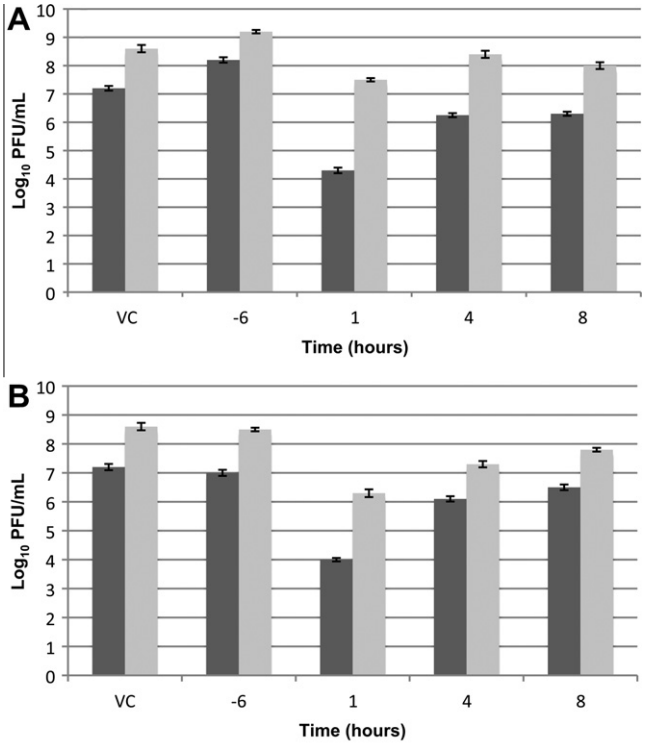


Fig. 3. Effect of time of DHEA or E2 addition on HSV production. Vero cells infected with HSV-1 (m.o.i. = 1) were incubated with MM (VC) or MM containing DHEA 180 μM (A) or E2 290 μM (B) added either at 6 h before infection or at 1, 4 or 8 h p.i. Cultures were further incubated at 37 °C until 30 h p.i and cell free (black bars) and total (gray bars) virus yields were determined by plaque assay. Values are means ± SD from three independent experiments, in each of which titrations were carried out in duplicate.

Fig. 4A, exposure of Vero cells to HSV-1 stimulated ERK1/2 phosphorylation. The highest levels of ERK phosphorylation were found at 40 min, 1 h and 3 h. According to HSV-1 multiplication cycle this ERK phosphorylation occurs due to early events of HSV-1 infection. At 30 h p.i., another peak was observed probably because of ERK activation by secondary infections occurring at this time.

3.4. Effect of DHEA and E2 on ERK1/2 phosphorylation

To further assess the potential role of Raf/MEK/ERK signaling pathway in the antiviral activity of DHEA, we compared its effect on ERK phosphorylation with that of UO126, a selective inhibitor of ERK, and anisomycin, an ERK activator. In all cases, treatment with the drugs was preformed during 30 h. Western blot analysis showed that treatment of cells with UO126 (50 μM) results in a complete inhibition of ERK phosphorylation and anisomycin (0.15 μM), as expected, increases ERK phosphorylation. Meanwhile, DHEA (180 μM) markedly increased ERK 1/2 phosphorylation (Fig. 5A). On the other hand, the effect of the synthetic analog E2 on ERK phosphorylation was also determined. This compound does not seem to modulate the Raf/MEK/ERK signaling pathway (Fig. 5B) probably due to its structural differences with DHEA, which enhances ERK phosphorylation in a dose dependent manner (Fig. 5C). Therefore, as E2 does not induce ERK phosphorylation, when it was added 6 h before infection, virus multiplication was not enhanced as it was when cells were treated with DHEA under the same conditions. Total expression levels of ERK1, used as a control, did not change under these treatments.

Furthermore, HSV-1 infected cells harvested at 30 h p.i. showed an increased ERK 1/2 phosphorylation compared with uninfected cells. Infected cells treated with DHEA and anisomycin during 30 h, showed enhanced phosphorylation of ERK1/2 compared with uninfected cells. Meanwhile, 30 h treatment with UO126 of HSV-1 infected cells, failed to completely inhibit phosphorylation of ERK 1/2. These differences found between the effects of the compounds in infected and uninfected cells are probably due to the combined effects of the virus-induced increase in ERK 1/2 phosphorylation and the particular effect caused by each compound. These results raise the question whether UO126 and anisomycin could affect HSV-1 viral titers in Vero cells.

