Dehydroandrographolide Succinic Acid Monoester as an Inhibitor against the Human Immunodeficiency Virus (43225)

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Abstract. **Dehydroandrographolide succinic acid monoester (DASM) is the dehydroandrographolyl ester of succinic acid; and andrographolide, from which DASM is made, is the major diterpenoid lactone found in the Chinese medicinal herb, *Andrographis paniculata*. DASM has been found to be an inhibitor against the human immunodeficiency virus (HIV) *in vitro*. It was nontoxic to the H9 cell at the concentrations of 50–200 (average, 108) μg/ml and was inhibitory to the HIV-1 (IIIB) at the minimal concentration of 1.6–3.1 (average 2.0) μg/ml. It was also inhibitory to two other strains of HIV-1 and a strain of HIV-2. This inhibitory effect could also be demonstrated in cultures of activated human blood mononuclear cells; the 50% toxic dose and the 50% HIV inhibitory dose were about 200–≥400 and 0.8–2 μg/ml, respectively. At the subtoxic concentration, DASM partially interfered with HIV-induced cell fusion and with the binding of HIV to the H9 cell. Presumably, it also interfered with HIV replication at another unidentified step(s).** [P.S.E.B.M. 1991, Vol 197]

Andrographis paniculata is a herb commonly used in China by herb doctors in the treatment of a large variety of illnesses which include acute hepatitis, bacillary dysentery, meningitis, choriocarcinoma, and many other acute inflammatory conditions (1, 2). It has been claimed that extracts of this herb are bacteriocidal to Leptospira and to many species of cocci and are also inhibitory to the growth of ECHO virus type II in human cell culture (1). The main components of this herb are the diterpenoid lactones of which andrographolide is the major component (3). The structure of andrographolide and of several minor related diterpenoid lactones have been recently deduced (3). We now report that a succinyl derivative of andrographolide, dehydroandrographolide succinic acid monoester (DASM), is inhibitory to the growth of human immunodeficiency viruses (HIV) at concentrations nontoxic to the human cells.

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Materials and Methods

Preparation of DASM. DASM was prepared by Pan's procedure with modifications (4). About 0.5 kg of dried *A. paniculata* (whole plant), purchased in San Francisco, was blended and then soaked in about 2.5 liters of ethanol at 22°C for 1 day. The alcohol extract was collected and the extraction was repeated five times. All alcohol extracts were combined and the volume was reduced to one third of its original volume. The concentrated alcohol extract was decolorized with about 50 g of activated charcoal. After removal of the charcoal, the alcohol extract was further reduced to 100 ml. Thin, needle-like crystals (K_2CO_3) formed in the extract were removed by filtration. The concentrated ethanolic extract was extracted five times with methylene chloride, with 500 ml being used per extraction. All methylene chloride extracts were combined and dried over anhydrous Na_2SO_4. Its volume was reduced to about 75 ml in a Buchi Rotovapor. The gold-colored granules formed in the concentrated methylene chloride extract were collected, dried, and dissolved in 1 liter of acetone at approximately 70°C. It was filtered and the filtrate reduced to 50 ml. The light gold-colored granules were collected and dried. Approximately 3.5 g of the granules were again dissolved in 700 ml of acetone in a beaker. The beaker was covered with plastic wrap and the acetone was allowed to evaporate at room temperature

until its volume was reduced to approximately 50 ml. Colorless needle-like crystals formed on the beaker's surface. The acetone was decanted. The crystals were washed with 5 ml of fresh acetone and then dried under vacuum at 50°C overnight. The crystals are the andrographolide.

Approximately 1 g of andrographolide was dissolved in 10 ml of pyridine. To this solution, 0.9 g of succinic anhydride was added. The mixture was kept at approximately 22°C with stirring for 17 hr and then evaporated to dryness under vacuum. To the dried material, 10% sodium carbonate aqueous solution was added dropwise until a clear brown solution formed. The solution was filtered and the filtrate was mixed with sufficient HCl (10%) to bring the pH to 1. The light gold-colored granules were collected by filtration, washed with 5 ml of water, and dried under vacuum at 50°C. This granular material is DASM.

