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Anti-Adenovirus Activity of Epiandrosterone and Dehydroepiandrosterone Derivatives

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Key Words
Antiviral activity · Dehydroepiandrosterone analogues · DHEA · Adenovirus · Anti-adenovirus activity

Abstract
Background: Dehydroepiandrosterone (DHEA) exhibits a wide range of biological functions including antiviral activity. In this work, we present in vitro anti-adenovirus (AdV) activity of seven DHEA and twelve epiandrosterone (EA) analogues. ***Methods:*** The cytotoxic effect of the compounds was determined by the MTT assay and the antiviral activity by a virus yield inhibition assay. The mode of antiviral activity was examined using time-of-addition experiments, adsorption and internalization assays and Western blot analysis. ***Results:*** EA, DHEA, and two synthetic derivatives inhibit virus replication with selectivity indices ranging between 42 and 83. Virus adsorption and internalization are not the target of the inhibitory action; meanwhile, AdV protein synthesis was diminished in the presence of DHEA. ***Conclusions:*** DHEA and some synthetic derivatives present antiviral activity similar to cidofovir, which was used as reference drug. These steroidal compounds adversely affect virus protein synthesis and viral mature particle formation.

Introduction

The incidence and severity of diseases caused by adenoviruses (AdVs) have increased in recent years mainly in immunocompromised individuals owing to the inability of the adaptive immune system to fight diseases [1–3].

Currently, there is no formally approved antiviral therapy for AdV infections. Several compounds of dissimilar chemical structures were evaluated for their effectiveness in vitro and in virus-infected patients, but the results appeared to be inconclusive [4–8]. The nucleotide analogue cidofovir was found to be beneficial in several small-scale studies involving patients with life-threatening AdV infections [5, 7, 9]. On the other hand, studies on the nucleoside analogue ribavirin have yielded conflicting results [7, 10, 11].

Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, DHEA) is one of the most abundant steroids in the 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 steroids [12, 13]. In addition, its positive effects in the treatment of several diseases, e.g. cancer, viral infections, and immune disorders such as systemic lupus erythematosus, have been proven [14–16]. Despite the possible clinical utility of this hormone, serious limi-

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tations restrict its usage because high doses have to be administered. Although it seems to be less cytotoxic than other drugs, administration for extended periods increases circulating testosterone and dihydrotestosterone, especially in women, and may cause masculinization [17, 18]. Many current investigations aim to identify DHEA analogues that may exert beneficial effects but lack the negative effects of sexual hormones; particularly, some analogues have been tested as antiviral compounds in vitro and in vivo [16, 19–24].

On the other hand, the 17-ketosteroid epiandrosterone (5α-androstane-3β-ol-17-one; EA) is an inactive isomer of androsterone; it is formed in the peripheral tissue, from which it is released into the circulation and is ultimately excreted into the urine. Similar to DHEA, it is also found in testicular and ovarian tissue, but in contrast to DHEA, EA is only a weak androgen [25].

In this work, we present the in vitro antiviral activity of seven DHEA and twelve EA analogues against AdV and their possible mode of action.

Materials and Methods

Compounds

DHEA and EA were purchased from Sigma-Aldrich (St. Louis, Mo., USA). The compounds D1–D7 (obtained from DHEA) and E1–E12 (obtained from EA) were prepared in our laboratory. They were purified by flash chromatography on silica and identified by electron impact mass spectra, infrared (IR) and ^{1}H and ^{13}C NMR spectroscopy. Electron impact mass spectra were measured in a VG Trio-2 or in a Shimadzu QP-5000 mass spectrometer at 70 eV by direct inlet. IR spectra were recorded in KBr pellets on a Nicolet Magna IR 550 FT spectrometer. ^{1}H and ^{13}C NMR were measured in $CDCl_3$ and $CDCl_3/D_3COD$ with tetramethylsilane as internal standard using a Bruker-AC-200 spectrometer. All solvents and reagents were of analytical grade.