3.5. Effect of ERK pathway modulators on viral multiplication

Next, we examined whether ERK modulators affected HSV-1 viral titers in Vero cells. As HSV-1 activates ERK 1/2, we expected that UO126 would reduce viral infectivity, while anisomycin would increase or at least not modify viral multiplication. At 30 h p.i. supernatants were collected and viral titers determined by a

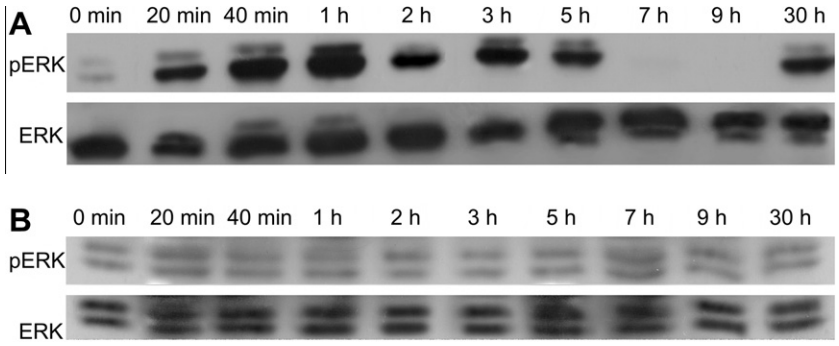


Fig. 4. Time course of ERK1/2 phosphorylation in HSV-1 infected cultures. Lysates from infected (A) or mock infected (B) cells were collected at the indicated times after infection and subjected to Western blotting to analyze ERK1/2 phosphorylation. Data are from one of three different experiments.

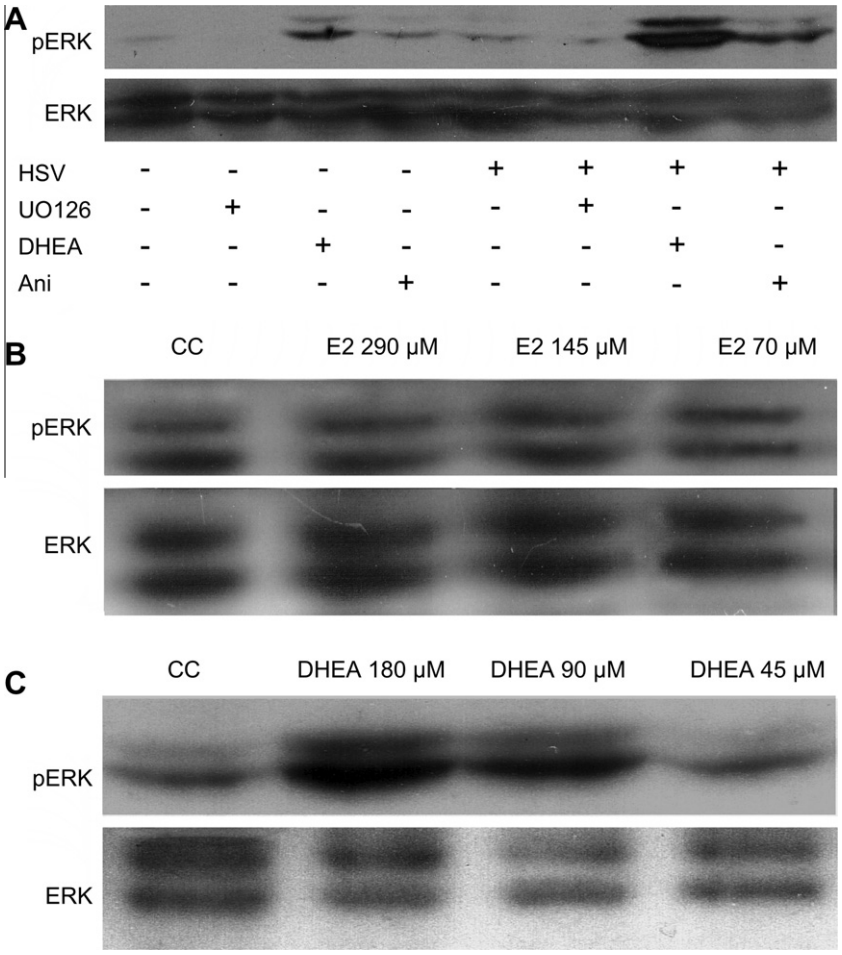


Fig. 5. Effect of DHEA, E2 and Raf/MEK/ERK pathway modulators on ERK1/2 phosphorylation in uninfected or HSV-1infected cells. (A) Vero cells were infected or not with HSV-1 at a m.o.i. of 1 and 1 h after infection the cultures were covered with MM (control) or MM containing DHEA (180 μM), UO126 (50 μM) or anisomycin (0.15 μM). Uninfected cultures were also treated with different concentrations of E2 (B) or DHEA (C). At 30 h p.i. cell lysates were collected and subjected to Western blotting. ERK1/2 activities were analyzed based on phosphorylated ERK1/2 (p-ERK1/2). To verify equal loading, Western blotting was performed with an antibody to ERK1. Data are representative of a set of three different experiments.

plaque assay. The results using different concentrations of anisomycin or UO126 are shown in Fig. 6 and Table 1. As expected, UO126 reduced viral titers in more than five logs when used at a concentration of 50 μM or higher, without exhibiting cytotoxic effect in any of the assayed concentrations. On the other hand, anisomycin increased viral titers in approximately 1 log when used at concentrations 0.15 μM or lower. As can be seen in Fig. 6A, anisomycin at a concentration of 22 μM produced an inhibition in viral titer of 99.9%. At such high concentrations, anisomycin is a well-known protein synthesis inhibitor; therefore, at this concentration, viral inhibition may be due to this activity (Ramabhadran and Thach, 1980).