DASM (20 mg) was solubilized by adding 0.16 ml of 12.5% diethanolamine and 0.2 ml of 25% propylene glycol. About 0.5 ml of water was added and the mixture was sonicated in an ultrasonicator until the mixture was clear. Water was added to a total volume of 1 ml. The solution was sterilized by filtration through a 0.45-μm membrane filter.

Analytic thin layer chromatography (reversed phase; solvent system 80% CH_3OH, 20% H_2O) showed four bands. Two close fast-running bands fluoresced under short wavelength UV light, whereas two slower moving bands fluoresced only with long wavelength UV light. Proton nuclear magnetic resonance spectra of the two faster moving bands were similar, and possessed additional vinyl proton peaks compatible with the presence of the dehydroandrographolide moiety. The two slow-moving chromatographic bands showed only solvent resonances in the proton nuclear magnetic resonance spectra.

We believe that the two close fast-running chromatographic bands consist of the two possible dehydroandrographolide monosuccinyl esters, B and C (Fig. 1). We are at present attempting to prove these structures by using standard analytic organic chemistry and to identify which isoler (B or C) is which.

HIV. Three strains of HIV-1 and one strain of HIV-2 were used. The HIV-1 strains were the IIIB (a gift from Dr. Robert C. Gallo, National Cancer Institute), the LAV (from Syntex Research, Mountain View, CA), and the UCD123 (from the AIDS Diagnostic Laboratory, the University of California, Davis, CA). The HIV-2 was the U937/HIV-2_{ms} that was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (given by P. Kanki, F. Barrin, and M. Essex). Pools of each strain were prepared using the following procedure. A culture of the H9 cell (5) was inoculated with the virus. One to 2 weeks later, the culture was centrifuged at 1500 rpm for 10 min. The upper nine-tenths of the supernatant fluid was collected, distributed into small portions, and stored at below −70°C. The potencies of the IIIB, UCD123, LAV, and U937/HIV-2 pools were, respectively, $10^{5.8}$, $10^{6.3}$, $10^{4.5}$, and 10^5 50% tissue culture infective dose ($TCID_{50}$)/ml when assayed by terminal dilution on the MT2 cell (6).

Determining the Subtoxic Concentration. Toxicity of DASM was tested on the H9 cell by a previously described procedure (7). Briefly, DASM was diluted 2-fold serially in medium. To 0.2 ml of diluted DASM in duplicate, 0.8 ml of H9 cell suspension (10^5 cells/ml) was added. After 72 hr at 37°C, the number of viable cells per culture was counted by dye exclusion. A control consisting of 0.8 ml of the H9 cell suspension and 0.2 ml of medium in quadruplicate was included in every assay. Cultures whose average viable cell count was 2 or more SD below, or less than 75% of, the mean of the medium control (whichever was the larger reduction) were considered to have evidence of cytotoxicity. In 15 consecutive assays, the average of 2 SD was equal to 25% of the mean. The subtoxic concentration was the highest concentration that did not cause cytotoxicity.

For determining toxicity against peripheral blood mononuclear cells (PBMC), PBMC were harvested from HIV-seronegative donors and suspended to 2×10^6 cells/ml in medium with phytohemagglutinin at 2 μg/ml. After 3 days at 37°C, the cell pellet was suspended to 2.5×10^6 cells/ml in medium containing recombinant interleukin 2 (a gift from the Cetus Corp., Emeryville, CA) at 36 IU/ml. The cell suspension was distributed in 80-μl portions in 96-well microtiter tissue culture plates and 20 μl of DASM in varying concentrations were added to each well. After 2 days at 37°C, 1 μCi of [^{3}H]thymidine in 20 μl was added to each well. After 5 hr (Assay 1) or 16 hr (Assay 2), radioactivity in the acid insoluble fraction from 100 μl of each of the cell suspensions was measured. Control A consisted of cells without DASM and Control B consisted of medium without DASM and cells. The assay was done in quadruplicate. Radioactivity was expressed as cpm above the value for Control B.