Descriptions of the synthesis of compounds E5, E12 [26], E10 and E11 [27] were revisited seeking to optimize yields. Briefly, bromination of the acetal E8 with pyridinium bromide perbromide in tetrahydrofuran provided the 16α-bromo acetal (16α-bromo-17,17-ethylenedioxy-5α-androstan-3β-yl acetate) with a 84% yield [28], and followed by deacetylation with KOH diluted in methanol-toluene water (5:1:1) resulting in E7 (16α-bromo-17,17-ethylenedioxy-5α-androstan-3β-ol) with a 90% yield. Dehydrobromination was achieved in 83% with potassium t-butoxide in dimethylsulfoxide at 40°C overnight to give E5 (17,17-ethylenedioxy-5α-androst-15-en-3β-ol). Mild hydrolysis of E5 with *p*-toluenesulfonic acid in aqueous acetone (10:1) at room temperature afforded 75% pure 15,16-dehydroketone E12 (3β-hydroxy-5α-androst-15-en-17-one) after column chromatography. Thus the overall yield for E12 was 48%; alternative synthesis provides poor results [26, 27]. Conjugated addition of water on compound E12 was achieved under vigorous hydrolytic conditions by recircling it overnight with *p*-toluenesulfonic acid in aqueous acetone (5:1). A mixture of two epimers E11 (3β,15α-dihydroxy-5α-androstan-17-one) and E10 (3β,15β-dihydroxy-5α-androstan-17-one) in a ratio of 1:9 (85% total yield) was obtained.

Compounds D1–D7, E1–E4, and E6–E9 were synthesized according to previous reports [29–33]. A complete ^{1}H NMR and ^{13}C NMR spectral characterization of all compounds was in accordance with previously published data. The chemical structures and code numbers are shown in table 1. Stock solutions were prepared in ethanol, stored at –20°C, and further diluted immediately before use.

(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir) was purchased from Sigma (St. Louis, Mo., USA).

Cells and Viruses

Human lung fibroblastic A549 cells were grown as monolayers in minimum essential medium (Gibco, Carlsbad, Calif., USA) supplemented with 10% inactivated fetal calf serum and 50 μg/ml gentamycin. Maintenance medium (MM) consisted of minimal essential medium containing 2% inactivated fetal calf serum.

AdV type 5 (AdV5) was obtained from American Type Culture Collection (Rockville, Md., USA). Virus stocks were prepared in A549 cells.

Cytotoxicity Assays

To determine cytotoxic concentrations of the compounds, monolayers of A549 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 examined by the ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; Sigma] by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) [34]. The precise MTT procedure has been described elsewhere [35, 36].

Another set of Vero cells was seeded in 96-well tissue culture plates and after 20 h (when the monolayer was not yet confluent) the medium was exchanged with growth medium containing different concentrations of DHEA. Cells were further incubated at 37°C and after 3 days, when the cell number in the control wells (counted with a hemocytometer) increased from 1.2 to 8.7 $\times$ 10^4 cells/well, the cytotoxicity of DHEA on growing cells was determined using the MTT assay, as described above.

The CC_{50} was defined as the compound concentration (μM) that reduced cell viability by 50%. These values were calculated by regression analysis.

Virus Yield Reduction Assay

Antiviral activity was evaluated by the virus yield reduction assay. For that purpose, A549 cells grown in 24-well culture plates for 48 h were infected with AdV at a multiplicity of infection (MOI) of 1. After 1 h of adsorption at 37°C, cells were covered with MM containing varying concentrations of the compounds. After 24 h of incubation at 37°C, AdV-infected cultures were subjected to two freeze-thawing cycles followed by centrifugation at low speed (10,000 *g*) and the supernatants were titrated by a plaque assay. Antiviral activity was expressed as EC_{50} (50% effective concentration) or compound concentration required to reduce AdV plaque formation after 7 days by 50% compared with the untreated infected cultures. The EC_{50} values were calculated by plotting percentages of inhibition versus different concentrations of each compound.

