



## *In Vitro* Inhibitory Effects of the Herbal-Marine Compound HESA-A Against Replication of Human Immunodeficiency Virus-1

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### ABSTRACT

**Background:** For more than 2 decades, human immunodeficiency virus (HIV) infection has been known to cause significant morbidity and mortality. Difficulties in treating HIV-infected patients include adverse effects and drug resistance and continue to limit the use of conventional anti-retroviral therapies.

**Objectives:** To find new anti-retroviral drugs from natural sources, we investigated the inhibitory effects and mechanism of action of HESA-A, a natural biological compound of herbal-marine origin, against HIV-1 replication *in vitro*.

**Materials and Methods:** In this study, we used a single-cycle replicable HIV-1 system in which co-transfection of human embryonic kidney (HEK)-293T cells with pmzNL4-3, psPAX2, and pM2G.2 plasmids was performed. Cytotoxicity and cytopathic protection assays were performed using the 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-(2H)-tetrazolium-5-carboxanilide method. Inhibition of p24 antigen production was analyzed, and time-of-drug-addition assay was conducted using quantitative enzyme-linked immunosorbent assay (ELISA).

**Results:** HESA-A inhibited HIV-1-induced cytopathic effect in MT2 and HEK293T cells, and the selectivity index values were 13.3 and 8, respectively. We performed quantitative p24 ELISA and added varying concentrations of HESA-A in cell culture supernatants at different times; we observed that HESA-A preserved its ability to inhibit viral replication even at 12 h post-infection.

**Conclusions:** These results suggest that HESA-A has potent anti-HIV activity, and its mechanism of action likely involves interference during the late stages of viral replication, such as virus maturation.

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### ► Implication for health policy/practice/research/medical education:

From a therapeutic point of view, our findings suggest that this herbal drug has relevant therapeutic potential for HIV infection.

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## 1. Background

In some cases, current chemotherapeutic retroviral agents have shown limited clinical efficacy, toxic side effects, and suboptimal pharmacokinetics (1). Therefore, researchers need to find and develop new anti-retroviral drugs. An effective way of discovering these potential new medicines is by screening anti-viral agents isolated

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from natural sources. A broad spectrum of natural products, such as alkaloids, lignans, and flavonoids, have been found to interfere with the unique enzymes and proteins vital to the life cycle of the human immunodeficiency virus (HIV) (2, 3).

HESA-A is a natural biological compound of herbal-marine origin consisting of *Penaeus (Melicertus) latisulcatus* (Penaeidae), *Carum carvi* L. (Apiaceae), and *Apium graveolens* L. (Apiaceae) and has hepatoprotective and antitumor effects (4, 5). HESA-A contains organic constituents (45%), mineral constituents (50%), and water (5%). The mineral constituents include magnesium sulfate, calcium carbonate, sodium sulfate, potassium phosphate, and sodium phosphate. Other elements such as Mn, Br, Ti, Sr, Ni, As, Ag, Cu, Zn, W, Tm, Lu, Er, Ba, Cs, Te, and Va are present in lower amounts in the salt or complex forms of the HESA-A compound (5). Previous studies have reported the antitumor properties of some of the elements in HESA-A (6), and these elements have also been shown to accelerate the healing process. Several *in vitro* and *in vivo* studies have evaluated the anticancer activities of HESA-A, and significant outcomes have been reported (7, 8).

## 2. Objectives

In present study, we investigated whether HESA-A could inhibit HIV-1 replication by using a recombinant HIV-1 system that was designed and tested in our laboratory.

## 3. Materials and Methods

### 3.1. Compound and Cell Lines

HESA-A of herbal-marine origin was provided and manufactured by Osveh Drug Co. Twenty grams of the active components of the drug were dissolved in 200 mL of 1 N HCl. After overnight shaking, the pH of the solvent was increased to 7.6 by using NaOH and then filtered (pore size, 0.45  $\mu$ m) and stored at 4°C. The human T-cell lymphotropic virus-1 (HTLV-1)-infected cell line MT2 and human embryonic kidney (HEK) 293T cells (American Type Culture Collection) were cultured at 37°C with 5% CO<sub>2</sub> in RPMI1640 medium (Biosera, England) and DMEM (Biosera, England), respectively. The media were supplemented with 10% fetal bovine serum (Biosera, England), 100 units/mL of penicillin G, and 100  $\mu$ g/mL of streptomycin (Sigma, USA).