Determining the Minimal HIV Inhibitory Concentration. The minimal inhibitory concentration (MIC) against the HIV was determined by a previously described procedure (7). Briefly, DASM was diluted 2-fold serially. To 0.2 ml of drug in duplicate were added 0.7 ml of H9 cells (10^5 cells/ml) and 0.1 ml of virus stock. Control (in quadruplicate) consisted of cells and virus without drug. Concentration of the virus stock was adjusted so that approximately 10–20% of the cells in cultures not treated with drug were positive for HIV antigens. After 72 hr at 37°C, cells in each culture were smeared and stained for HIV antigens by indirect immunofluorescence using a pool of sera from 18 homo-

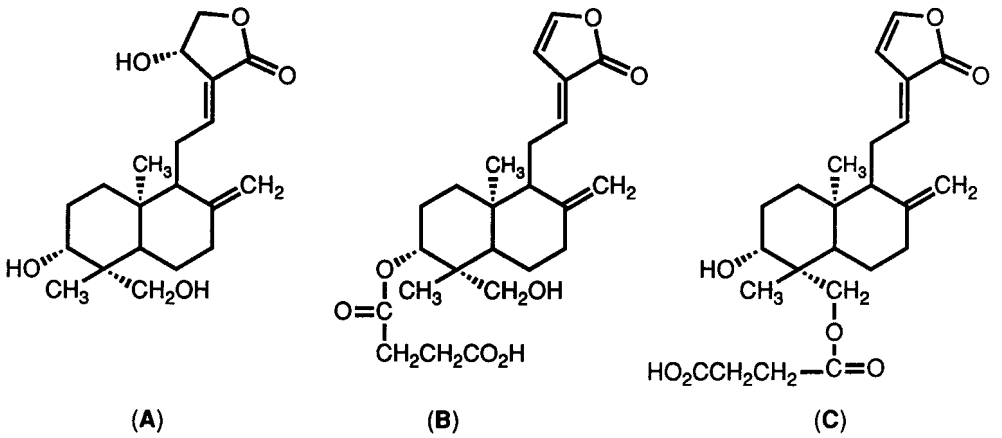

Figure 1. Structures of andrographolide (A) and two possible DASM (B and C).

sexual males suffering from persistent generalized lymphadenopathy (18). This serum had a titer of >1/320 in the indirect immunofluorescent test and contained antibodies to the p24, gp41, gp120, and other HIV antigens by Western blotting; it was used at 1/10 dilution in our assay. Smears were given code numbers and scored for fluorescent cells. At least 500 cells in each smear were scored. A culture whose percentage of HIV antigen-positive cells was ≥3 SD below, or less than 50% of, the mean of the virus control (whichever was the larger reduction) was considered to have shown a significant inhibition of virus growth. The average of 3 SD in 15 consecutive assays was equal to 45% of the mean. In this assay, the percentage of HIV antigen-positive cells showed good relation to the amount of infectious HIV released (Fig. 2). The MIC is the least amount of DASM that significantly inhibits virus growth. Several assays were also performed with activated blood mononuclear cells rather than the H9 cell. The procedure described by Yamamoto *et al.* (9) was used except that the assays were terminated on Day 3 of postinfection and that 15 μl of culture fluid was used in determining the reverse transcriptase activity.

Quantifying Infectious HIV or HIV-Infected Cells. Cultures to be tested for extracellular infectious HIV were centrifuged at 1500 rpm for 10 min. The supernatant fluid was diluted 10-fold serially. Diluted material (0.1 ml) was inoculated into a culture of MT2 cells; four cultures were used for each dilution. Inoculated cultures were fed twice a week and examined for cytopathic changes on the Day 10. The results were expressed as log $TCID_{50}$ according to the formula of Reed and Muench (10); a difference of ≥1 between two preparations is statistically significant (11). For titrating infected cells, the same procedure was used except that cells in the suspension were dispersed rather than pelleted.

Inactivation of Extracellular Virions. To 0.1 ml of HIV, 0.7 ml of medium and 0.2 ml of DASM (to yield a final concentration of 100 μg/ml) were added. Control consisted of 0.1 ml of HIV and 0.9 ml of medium. Both were incubated at 37°C for 1 hr and then titrated for residual infectivity.