Table 1. Cytotoxicity and antiviral activity of DHEA, EA, and their synthetic derivatives against AdV

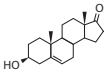
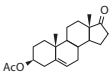
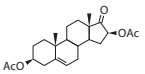
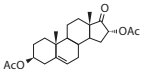
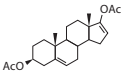
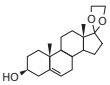
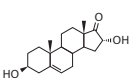
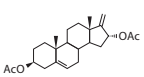
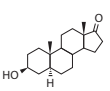
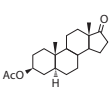
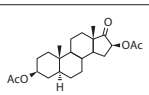
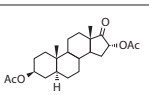
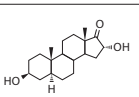
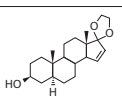
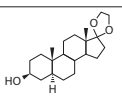
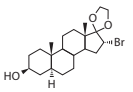
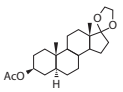
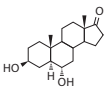
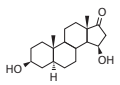
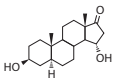
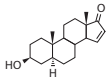
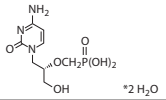
Compound	Structural formula	IUPAC name	CC_{50}, μM	EC_{50}, μM	SI
DHEA		3β-hydroxyandrost-5-en-17-one	5,530	88	63
D1		17-oxoandrost-5-en-3β-yl acetate	1,846	146	13
D2		17-oxoandrost-5-en-3β,16α-diyl diacetate	2,172	263	8.2
D3		17-oxoandrost-5-en-3β,16α-diyl diacetate	1,546	73	21
D4		androsta-5,16-dien-3β,17-diyl diacetate	774	43	18
D5		17,17-ethylendioxyandrost-5-en-3β-ol	>3,399	>1,804[a]	I
D6		3β,16α-dihydroxyandrost-5-en-17-one	821	39	21
D7		21-norpregna-5,17(20)-dien-3β,16α-diyl diacetate	183	2.3	79
EA		3β-hydroxy-5α-androstan-17-one	6,766	81	83
E1		17-oxo-5α-androstan-3β-yl acetate	534	69	7.7
E2		17-oxo-5α-androstan-3β,16β-diyl diacetate	645	289	2.2
E3		17-oxo-5α-androstan-3β,16α-diyl diacetate	1,933	90	21
E4		3β,16α-dihydroxy-5α-androstan-17-one	489	304	1.6
E5		17,17-ethylendioxy-5α-androst-15-en-3β-ol	3,002	127	24
E6		17,17-ethylendioxy-5α-androstan-3β-ol	1,973	>60[a]	I

Table 1 (continued)

Compound	Structural formula	IUPAC name	CC_{50}, μM	EC_{50}, μM	SI
E7		17,17-ethylendioxy-5α-androstan-16α-bromo-3β-ol	3,938	443	8.9
E8		17,17-ethylendioxy-5α-androstan-3β-yl acetate	876	38	23
E9		3β,6α-dihydroxy-5α-androstan-17-one	2,907	163	18
E10		3β,15β-dihydroxy-5α-androstan-17-one	972	104	9.4
E11		3β,15α-dihydroxy-5α-androstan-17-one	751	117	6.4
E12		3β-hydroxy-5α-androst-15-en-17-one	304	7.3	42
Cidofovir		(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine	2,264	34	67

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 = CC_{50}/EC_{50} ratio; I = inactive. Data are means from two independent experiments.

[a] Virus yield inhibition was <50% within the range of compound concentrations tested.

Virucidal Effect

To establish if DHEA and EA produced a virucidal effect, 10^7 plaque-forming units (PFU) of AdV were diluted in culture medium containing or not each compound (2,000 μM) and incubated for 0, 30, 60 or 90 min at 37°C. At the indicated times, aliquots were diluted in MM to a non-inhibitory drug concentration and titrated by plaque assay.

Effect of Time of DHEA Addition on AdV Production

DHEA (200 μM) was added to confluent monolayers of A549 cells infected with AdV at an MOI of 1 PFU/cell, at time 0 of infection (simultaneously with virus inoculum), or 2, 4, 6 and 24 h after infection. Cultures were further incubated at 37°C till 48 h after infection and at that time, extracellular virus yields were measured from supernatants by plaque assay on A549 cells. To determine total infectivity, cells were subjected to two cycles of freeze-thawing followed by centrifugation at low speed, and the supernatants obtained were titrated by plaque assay.