3.6. Effect of DHEA, UO126 and anisomycin on the expression of HSV-1 antigens

In order to investigate the effect of the Raf/MEK/ERK pathway modulators and DHEA on the expression of HSV-1 proteins,

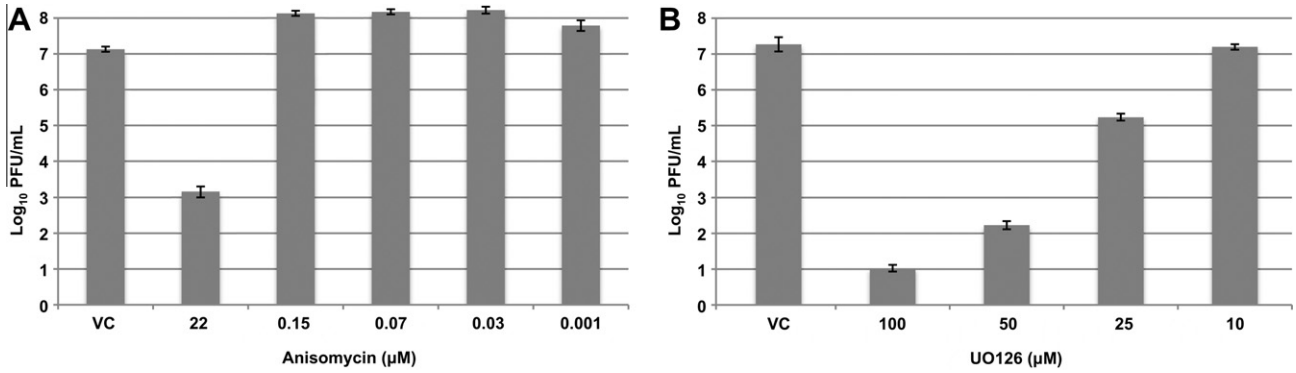


Fig. 6. Effect of UO126 and anisomycin on HSV-1 multiplication. Vero cells infected with HSV-1 (m.o.i. = 1) were incubated with different concentrations of UO126 (A) or anisomycin (B) at 30 h p.i. Supernatants were harvested and virus yields were determined by a plaque assay. Values are means ± SD from three independent experiments, in each of which titrations were carried out in duplicate.

mock-infected or infected Vero cells were treated with the compounds at 1 h p.i. and 30 h later an IFI assay was performed using a polyclonal antiserum against HSV-1 proteins. The number of HSV-1 infected cells were counted and expressed as the percentage of viral antigen positive cells. HSV-1 infected cultures, treated or not with anisomycin (0.15 μM), showed a characteristic pattern of small fluorescent foci and viral cytopathic effect (cellular rounding and detachment) (Fig. 7, Table 2). On the other hand, the number of fluorescent foci in UO126 (50 μM) and DHEA (180 μM) treated infected cultures, was reduced by 95.9%, 92.8%, respectively. Both compounds also prevented virus-induced cytopathic effect.

4. Discussion

In this study, we found that, two synthetic DHEA analogs (D3 and D6) and three synthetic EA analogs (E2, E5 and E9) exhibit selective inhibitory effect against HSV-1. Although in comparison with other antiviral agents like ACV the effective concentrations of the assayed compounds are high, considering the incidence of HSV infections and the reported emergence of ACV, PCV and FOS resistant mutants, the research to elucidate the molecular mechanism underlying the antiviral activity of these and other synthetic compounds is of great importance.

HSV-1 and other members of the *Herpesviridae* family induce ERK 1/2 phosphorylation (Sharma-Walia et al., 2005; Zhang et al., 2010; Qin et al., 2011). Our results are in accordance with these previous reports and supporting our findings, UO126 reduced HSV-1 titers in a concentration dependent manner and anisomycin, at concentrations that do not affect cellular protein synthesis, favored viral multiplication. According to Perkins et al. (2002), ICP10 HSV-2 protein has antiapoptotic activity in hippocampal neurons. MAPK activation induced by HSV-2 is required to block the apoptotic pathway since more than 50% of HSV-2 infected hippocampal neurons underwent apoptosis when treated with UO126. On the contrary, it is known that HSV-1 induces apoptosis in Vero cells (Nguyen et al., 2005). The inhibitory effect of UO126 in HSV-1 infected Vero cells could be due to apoptosis induction, which would result in a decrease in virus production without really affecting viral replication. However, the lack of cytopathic effect in these treated cultures (Fig. 7) suggests that apoptosis would not be responsible of the inhibitory effect of UO126. It should also be taken into account that several experimental evidences show that UO126 used at high concentrations would have other effects not directly related to inhibition of MEK protein kinases, for example, treatment with UO126 (12.5–50 μM) would promote the induction of cytochrome P450 in hepatocytes, whereas UO126 (80 μM) produces an increase in cellular AMP: ATP ratio in HEK cells, thus we cannot rule out that these effects would contribute to the inhibition of viral multiplication in Vero cells (Dokladda et al., 2005; Andrieux et al., 2004; Lee and Duesbery, 2010).