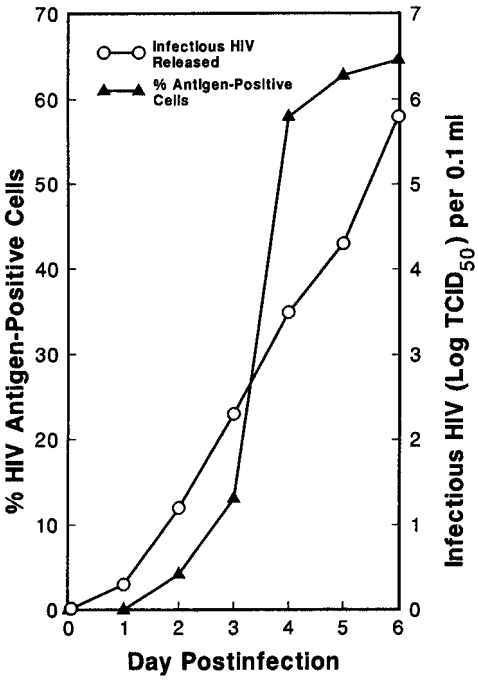

Figure 2. Relation between percentages of HIV antigen-positive cells and amount of infectious HIV released. (H9 cells were mixed with the IIIB strain of HIV at a multiplicity of infection 0.2. After 2 hr at 37°C, the cells were washed six times and then suspended in medium to 10^5/ml. The culture was sampled for determination of HIV antigen-positive cells and infectious HIV in cell-free fluid.)

Interference of HIV Infection. H9 cells (10^5) were suspended in 1 ml of HIV with and without DASM (100 μg/ml). The cell suspensions were incubated at 37°C for 2 hr with agitation once every 15 min. The cells were washed six times with medium to remove unadsorbed virus and the number of infected cells was titrated by terminal dilution. Two additional controls

(HIV in dextran sulfate at 100 μg/ml and zidovudine at 5 μg/ml) were also included. Medium used in washing contained the appropriate drug at the specified concentration.

Interference with Cell Fusion. The procedure of Nakashima *et al.* (12) was used with minor modifications. Briefly, suspensions of the Molt-4B cell and the Molt-4B cell persistently infected by the HIV (IIIB strain) were adjusted to the concentrations of 3×10^5/ml. To 144 μl of Molt-4B cell suspension in sextuplet, 40 μl of DASM and 16 μl of HIV-infected Molt-4B cell suspension were added. The mixture was centrifuged at 1000*g* for 1 min. in a Fisher centrifuge and incubated at 37°C for 16 hr. The cell pellet was dispersed and the cell suspension transferred to a 96-well microtiter plate. Four hours later, the number of giant cells in 24 microscopic fields was scored by a person who did not know the content in each well. A giant cell is defined as a cell whose diameter is at least twice the diameter of a Molt-4B cell. Key controls consisted of the cell mixture with dextran sulfate at 100 μg/ml, with zidovudine at 5 μg/ml, and with no drug.

Inhibition after Cell Infection. Two types of assay were performed to determine whether DASM was still inhibitory to the HIV after the cell had already been infected. The first type of assay was the same as the determination of MIC except that DASM was added to final concentrations of 50–100 μg/ml at 0, 2, 4, and 20 hr after the addition of HIV. The procedure for the second type of assay was as follows: a suspension of H9 cells was mixed with the HIV at a multiplicity of infection of approximately 2; after 2 hr at 37°C, the cells were washed six times with 10 ml of medium/wash; the washed cells were suspended in medium to 2×10^5 cells/ml and distributed into 1-ml portions; equal volumes of medium and medium with DASM, dextran sulfate, or zidovudine were added to the washed cells; and, 24, 48, and/or 72 hr later, the cell suspensions were centrifuged at 1000*g* for 2 min in a Fisher centrifuge and the supernatant fluid was assayed for infectious HIV.