Adsorption and Penetration Assay

To determine the effect of DHEA (200 μM) on viral adsorption and penetration, about 100 PFU of AdV were adsorbed for 1 h at 4°C on confluent A549 cells grown in 24-well plates (2×10^5 cells) in the presence or absence of the compound. 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 time periods in the presence or absence of DHEA. 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 washing twice with PBS, cultures were overlaid with MM containing 0.7% methylcellulose, and after 7 days of incubation at 37°C the plaque numbers were counted.

Western Blotting

Monolayers of confluent A549 cells were infected with AdV type 5 (MOI = 5 PFU/cell), and cultures were incubated at 37°C for 2 h to allow virus internalization. After removal of the inocula, monolayers were covered with MM or MM containing DHEA (200 μM) and incubated at 37°C for 24 h. Immediately after the 24-hour incubation with or without the compound, cells in duplicate wells were lysed with SDS-PAGE loading buffer

(0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue). Samples were heated for 2 min in boiling water before loading onto 10% acrylamide gels. Following electrophoresis, the resolved proteins were transferred to a PVDF membrane (Perkin Elmer Life Sciences, Waltham, Mass., USA) in a dry system (LKB, Multiphor II). Viral core protein (V) was revealed with mouse anti-AdV monoclonal antibody 805 blend (Chemicon, Temecula Calif., USA) and a peroxidase anti-mouse IgG (ICN Biologicals, Aurora, Ohio, USA) as secondary antibody and visualized using a chemiluminescence kit (Perkin Elmer Life Sciences). After performing the stripping procedure according to the manufacturer's instructions, the detection of actin in each sample was done using a mouse monoclonal antibody anti-actin (JLA20, Calbiochem, Darmstadt, Germany) and a peroxidase anti-mouse IgG (ICN Biologicals) as secondary antibody.

Results

Anti-AdV Activity of DHEA, EA, and Their Derivatives

The synthetic EA and DHEA derivatives were tested for their potential to inhibit AdV multiplication in cell cultures (table 1). For comparative purposes, cidofovir, reported to be active against AdV [5–7, 9], was tested as positive control.

First, the CC_{50} was determined using confluent non-growing A549 cells after 48 h of incubation at 37°C (table 1). The cytotoxicity of the derivatives was determined by the MTT colorimetric assay.

DHEA, EA, and E7 were less cytotoxic than the other compounds tested. The presence of other functional groups on C3, C15, C16, or C17 increased cytotoxicity; however, 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 (compounds D2 and E2) did not significantly affect cytotoxicity, but higher compound concentrations were needed to inhibit AdV. However, replacement of the β-acetyl group by an α-acetyl group on C16 (compounds D3 and E3**)** restored CC_{50} to values which were about 2.6 times lower than those obtained with DHEA and EA, although a slight increase in EC_{50} values was also observed. The presence of a β-acetyl group on C-17 instead of a ketone group and a double bond in C16–C17 also increased cytotoxicity 5.5 times (compound D4) compared with DHEA. On the other hand, the presence of 3β and 16α acetyl groups and a methylene group in C17 (compound D7) significantly reduced the CC_{50} and EC_{50} values resulting in a selectivity index (SI) which is slightly higher than those obtained with DHEA and cidofovir. The presence of an α-hydroxy group in C16 also resulted in a decreased CC_{50} value with respect to DHEA and EA, respectively (compounds D6 and E4). D5 and E6, which are protected C17 ketone derivatives of DHEA and EA, respectively, were inactive. However, compounds E7 with a 16α bromo group and E8 with a 3β acetyl group (both protected C17 ketone derivatives) presented SI values of 8.9 and 23, respectively. The presence of a hydroxyl group in different C in α or β position (compounds E9–E11) did not increase the SI with respect to EA. Meanwhile, a double bond between C15–C16 (compound E12) gave a low CC_{50} value but also a high reduction in the EC_{50} value. We determined, under our experimental conditions, that EA, DHEA, D7, and E12 presented similar or higher SI values than those obtained with cidofovir used as reference drug (table 1).

In order to analyze the cytotoxicity of DHEA using a more rigorous test, we determined the concentration of the compound required to inhibit cellular replication using cells in a growing state. The CC_{50} value obtained was 2,800 μM, which is two times lower than the CC_{50} value calculated using confluent non-growing cells (table 1), indicating that DHEA exhibits a moderate inhibitory effect on cell growth. However, under cell-growing conditions DHEA displays similar cytotoxicity to cidofovir on confluent monolayers.