In addition, we found that ERK phosphorylation was induced mainly within the first hours after infection with HSV-1 and a sudden drop in ERK activation was evident at 7 h p.i. (Fig. 4). Recently, Qin et al. (2011) showed an increase in ERK phosphorylation at 12 h p.i. in HSV-1 infected BCBL-1 cells (Kaposi's Sarcoma-associated herpesvirus positive). Unfortunately, the authors have not examined earlier times p.i., thus it is difficult to compare both studies. Taking together these results, our findings suggest that the relationship between HSV-1 infection and the Raf/MEK/ERK pathway is of great importance for a productive infection.

Although present understanding of the antiviral activity of DHEA is limited, many in vivo and in vitro reports have demonstrated the ability of DHEA to inhibit the replication of both DNA and RNA viruses, such as feline immunodeficiency virus (Bradley et al., 1995), Epstein-Barr virus (Henderson et al., 1981), HIV-1 (Diallo et al., 2000; Mavoungou et al., 2005), coxackievirus B4 and influenza virus (Loria, 2002). However, they do not speculate about a possible mechanism of DHEA's antiviral activity. Chang et al. (2005) referred to the ability of DHEA to inhibit the in vitro replication of Japanese encephalitis virus (JEV). In this case, JEV infection resulted in an inhibition of the Raf/MEK/ERK signaling cascade, so the antiviral activity of DHEA was attributed to the ability of this compound to restore MAPK signaling functions. In this work, we found evidences suggesting that DHEA, despite being Raf/MEK/ERK activator, may not exert its antiviral activity against HSV-1 through the modulation of this pathway. We also demonstrated that, as well as DHEA, the synthetic analog E2 inhibits viral multiplication in a dose dependent manner without inhibiting early stages of viral replication. Western blot analysis showed a reduction in the expression of HSV-1 protein gD in infected cultures treated with DHEA or E2. Moreover, in our study we determined that the inhibition by DHEA and E2 of extracellular viral titers was stronger than the inhibition of total viral infectivity, suggesting that the antiherpetic activity of DHEA and its derivative may be in part due to an inhibition in virus release. Additionally, IFI analysis of DHEA and UO126 treated infected cells showed not only a reduction of viral titers, but also a diminishment of cytopathic effect and viral protein synthesis. Although DHEA and E2 seem to exhibit a similar mechanism of antiviral activity, the analog lacks the ability to modulate the Raf/MEK/ERK signaling pathway.

We previously showed that DHEA and E2 inhibit the multiplication of Junin arenavirus (Acosta et al., 2008), vesicular stomatitis virus (Romanutti et al., 2009) and adenovirus (Romanutti et al., 2010). Steroidal compounds modulate host cell metabolism