Interferon Induction. The 50% plaque reduction test was used (13). Briefly, 2 ml of medium containing DASM at 100 μg/ml were added to a confluent culture of human fibroblasts in a petri dish (3.5 cm in diameter). After 20 hr at 36°C, the culture was tested for susceptibility to vesicular stomatitis virus (VSV) by the plaque assay. Positive poly(I·C) and negative (medium) controls were included in each assay. In testing the induction in the H9 cell of resistance to the VSV, 0.8 ml of H9 cell suspension (10^5/ml) was mixed with 0.2 ml of DASM (500 μg/ml). After 24 hr at 37°C, 0.1 ml of VSV containing approximately 500 plaque-forming units (as assayed on human fibroblasts) was added. At 48 and 72 hr postinfection, cultures were examined for cell degeneration induced by the VSV.

Reverse Transcriptase Assay. Reverse transcriptase from HIV-1 was prepared and assayed according to the procedure of Rey *et al.* (14). For assay of inhibition, equal volumes of DASM and reverse transcriptase were mixed, kept at 0°C for 30 min and 37°C for 10 min, and then assayed for reverse transcriptase activity. Appropriate controls were included in each assay.

Results

Cytotoxicity in H9 Cells. Results of two representative assays of cytotoxicity of DASM to the H9 cell are shown in Table I; the subtoxic concentrations in both assays were 100 μg/ml. In four additional assays, they were 100, 50, 100, and 200 μg/ml. At this subtoxic concentration, the H9 cell increased in number at least 6-fold in 72 hr. The average of the six assays was 108. In seven similar assays of zidovudine, the subtoxic concentrations varied from 1.3 to 5.0 (average, 2.7) μg/ml. In five assays of a DASM preparation from China, the subtoxic concentrations also varied from 50 to 200 (average, 110) μg/ml.

MIC against the HIV. Results of two representative assays of the inhibition of HIV (IIIB) by DASM in the H9 cell are shown in Table II. The MIC for DASM was 3.1 and 1.6 μg/ml in Assays 1 and 2, respectively. In four additional assays, the MIC were 1.6, 3.1, 0.8, and 1.6 μg/ml. The average of these six assays was 2.0 ± 0.9. The MIC for zidovudine in nine consecutive assays

Table I. Cytotoxicity Assays of DASM

Concentration (μg/ml)	Viable H9 cells/culture	
	Assay 1	Assay 2
0	129 ± 12[a]	176 ± 15
25	121	165
50	106	205
100	124	154
200	91	48
400	48	4

[a] Multiplied by 4×10^3 to give actual number of viable cells per culture.

Table II. Inhibition of HIV-1 (IIIB Strain) by DASM in H9 Cells

Concentration (μg/ml)	% HIV antigen-positive cells	
	Assay 1	Assay 2
0	16.8 ± 1.2	16.9 ± 3.4
0.8	16.1 (4)[a]	
1.6	13.5 (20)	5.9 (65)
3.1	6.8 (59)	2.9 (83)
6.3	4.7 (72)	0.9 (95)
12.5	0.8 (95)	0 (100)
25	0.1 (99)	
50	0 (100)	

[a] Values are percentage of HIV antigen-positive cells with percentage inhibition in parentheses.

Table III. Inhibition of HIV Replication in H9 Cells by DASM as Determined by HIV Antigen and Infectivity Assays

Day	Assay[a]	% HIV Ag-pos cell and infectivity in infected cultures			
		Experiment 1[b]		Experiment 2[b]	
		Control	DASM[c]	Control	DASM[c]
3	% Ag-pos	9.4	0	8.5	0
	Infectivity	10^3	0		
6	% Ag-pos	50.3	0	81.4	0.2
	Infectivity	$10^{4.5}$	0	$10^{5.5}$	$10^{2.8}$
9	% Ag-pos	91.4	0	97.3	0.6
	Infectivity	$10^{5.8}$	0	10^6	$10^{3.5}$
	Cell growth	49	68	6	70

[a] % Ag-pos, (percentage of cells positive for HIV-1 antigen as determined by immunofluorescence. Infectivity is expressed as $TCID_{50}$/ml of cell-free medium (0, none detected in 0.4 ml). Cell growth is expressed as fold increase in viable cells from Day 0 to 9.
[b] Experimental procedures were the same as those described in Table II, except that cultures were tested for HIV growth with infectivity assay in addition to immunofluorescent assay on the third, sixth, and ninth days after inoculation of cultures with HIV, and that culture volumes were doubled with fresh medium (control) or medium with DASM at 50 μg/ml. The IIIB strain was used in Experiment 1 and the UCD123 in Experiment 2.
[c] Concentration of DASM, 50 μg/ml.