### 3.2. Cytopathic Inhibition Assay

The ability of HESA-A to reduce the lytic activity of HIV virion replication was assayed using HEK293T and MT2 cells. The MT2 or HEK293T cells ( $20 \times 10^3$  cells per well) were infected with 1800 ng of p24 from pseudotyped HIV-1 virions in each well of a 96-well plate containing 60  $\mu$ L of fresh medium and different concentrations of HESA-A. BMS-232632 (an HIV-1 protease inhibitor) was used as positive control. After 16 h, 250  $\mu$ L of fresh complete growth medium was added to each well. One hun-

dred microliters from each well was replaced with fresh medium every 48 h during the days following infection. Seven days after infection, 50  $\mu$ L of 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-(2H)-tetrazolium-5-carboxanilide (XTT; Roche Diagnostics GmbH, Germany) was added to each well, and then the plate was incubated for 4 h at 37°C. Plates were evaluated using a BioTek ELx800 enzyme-linked immunosorbent assay (ELISA) reader (Bio-Hit BP800, Finland) at 450 nm/630 nm.

### 3.3. XTT-Based Cytotoxicity Assay

The cellular toxicity of HESA-A in HEK293T and MT2 cells was assessed using a cell proliferation XTT kit (Roche Diagnostics, Germany), as described previously (9). Briefly, cells were plated in triplicate in 96-well plates in the presence or absence of various concentrations of HESA-A. After incubation at 37°C with 5% CO<sub>2</sub> for 3 days, 50  $\mu$ L of prepared XTT mixture was added to each well. The cells were incubated for an additional 4 h to allow the production of XTT formazan. Absorbance was measured using an ELISA plate reader (BioTek ELx800) at a test wavelength of 450 nm and a reference wavelength of 690 nm. Percent inhibition was calculated using the following formula: Inhibition (%) =  $[100 - (At/As)] \times 100$ , where As is the absorbance of the solvent and At, of the test sample, respectively. The cytotoxic concentration that resulted in a reduction of the number of viable cells by 50% (CC<sub>50</sub>) was calculated from dose-response curves.

### 3.4. Production of Pseudotyped Single-Cycle Replicable HIV Virions

Single-cycle replicable HIV-1 (SCR HIV-1) virions were constructed by deleting a 2-kb segment within the Pol region of the HIV-1 genome from the pNL4-3 strain (provided by Dr. Navid Madani). Pseudotyped SCR HIV-1 virions were produced by co-transfection of HEK293T cells with pmzNL4-3 (containing the mutated genome), psPAX2, and pMD2G plasmids obtained from Addgene (www.addgene.org) (10, 11). The pmzNL4-3 plasmid encodes the HIV-1 full-length RNA, with packaging ability containing the above-mentioned deletion in the Pol region; the psPAX2 plasmid encodes HIV Gag and Gag-Pro-Pol polyproteins, in addition to all the viral accessory proteins; and the pMD2G plasmid encodes the vesicular stomatitis virus surface glycoprotein (VSVG), which is necessary for virion assembly and the budding process. These pseudotyped virions are able to infect a broad spectrum of cells, even without the CD4 receptor. After co-transfection of the HEK293T cells with the above-mentioned plasmids by using the Polyfect reagent (Qiagen, Germany), supernatant containing the virions was harvested at 24, 48, and 72 h. Virus stock was concentrated 20 times by ultracentrifugation, p24 load was quantified (HIV p24 ELISA, Biomerieux, France), and the stock was stored at -70°C (10, 11).

### 3.5. Inhibition of HIV p24 Core Antigen Production (HIV Replication)

HEK293T cells, which were used as target cells in this experiment, were seeded at a density of  $6 \times 10^4$  cells per well in 24-well plates. Each well was infected with 600 ng of p24 SCR HIV virions. After 2 h of virus adsorption, cells were washed 3 times with pre-warmed DMEM to remove free virus particles. Cells were then incubated for 48 h in a total volume of 500  $\mu$ L per well of fresh medium containing various concentrations of HESA-A. Nevirapine (an HI-1/2 RT inhibitor) was used as positive control. After 48 h, the p24 antigen (Ag) assay was performed on the supernatants by using a quantitative p24 ELISA method (HIV p24 ELISA, Biomerieux, France), according to the manufacturer's protocol. The  $CC_{50}$  of HESA-A was calculated according to the method described by Cheng *et al* (12). The selectivity index (SI) was evaluated as the ratio of  $CC_{50}$  to  $IC_{50}$ .