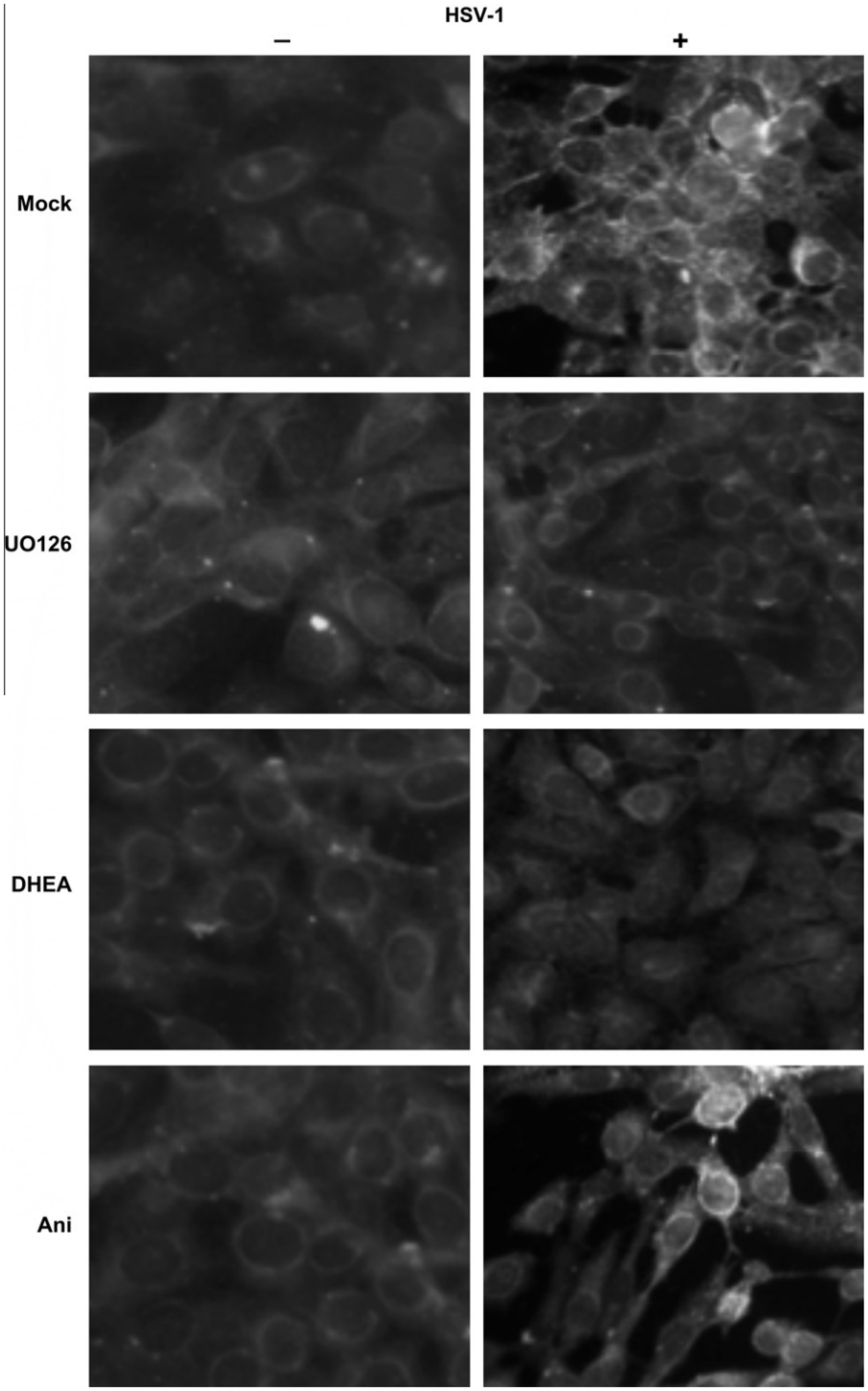


Fig. 7. Immunofluorescence analysis of HSV-1 infected cells treated with DHEA, UO126 or anisomycin. Vero cells infected with HSV-1 (m.o.i. = 1) were treated or not with DHEA (180 μM), UO126 (50 μM) or anisomycin (Ani) (0.15 μM). At 30 h p.i. monolayers were fixed and viral proteins were detected by total IFI. Data are from one of three different experiments.

through the regulation of different cell signaling pathways (Ashida et al., 2005; Jiang et al., 2005). Taking into account the broad antiviral spectrum of DHEA and its synthetic analog, we think that this kind of molecules could exert its antiviral activity affecting a cellular function required for viral replication and, to a lesser degree, a specific viral factor. Despite the fact that in this work we found that the antiviral activity of DHEA and one of the assayed derivatives seems to be similar, this does not imply that all the active derivatives have the same mechanism of action. The findings described here will support further study and design of new more effective derivatives.

Even though DHEA is a native steroid that has been used clinically with minimal side effects, administration of the steroid for extended time periods increases circulating testosterone and dihydrotestosterone manifold above normal levels, especially in women, and may cause masculinization (Labrie et al., 2003). Therefore, the evaluation of the antiviral activity of structural related compounds is useful in pursuit of an effective treatment for virus infections.

Table 2
Immunofluorescene analysis of HSV-1 infected Vero cells with different treatments.

Treatment	Fluorescent cells[a]/total cells	Percentage of fluorescent cells	Percentage inhibition of fluorescent cells
Mock	629/632	99.5	–
U0126 (50 μM)	34/857	4.0	96.0
DHEA (l80 μM)	75/1048	7.2	92.8
Anisomycin (0.15 μM)	647/652	99.2	–

[a] The number of fluorescent cells was obtained by counting 20 randomly selected fields.

5. Conclusions

We have found that, three synthetic EA analogs and two synthetic DHEA analogs exhibit a selective inhibitory effect against HSV-1. Additionally, HSV-1 manipulation of the Raf/MEK/ERK signaling pathway was studied. In this context, we found that UO126 and anisomycin, two Raf/MEK/ERK signaling pathway modulators, could inhibit or enhance viral multiplication, respectively. DHEA or E2 treatment resulted in an inhibition of viral protein synthesis and a reduction in virus release. DHEA, but not E2, showed a Raf/MEK/ERK modulator activity, however its antiviral activity against HSV-1 occurs via a mechanism independent of its ability to modulate ERK phosphorylation. Besides, DHEA showed a cytoprotective effect in infected cultures. Though additional studies are needed to understand the precise molecular mechanism involved in the antiviral activity of DHEA and its synthetic analogs, our findings provide a first step for future research.