Table IV. [^{3}H]Thymidine Uptake by Activated PBMC in Varying Concentrations of DASM

DASM (μg/ml)	cpm/PBMC suspension[a]		
	Assay 1	Assay 2	Assay 3
0	2,200	14,128	18,020
6.3	2,515 (0)	11,254 (20)	21,601 (0)
12.5	2,430 (0)	12,281 (13)	20,323 (0)
25	2,615 (0)	9,450 (33)	21,243 (0)
50	2,603 (0)	14,047 (6)	21,506 (0)
100	1,967 (11)	10,004 (29)	19,500 (0)
200	1,169 (47)	7,618 (46)	18,950 (0)
400	253 (89)	Not tested	13,842 (23)

[a] The cpm values are averages of quadruplicates. Numbers in parentheses are percentages of inhibition. PBMC from three donors were used in the three assays. Cells were labeled with [^{3}H]thymidine for 5 hr in Assay 1 and 16 hr in Assays 2 and 3.

varied from 0.02 to 0.08 (average 0.06) μg/ml, which is close to the value ($IO_{90} \leq 0.13$ μg/ml) reported by the manufacturer. In five assays of a DASM preparation from China, the MIC varied from 3.1 to 12.5 (average, 6.9 ± 3.0) μg/ml.

The HIV inhibitory activity of DASM was also established with a modified procedure. Modifications consisted of the quantification of HIV growth with infectivity assays in addition to immunofluorescent assay on the sixth and ninth days postinoculation as well as on the third day. Table III summarizes the results of two such experiments. DASM at 50 μg/ml completely inhibited the growth of the IIIB strain of

Table V. Inhibiton by DASM of HIV Growth in Human Blood Mononuclear Cells

Assay[a]	RT activity[b] in cultures with		
	No drug	DASM[c]	Zidovudine[c]
1	2535	24	20
2	3852	81	85
3	3319	413	173
4	955	161	97

[a] The HIV-1 (IIIB), HIV-1 (LAV), HIV-1 (UCD123), and U937/HIV-2_{ms} were used in Assays 1, 2, 3, and 4, respectively.
[b] Reverse transcriptase activities in cell-free medium all expressed as cpm above that of uninfected controls. Values are averages of duplicate samples. (To convert into cpm/ml, multiply the number of 1/0.015.)
[c] Concentrations of DASM and Zidovudine were 50 and 2.5 μg/ml, respectively.

Table VI. Interference with HIV-Induced Cell Fusion by DASM

Assay	No. of giant cells in cell mixture containing					
	No drug	DASM[a]			Dextran Sulfate[a]	Zidovudine[a]
		100	25	6.3		
1	30	3	8	20	0	36
2	42	8	20	31	8	34
3	62	10	27	25	0	58
4	54	0	0	7	0	34

[a] The final concentrations of DASM used in the assays were 100, 25, and 6.3 μg/ml; that of dextran sulfate, 100 μg/ml; and that of zidovudine, 2.5 μg/ml.

Table VII. Interference by DASM of HIV Infections of the H9 and Molt-4 Cells

Assay[a]	No. (log) of HIV-infected cells/ml			
	Control	DASM	Dextran sulfate	Zidovudine
1	4.3	3.3	3.3	4.8
2	3.3	2.8	2.8	3.3
3	4.8	3.5	3.3	4.8
4	4.8	4.0	3.8	3.8
5	3.5	3.3	3.3	4.3
6	4.8	3.5	3.3	4.8
7	4.0	3.0	2.0	
8	3.5	2.8	2.8	
Average	4.1	3.3	3.1	4.3

[a] The IIIB strain was used in the first three assays and the UCD123 strain, in Assays 4 to 8. The Molt-4 cell was used in the last two assays and the H9 cell line in the first six. The concentrations of DASM, dextran sulfate, and zidovudine were 100, 100, and 5 μg/ml, respectively.