Effect of DHEA on AdV Infectious Particle Production

DHEA and EA inhibited the multiplication of AdV in confluent A549 cells in a dose-dependent manner. To establish if these compounds produced a direct effect on the viral particle, we performed a virucidal assay. Treatment of viral suspensions with DHEA or EA did not affect viral infectivity, indicating that the antiviral action of these compounds is not due to the inactivation of virus particles (data not shown).

To further characterize the inhibitory action, a time-of-addition experiment was performed. For that purpose, DHEA (200 μM) was added to AdV-infected A549 cells at different times after infection, and 48 h after infection, cell-free and total infectivity were determined. In control untreated cultures, 24 h after infection (last time point at which DHEA was added) only few cells showed a cytopathic effect (cell rounding and nuclear inclusion bodies observed in Giemsa-stained cultures) whereas 48 h after infection (time at which supernatants were harvested), 90% of the cells displayed a cytopathic effect (data not shown). As can be seen in figure 1, yields of AdV-released virus were reduced by 2 logs when the compound was

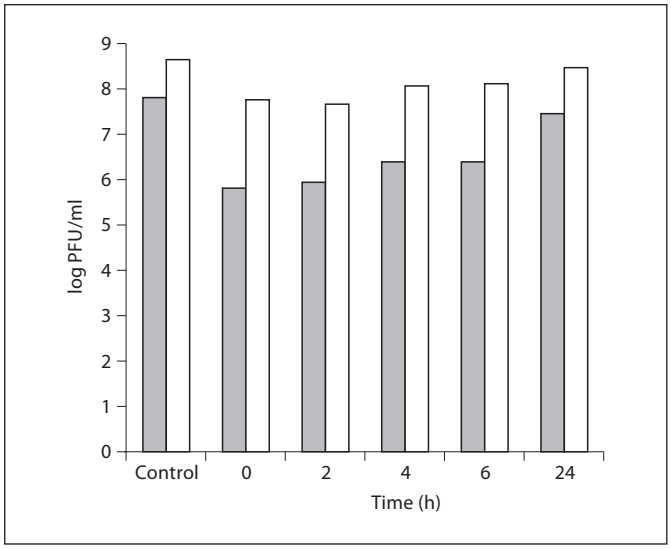


Fig. 1. Effect of time of DHEA addition on AdV replication. A549 cells infected with AdV (MOI = 1) were incubated with MM (control) or MM containing DHEA (200 μM) at different times: time 0 (with virus inoculum), and 2, 4, 6 or 24 h after infection; 48 h after infection, cell-free (gray bars) and total (white bars) virus yields were determined by plaque assay.

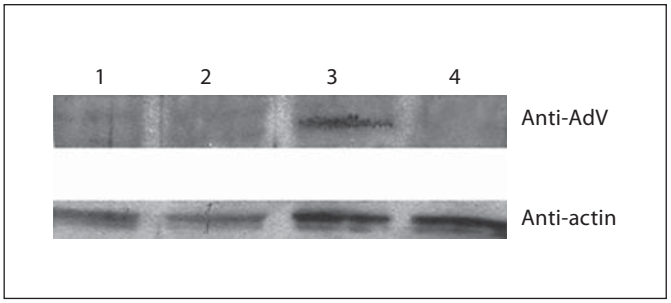


Fig. 2. Effect of DHEA on AdV protein synthesis. A549 cells were infected with AdV (MOI = 5 PFU/cell) and cultures were incubated at 37°C for 2 h to allow virus internalization. After removal of the inocula, monolayers were covered with MM or MM containing DHEA (200 μM) and incubated at 37°C for 24 h. Immediately after the 24-hour incubation in the presence or absence of the compound, cells were lysed with SDS-PAGE loading buffer. All samples were subjected to SDS-PAGE and viral core protein and cellular actin were revealed by Western blot. 1 = Uninfected cells; 2 = uninfected treated cultures; 3 = untreated infected cultures; 4 = DHEA-treated infected cultures.

added during or 2 h after infection, and by approximately 1 log when DHEA was added 4 h after infection or later. Meanwhile, total virus yields were also reduced mainly when DHEA was present at time 0 or 2 h after infection (fig. 1), indicating that the compound may affect both the formation and the release of infectious particles to the extracellular medium.