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References

Acosta, E., Bruttomesso, A., Bisceglia, J., Wachsman, M., Galagovsky, L., Castilla, V., 2008. Dehydroepiandrosterone, epiandrosterone and synthetic derivatives inhibit Junin virus replication in vitro. Virus Res. 135, 203–212.

Andrade, A., Silva, P., Pereira, A., De Sousa, L., Ferreira, P., Gazzinelli, R., Kroon, E., Ropert, C., Bonjardim, C., 2004. The vaccinia virus-stimulated mitogen-activated protein kinase (MAPK) pathway is required for virus multiplication. Biochem J. 381, 437–446.

Andrieux, L., Langouët, S., Fautrel, A., Ezan, F., Krauser, J., Savouret, J., Guengerich, F., Baffet, G., Guillouzo, A., 2004. Aryl hydrocarbon receptor activation and cytochrome P450 1A induction by the mitogen-activated protein kinase inhibitor U0126 in hepatocytes. Mol. Pharmacol. 65, 934–943.

Ashida, K., Goto, K., Zhao, Y., Okabe, T., Yanase, T., Takayanagi, R., Nomura, M., Nawata, H., 2005. Dehydroepiandrosterone negatively regulates the p38 mitogen-activated protein kinase pathway by a novel mitogen-activated protein kinase phosphatase. Biochim. Biophys. Acta 1728, 84–94.

Azwa, A., Barton, S., 2009. Aspects of herpes simplex virus: a clinical review. J. Fam. Plann. Reprod. Health Care 35, 237–242.

Bradley, W., Kraus, L., Good, R., Day, N., 1995. Dehydroepiandrosterone inhibits replication of feline immunodeficiency virus in chronically infected cells. Vet. Immunol. Immunopathol. 46, 159–168.

Cai, Y., Liu, Y., Zhang, X., 2007. Suppression of Coronavirus Replication by Inhibition of the MEK Signaling Pathway. J. Virol. 81, 446–456.

Chambard, J., Lefloch, R., Pouysségur, J., Lenormand, P., 2006. ERK implication in cell cycle regulation. Biochim. Biophys. Acta 1773, 1299–1310.

Chang, C., Ou, Y., Raung, S., Chen, C., 2005. Antiviral effect of dehydroepiandroster one on Japanese encephalitis virus infection. J. Gen. Virol. 86, 2513–2523.

Chang, N., Schultz, L., Heath, J., 2000. Suppression of IκBα expresión is necessary for c-Jun N-terminal kinase-mediated enhancement of Fas cytotoxicity. Biochem. Biophys. Res. Commun. 274, 4–10.

Chilukuri, S., Rosen, T., 2003. Management of acyclovir-resistant herpes simplex virus. Dermatol. Clin. 21, 311–320.

Dalla Valle, L., Couet, J., Labrie, Y., Simard, J., Belvedere, P., Simontacchi, C., 1995. Occurrence of cytochrome P450c17 mRNA and dehydroepiandrosterone biosynthesis in the rat gastrointestinal tract. Mol. Cell Endocrinol. 111, 83–92.

Dawson, W., Laverick, L., Morris, M., Tramoutanis, G., Young, L., 2008. Epstein-Barr virus-encoded LMP1 regulates epithelial cell motility and invasion via the ERK–MAPK pathway. J. Virol. 82, 3654–3664.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. J. Immunol. Methods 89, 271–277.

Dhawan, P., Bell, A., Kumar, A., Golden, C., Mehta, K., 1999. Critical role of p42/44 (MAPK) activation in anisomycin and hepatocyte growth factor-induced LDL receptor expression: activation of Raf-1/MEK-1/p42/44 (MAPK) cascade alone is sufficient to induce LDL receptor expression. J. Lipid Res. 40, 1911–1919.

Diallo, K., Loemba, H., Oliveira, M., Mavoungou, D., Wainberg, M., 2000. Inhibition of human immunodeficiency virus type-1 (HIV-1) replication by immunor (IM28), a new analog of dehydroepiandrosterone. Nucleosides Nucleotides Nucleic Acids 19, 2019–2024.

Dokladda, K., Green, K., Pan, D., Hardie, D., 2005. PD98059 and U0126 activate AMP-activated protein kinase by increasing the cellular AMP:ATP ratio and not via inhibition of the MAP kinase pathway. FEBS Lett. 579, 236–240.

Favata, M., Horiuchi, K., Manos, E., Daulerio, A., Stradley, D., Feeser, W., Van Dyk, D., Pitts, W., Earl, R., Hobbs, F., Copeland, R., Magolda, R., Scherle, P., Trzaskos, J., 1998. Identification of a novel inhibitor of mitogen-activated protein kinase. J. Biol. Chem. 273, 18623–18632.