HIV-1 (Experiment 1) and partially inhibited that of the UCD123 strain (Experiment 2).

Cytotoxicity and HIV Inhibitory Activity in Activated PBMC Cultures. Results of three assays on the

Table VIII. Percentages of HIV Antigen-Positive Cells in Cultures to which DASM (100 μg/ml) Was Added at Specified Times after the Addition of HIV

DASM added hours after HIV	% HIV antigen-positive cells[a]			
	Assay 1	Assay 2	Assay 3	Assay 4
0	0	0	0.1	0
2	0	0.8	0.5	1.0
4	0.2	2.2	0.9	3.3
20	3.3	11.7	3.0	5.6
No DASM	6.5	17.8 ± 4.8	8.3 ± 1.6	10.9 ± 0.9

[a] The UCD123 strain was used in Assays 1 and 3; and, the IIIB strain, in Assays 2 and 4. Assay 1 was done in duplicate; therefore, standard deviation of the mean of the untreated control could not be calculated. The percentage positive for the "no DASM" in Assay 1 were 7.5 and 5.5.

Table IX. Effect of Drugs on the Amount of Infectious HIV Released from H9 Cells Infected with the HIV at a Multiplicity of Infection of Approximately Two and Treated with Drugs Immediately after Infection

Assay[a]	Tested on day postinfection	HIV released (log $TCID_{50}$/0.1 ml) in culture with			
		No drug	DASM[b]	Zidovudine	Dextran sulfate[b]
1	0	1.8			
	1	1.5	1.3	<1.0	1.5
	2	4.0	3.3	2.5	3.5
	3	5.3	4.3	3.3	4.5
2	0	1.0			
	1	0.5	0.5		
	2	2.0	1.5	0.8	1.8
	3	3.0	1.8	<0.5	2.8
3	3	4.3	3.5		
4	3	2.8	2.8		
5	3	4.3	3.3		
6	3	2.8	2.0		

[a] The IIIB strain was used in assays 1, 3, and 5; and, the UCD123 in assays 2, 4, and 6.
[b] DASM, zidovudine, and dextran sulfate were used at the final concentrations of 100, 2.5, and 100 μg/ml, respectively.

cytotoxicity of DASM to phytohemagglutinin-activated PBMC suspensions are shown in Table IV. The 50% toxic dose was 200 μg/ml in the first two assays and ≥400 μg in the third assay.

Data in Table V show that DASM (50 μg/ml) is effective in suppressing the growth of four strains of HIV in cultures of activated PBMC from HIV-seronegative persons. The minimal amount of DASM required to reduce the growth of HIV-1 in activated blood mononuclear cells by 50% was also determined. In one assay, the 50% inhibitory concentrations were 2 and <0.8 μg/ml against the IIIB and UCD123 strains, respectively. In a second assay using the blood cell of another subject, the 50% inhibitory concentrations were <0.8 and 1.4 μg/ml against the IIIB and UCD123 strains, respectively.

Failure of DASM to Inactivate Extracellular HIV. Four assays with the IIIB and UCD123 strains were completed. There was no significant difference in residual infectivity of HIV suspensions containing 0 or 100 μg/ml DASM. The differences, in log $TCID_{50}$, were 0.2, −0.2, −0.3, and 0.2.

Interference with HIV-Induced Cell Fusion. Results of four experiments are summarized in Table VI. DASM, at the concentrations of 100 and 25 μg/ml, regularly reduced the number of giant cells by 50% or more. As expected, dextran sulfate, but not zidovudine, interfered with HIV-induced cell fusion.