To determine if DHEA interferes with early events of the virus multiplication cycle, we analyzed the effect of the compound on virus adsorption and penetration, as described in Materials and Methods. No differences in the amount of adsorbed or internalized virus were observed in both treated and untreated infected control cells (data not shown).

Effect of DHEA on AdV Protein Synthesis

Next, we studied the effect of DHEA on AdV protein synthesis. To this end, cells infected with AdV at an MOI of 5, treated or not with the compound (200 μM), were analyzed by Western blot assay using a monoclonal antibody for the detection of AdV core protein (V). As can be seen in figure 2, the viral protein level was markedly decreased in DHEA-treated cultures (lane 4) compared with untreated infected ones (lane 3), indicating that DHEA affects viral particle formation.

Discussion

In this study, we determine that DHEA, EA, D7, and E12 exhibited in vitro SI values for AdV which were similar to those obtained with cidofovir used as reference drug. The low oral bioavailability and the nephrotoxicity associated with cidofovir are reasons to spur the quest for alternative therapies for AdV infections. The SI values obtained with these four compounds are higher than those reported by other authors using compounds with dissimilar chemical structures [5–8], indicating that these steroids show promise. Other studies on the antiviral activity of this kind of steroids have also been reported. Pedersen et al. [16] demonstrated that a 16α bromine EA analogue was effective for the treatment of acute feline immunodeficiency virus infection in laboratory cats. It has also been reported that DHEA treatment results in a significantly reduced mortality in infected mice with coxsackie B virus, herpes simplex type 2, or influenza virus [14, 37, 38]. In more recent reports, a potent new analogue of DHEA was able to prevent cell-cell fusion, an important step at the beginning of human immunodeficiency virus infection of naive cells [21, 22]. It was also demonstrated that DHEA inhibited the multiplication of Japanese encephalitis virus, Junin virus (JUNV) and vesicular stomatitis virus (VSV) in cell cultures. Furthermore, beside DHEA, EA and E3 inhibited JUNV, VSV

and AdV in a similar way. We have previously demonstrated that D7 and E12 exhibited an inhibitory effect against VSV and JUNV, respectively, indicating that the most active anti-AdV analogues display a broad spectrum of antiviral action [23, 24, 39].

This study evidenced that the antiviral effect of DHEA and EA could not be attributed to direct inactivation of virus particles. Time-of-addition experiments revealed that DHEA exerts an adverse effect on viral release and prevents viral particle formation. The observed reduction was not caused by an impairment in virus adsorption or penetration into the cell. In contrast, we determined that DHEA inhibits AdV protein synthesis at concentrations that do not affect cellular protein synthesis.

We cannot rule out that DHEA, EA, or their derivatives would exert their antiviral action on a specific viral factor and/or – to a lesser degree – a cellular function required for viral replication. An interesting possibility to analyze is the study of the effect of DHEA on the cell signal transduction system, such as mitogen-activated protein kinases (MAPKs). It has been reported that DHEA induces a p38 MAPK phosphatase that suppresses the p38-MAPK cascade [40]. Controversial results have been reported about the action of DHEA on the extracellular stimulus-regulated kinases (ERK)/MAPK pathway in different cell systems. In certain cell lines, DHEA induces ERK phosphorylation [39] whereas in other cell types DHEA treatment inhibits ERK activation [41]. Several viruses, including AdVs, not only induce ERK phosphorylation but also seem to depend on it, and it was proven that the levels of AdV protein synthesis are strongly reduced by inhibitors of ERK activation [42], so a possible explanation for anti-AdV DHEA activity may be related with the ability of the steroid to modulate MAPK activation induced by virus infection. Studies to explore this possibility are underway in order to elucidate this indirect antiviral effect of DHEA against AdV.

In conclusion, EA, DHEA, and some of their derivatives evaluated in this study represent an important class of compounds that have been found to be active against AdV at concentrations that were not toxic to the host cells. However, further modifications, based on structure-activity relationship studies, are needed to develop more potent and selective viral inhibitors. The mechanism underlying their antiviral mode of action and their relation with the host cell signal transduction system should also be further explored.

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