Field, H., 2001. Herpes simplex virus antiviral drug resistance-current trends and future prospects. J. Clin. Virol. 21, 261–269.

Formoso, G., Chen, H., Kim, J., Montagnani, M., Consoli, A., Quon, M., 2006. Dehydroepiandrosterone mimics acute actions of insulin to stimulate production of both nitric oxide and endothelin 1 via distinct phosphatidylinositol 3-kinase- and mitogen-activated protein kinase-dependent pathways in vascular endothelium. Mol. Endocrinol. 20, 1153–1163.

Freeman, E., Weiss, H., Glynn, J., Cross, P., Whitworth, J., Hayes, R., 2006. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. AIDS 20, 73–83.

Hansen, J., Moore, P., Steitz, T., 2003. Structures of Five Antibiotics Bound at the Peptidyl Transferase Center of the Large Ribosomal Subunit. J. Mol. Biol. 330, 1061–1075.

Hazzalin, C., Le Panse, R., Cano, E., Mahadevan, L., 1998. Anisomycin Selectively Desensitizes Signalling Components Involved in Stress Kinase Activation and fos and jun Induction. Mol. Cell Biol. 18, 1844–1854.

Henderson, E., Schwartz, A., Pashko, L., Abou-Gharbia, M., Swern, D., 1981. Dehydroepiandrosterone and 16 alpha-bromo-epiandrosterone: inhibitors of Epstein-Barr virus-induced transformation of human lymphocytes. Carcinogenesis 2, 683–686.

Henderson, E., Yang, J., Schwartz, A., 1992. Dehydroepiandrosterone (DHEA) and synthetic DHEA analogs are modest inhibitors of HIV-1 IIIB replication. AIDS Res. Hum. Retroviruses 8, 625–631.

Hill, C., McKinney, E., Lowndes, C., Munro, H., Murphy, G., Parry, J., Gill, O., the GUM Anon Network, 2009. Epidemiology of herpes simplex virus types 2 and 1 amongst men who have sex with men attending sexual health clinics in England and Wales: implications for HIV prevention and Management. Euro Surveill 14 (47), pii = 19418. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19418>.

Jiang, Y., Miyazaki, T., Honda, A., Hirayama, T., Yoshida, S., Tanaka, N., Matsuzaki, Y., 2005. Apoptosis and inhibition of the phosphatidylinositol 3-kinase/Akt signaling pathway in the anti-proliferative actions of dehydroepiandrosterone. J. Gastroenterol. 40, 490–497.

Labrie, F., Luu-The, V., Labrie, C., Bélanger, A., Simard, J., Lin, S., Pelletier, G., 2003. Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. Endocr. Rev. 24, 152–182.

Lee, C., Duesbery, N., 2010. Highly Selective MEK Inhibitors. Curr. Enzyme Inhib. 6, 146–157.

Lee, Y., Lee, C., 2010. Porcine reproductive and respiratory syndrome virus replication is suppressed by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. Virus Res. 152, 50–58.

Liu, D., Iruthayanathan, M., Homan, L., Wang, Y., Yang, L., Wang, Y., Dillon, J., 2007. Dehydroepiandrosterone stimulates endothelial proliferation and angiogenesis through extracellular signal-regulated kinase 1/2-mediated mechanisms. Endocrinology 149, 889–898.

Loria, R., Inge, T., Cook, S., Szakal, A., Regelson, W., 1988. Protection against acute lethal viral infections with the native steroid dehydroepiandrosterone (DHEA). J. Med. Virol. 26, 301–314.

Loria, R., 2002. Immune up-regulation and tumor apoptosis by androstene steroids. Steroids 67, 953–966.

Mavoungou, D., Poaty-Mavoungou, V., Akoume, M., Ongali, B., Mavoungou, E., 2005. Inhibition of human immunodeficiency virus type-1 (HIV-1) glycoprotein-mediated cell–cell fusion by immunor (IM28). Virol. J. 11, 9–10.

McCubrey, J., Steelman, L., Chappell, W., Abrams, S., Wong, E., Chang, F., Lehmann, B., Terrian, D., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A., Franklin, R., 2007. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim. Biophys. Acta 1773, 1263–1284.