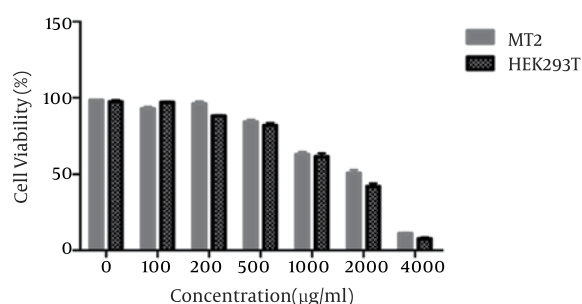
### 3.6. Time-of-Addition Assay

To determine the viral replication mechanism inhibited by HESA-A, a time-of-addition assay was performed as described previously (13, 14). Similar to previous experiments, HEK293T cells were infected with SCR HIV-1 virions; however, in this experiment, 250  $\mu$ g/mL of HESA-A was added to the cell culture medium at different times after infection. HIV p24 Ag production was examined at 48 h after infection by using quantitative HIV p24 Ag ELISA (Biomerieux, France), according to the manufacturer's protocol.

## 4. Results

### 4.1. Cytotoxic Effects of HESA-A on MT2 and HEK293T Cells

First, we evaluated the cytotoxicity of HESA-A on both HEK293T and MT2 cells by using the XTT assay. The data indicated that HESA-A had no cytotoxic effects against either MT2 or HEK293T cells at concentrations 200  $\mu$ g/mL or 330  $\mu$ g/mL, respectively (Figure 1). The  $CC_{50}$  values of HESA-A for MT2 and HEK293T cells were observed to be approximately 300  $\mu$ g/mL and 420  $\mu$ g/mL, respectively.



**Figure 1.** Effects of HESA-A on the Viability of MT2 and HEK293T Cells. Cells were treated with various concentrations of HESA-A (0, 100, 200, 500, 1000, 2000, and 4000  $\mu$ g/mL) for 72 h, and cell viability was determined using the XTT assay

### 4.2. Cytopathic Inhibition Assay

We examined the ability of HESA-A to protect MT2 and HEK293T cells from HIV-1-induced cell lysis. The concentration of HESA-A leading 50% inhibition of HIV-induced cytopathic effect (CPE) was determined using dose-response curves (not shown). The  $IC_{50}$  of HESA-A in infected MT2 cells was 150  $\mu$ g/mL; the  $CC_{50}$  was 2000  $\mu$ g/mL, resulting in a SI greater than 13.3. The SI of HESA-A measured at 8 by using the same calculation methods in HEK293T HIV-1-infected cells was much lower (Table 1).

### 4.3. Inhibition of HIV p24 Production (Replication Assay) by HESA-A

The HIV replication assay was performed using VSVG pseudo-typed SCR HIV-1 virions, which are only able to replicate for 1 cycle. This experiment demonstrated the ability of the test drug HESA-A to inhibit the replication of HIV virions from the time of entry to that of release from the host cells.

**Table 1.** Anti-retroviral Activity of HESA-A on HIV-1 Virions Measured Using a Cytopathic Effect Inhibition Assay

|                                       | HESA-A | Ritonavir <sup>d</sup> |         |
|---------------------------------------|--------|------------------------|---------|
| Host cells                            | MT2    | HEK293T                | HEK293T |
| $CC_{50}$ <sup>a</sup> ( $\mu$ g/mL)  | 2000   | 2200                   | 1800    |
| $IC_{50}$ <sup>b</sup> ( $\mu$ g/mL)  | 150    | 275                    | <17     |
| SI <sup>c</sup> ( $CC_{50}/IC_{50}$ ) | >13.3  | 8                      | >1000   |

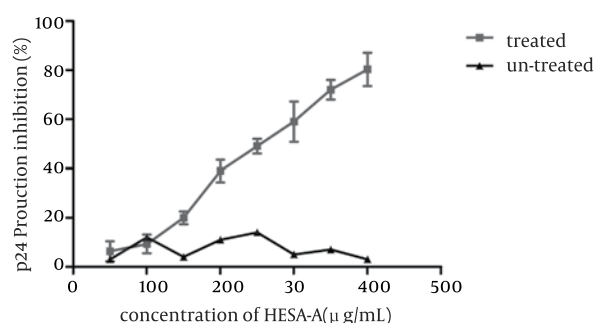
<sup>a</sup>  $CC_{50}$  is the drug concentration that causes 50% cytotoxic effect

<sup>b</sup>  $IC_{50}$  is the concentration of the drug required to inhibit 50% of virus-induced cytopathic effect

<sup>c</sup> Selectivity index (SI) =  $CC_{50}/IC_{50}$

<sup>d</sup> A potent HIV-1 protease inhibitor

At a concentration of 250  $\mu$ g/mL, HESA-A inhibited the production of HIV-1 p24 Ags by more than 50% (Figure 2), although the cytotoxicity of HESA-A at this concentration was less than 5% (Figure 1).