Morfin, F., Thouvenot, D., 2003. Herpes simplex virus resistance to antiviral drugs. J. Clin. Virol. 26, 29–37.
Mujugira, A., Magaret Am Baeten, J., Celum, C., Lingappa, J., 2011. Risk factors for HSV-2 infection among sexual partners of HSV-2/HIV-1 co-infected persons. BMC Res. Notes 4, 64.
Newton, R., Cambridge, L., Lorraine, A., Stevens, D., Lindsay, M., Barnes, P., 2000. The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1β-dependent PGE_2 release *via* mechanistically distinct processes. Br. J. Pharmacol. 130, 1353–1361.
Nguyen, M., Kraft, R., Blaho, J., 2005. African green monkey kidney Vero cells require de novo protein synthesis for efficient herpes simplex virus 1-dependent apoptosis. Virology 336, 274–290.
Pan, W., Bodempudi, V., Esfandyari, T., Farassati, F., 2009. Utilizing Ras Signaling Pathway to Direct Selective Replication of Herpes Simplex Virus-1. PLoS One 4 (8), e6514. http://dx.doi.org/10.1371/journal.pone.0006514.
Pedersen, N., North, T., Rigg, R., Reading, C., Higgins, J., Leutenegger, C., Henderson, G., 2003. 16alpha-Bromo-epiandrosterone therapy modulates experimental feline immunodeficiency virus viremia: initial enhancement leading to long-term suppression. Vet. Immunol. Immunopathol. 94, 133–148.
Perkins, D., Pereira, E., Gober, M., Yarowsky, P., Aurelian, L., 2002. The herpes simplex virus type 2 R1 protein kinase (ICP10 PK) blocks apoptosis in hippocampal neurons, involving activation of the MEK/MAPK survival pathway. J. Virol. 76, 1435–1449.
Piret, J., Boivin, G., 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrob. Agents Chemother. 55, 459–472.
Qin, D., Feng, N., Fan, W., Ma, X., Yan, Q., Lv, Z., Zeng, Y., Zhu, J., Lu, C., 2011. Activation of PI3K/AKT and ERK MAPK signal pathways is required for the induction of lytic cycle replication of Kaposi's Sarcoma-associated herpesvirus by herpes simplex virus type 1. BMC Microbiol. 11, 240.
Ramabhadran, T., Thach, R., 1980. Specificity of protein synthesis inhibitors in the inhibition of encephalomyocarditis virus replication. J. Virol. 34, 293–296.
Romanutti, C., Bruttomesso, A., Bisceglia, J., Castilla, V., Galagovsky, L., Wachsman, M., 2009. In vitro antiviral activity of dehydroepiandrosterone and synthetic derivatives against vesicular stomatitis virus. Vet. J. 182, 327–335.
Romanutti, C., Bruttomesso, A., Castilla, V., Galagovsky, L., Wachsman, M., 2010. Anti-adenovirus activity of epiandrosterone and dehydroepiandrosterone derivatives. Chemotherapy 56, 158–165.
Sharma-Walia, N., Krishnan, H., Naranatt, P., Zeng, L., Smith, M., Chandran, B., 2005. ERK1/2 and MEK1/2 Induced by Kaposi's Sarcoma-associated herpesvirus (human herpesvirus 8) early during infection of target cells are essential for expression of viral genes and for establishment of infection. J. Virol. 79, 10308–10329.
Shin, J., Cai, G., Weinberg, A., Leary, J., Levin, M., 2001. Frequency of acyclovir-resistant herpes simplex virus in clinical specimens and laboratory isolates. J. Clin. Microbiol. 39, 913–917.
Steiner, I., Kennedy, P., Pachner, A., 2007. The neurotropic herpes viruses: herpes simplex and varicella-zoster. Lancet Neurol. 6, 1015–1028.
Sturgill, T., 2008. MAP kinase: it's been longer than fifteen minutes. Biochem. Biophys. Res. Commun. 371, 1–4.
Talarico, L., Ramirez, J., Galagovsky, L., Wachsman, M., 2002. Structure–activity relationship studies in a set of new brassinosteroid derivatives assayed against herpes simples virus type 1 and 2 in cell cultures. Med. Chem. Res. 11, 434–444.
Talarico, L., Castilla, V., Rámirez, J., Galagovsky, L., Wachsman, M., 2006. Synergistic in vitro interactions between (22S,23S)-3-bromo-5,22,23-trihydroxystigmastan-6-one and foscarnet against herpes simples virus type 1. Chemotherapy 52, 38–42.
Van Vollenhoven, R., 2002. Dehydroepiandrosterone for the treatment of systemic lupus erythematosus. Expert Opin. Pharmacother. 3, 23–31.
Wachsman, M., López, E., Ramírez, J., Galagovsky, L., Coto, C., 2000. Antiviral effect of brassinosteroids against herpes virus and arena viruses. Antiviral Chem. Chemother. 11, 71–77.
Wachsman, M., Castilla, V., Talarico, L., Ramirez, J., Galagovsky, L., Coto, C., 2004. Antiherpetic mode of action of (22S,23S)-3β-bromo-5α,22,23-trihydroxystigmastan 6-one in-vitro. Int. J. Antimicrob. Agents 23, 525–527.
Whitley, R., Roizman, B., 2001. Herpes simplex virus infections. Lancet 357, 1513–1518.
Zhang, H., Feng, H., Luo, L., Zhou, Q., Luo, Z., Peng, Y., 2010. Distinct effects of knocking down MEK1 and MEK2 on replication of herpes simplex virus type 2. Virus Res. 150, 22–27.