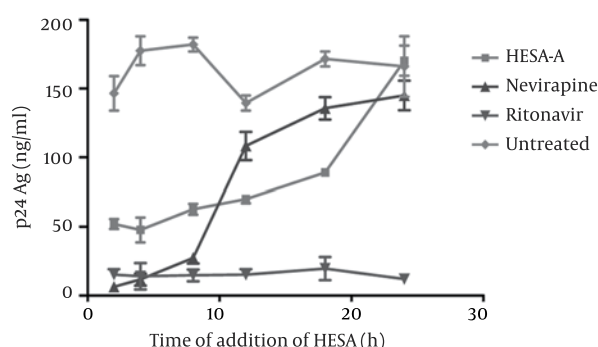


**Figure 2.** Effects of Increasing Concentrations of HESA-A On P24 Ag Production

### 4.4. Time of Intervention by HESA-A

A time-of-addition assay was conducted to determine the steps in HIV-1 replication that may be inhibited by

HESA-A. Therefore, we estimated HIV-1 p24 core Ag levels in culture supernatants of cells treated with HESA-A at various times post-infection by using quantitative p24 ELISA (Figure 3). Levels of p24 Ag in the culture supernatants of infected HESA-A-treated cells were significantly low, even when the drug was added 12 h post-infection.



**Figure 3.** Time-Dependent Effects of HESA-A on HIV-1 Replication

## 5. Discussion

Many studies have focused on developing new anti-retroviral agents because of the increasing emergence of drug-resistant viruses in HIV-infected patients and the cellular toxicity of conventional anti-HIV drugs (15). HESA-A, a versatile drug with herbal-marine origin whose antitumor effects have been reported in several studies (4, 8), has recently attracted great attention on account of the potential antimicrobial properties of its organic and non-organic elements. We were motivated to study the anti-retroviral activity of HESA-A *in vitro*, since various elemental components of HESA-A have been reported to interfere with and suppress HIV enzymes (16).

In the present study, HESA-A showed significant anti-viral activity. First, we observed that HESA-A inhibited the CPE in SCR HIV-1 virion-infected MT2 and HEK293T cells, with  $IC_{50}$  values of 150  $\mu$ g/mL and 275  $\mu$ g/mL, respectively (Table 1). Taking into account the  $CC_{50}$  of HESA-A in MT2 and HEK293T cells (Figure 1), the SI values of HESA-A for MT2 and HEK293T cells were calculated to be 13.3 and 8, respectively (Table 1). When compared to the effects of current synthetic anti-retroviral drugs commonly used in the clinic, such as ritonavir (an HIV protease inhibitor) and nevirapine (RT inhibitor), the SI value of HESA-A indicates that the effects of HESA-A are much weaker than those of these other clinical drugs.

In the time-of-addition assay, we observed that the addition of HESA-A can be postponed more than 6 h after infection without decreasing its ability to inhibit p24 production *in vitro*. The production of p24 core Ags can be used as an indicator of viral replication, and we identified the possible mode of action through which HESA-A inhibits HIV-1 replication by adding HESA-A, nevirapine, or ritonavir to parallel cultures at various times post-infection (Figure 3). The level of HIV-1 p24 Ags observed in the supernatant of HESA-A-treated cultures was quite low, even when the drug was added 12 h post-infection,

and the mechanism of action was comparable to that of ritonavir, a potent viral protease inhibitor (Figure 3); this suggests that the HIV protease and viral maturation are both possible targets of HESA-A. Furthermore, HESA-A does not appear to inhibit the early stages of the HIV-1 life cycle. Despite these findings, the exact mechanism by which HESA-A exerts its anti-HIV activity is still unclear. However, our findings have hinted at a possible mechanism for HESA-A-induced inhibition of HIV-1 replication.

Considering the lack of potent anti-retroviral drugs, other than the so-called highly active anti-retroviral therapy regimens that cause many potential adverse effects, exploring potential natural medicine-based anti-HIV drugs is of great importance. In this study, we found that HESA-A has noticeable anti-retroviral activity. Moreover, on the basis of the  $CC_{50}$  and  $IC_{50}$  values of the drug in the cells, the SI values of HESA-A was calculated to be 13.3 and 8 for MT2 and HEK293T cells, respectively.

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