Interference with HIV Infection of the H9 Cell. Results of eight experiments are summarized in Table VII. DASM consistently reduced the number of infected cells. The average difference in the number of infected cells (in log) in medium with and without DASM was 0.8, which is statistically significant ($P < 0.0001$) as determined by the t test for paired samples (15). As expected, dextran sulfate also reduced the number of HIV-infected cells by an average of 1 log, which is also significant ($P < 0.0001$). Zidovudine increased the number of infected cells by an average of 0.2 log, which is not significant ($P > 0.8$).

Inhibition of HIV in HIV-Infected Cultures. To determine if DASM was still inhibitory to HIV in cells that were already infected by the HIV, we performed the two types of experiments described in Materials and Methods. Table VIII summarizes results of those experiments in which DASM was added to the culture at specified intervals after the addition of virus; DASM consistently reduced the percentage of HIV antigen-positive cells by over 85% even when it was added 4 hr after the virus. In experiments in which the cells were infected with the HIV at a multiplicity of infection of approximately two and then treated with a specified drug, DASM consistently reduced the amount of infectious HIV released into the medium. Results of six experiments are summarized in Table IX. On the third postinfection day, DASM reduced the amount of infectious HIV released into the medium by an average of 0.8 log $TCID_{50}$. This reduction is significant based on the t test for paired samples ($P < 0.0001$) (15). DASM appeared to be as inhibitory as dextran sulfate but less inhibitory than zidovudine.

Interferon Induction. DASM at 100 μg/ml failed to induce in human fibroblasts or in the H9 cell a state of resistance to the growth of the VSV. In a typical assay on monolayer cultures of human fibroblasts, the number of plaques in medium control, DASM (100 μg/ml), and poly(I·C) (10 μg/ml) were 64 ± 8, 67, and 0, respectively. In H9 cell suspensions, there was complete cell degeneration in VSV-inoculated cell suspensions containing no drug or DASM at 100 μg/ml, but no visible degeneration in uninfected cell suspension containing no drug or 100 μg/ml of DASM.

Interference with HIV Reverse Transcriptase Reaction. DASM at concentrations up to 400 μg/ml failed to reduce significantly the activity of HIV reverse transcriptase. In a typical assay, the enzyme activities (expressed as cpm above background) were 14,234, 14,698, 13,672, 13,757, 11,742, and 10,806 for 0, 25, 40, 100, 200, and 400 μg/ml of DASM, respectively.

Failure to Detect HIV Inhibitory Activity of Andrographolide. Andrographolide, from which DASM was derived through succination, was not inhibitory to HIV in the H9 cell at the subtoxic concentration of 50 μg/ml. Dehydroandrographolide was also inactive against the HIV.

Discussion

Data are presented that DASM at the concentration nontoxic to human cells is inhibitory to the growth of HIV *in vitro*. This inhibition can be demonstrated for all of the four strains of HIV tested. It is active in a T helper cell line (H9) and in phytohemagglutinin-activated blood mononuclear cells from HIV-seronegative persons. The average *in vitro* therapeutic index (subtoxic concentration divided by MIC) of DASM for the IIIB strain in the H9 cell is 54 (108/2.0); that of zidovudine in the same type of assay in our laboratory was 45 (2.7/0.06). In activated PBMC, the 50% toxic concentrations and 50% HIV inhibitory concentrations versus the IIIB or UCD123 strains) were 200–>400 and 0.8–2 μg/ml, respectively. The therapeutic index of DASM in PBMC was, therefore, bigger than that in the H9 cell.

Because interferon inducers are sometimes found among natural products (16) including Chinese medicinal herbs (17), we have studied the possibility that DASM inhibited HIV growth through interferon induction. Our data established conclusively that DASM at the subtoxic concentration was incapable of inducing resistance to the growth of VSV under the prescribed condition in human fibroblasts or the H9 cell. We concluded that DASM interfered with the growth of HIV by a mechanism other than interferon induction.

At the subtoxic concentration, DASM (i) did not inactivate extracellular HIV, (ii) did not interfere with the reverse transcriptase reaction, (iii) partially interfered with HIV-induced cell fusion and with binding of HIV to the H9, and (iv) partially inhibited HIV replication after the virus had attached to cell receptors (Tables VIII and IX). These findings suggest that DASM exerts its suppressive effect on HIV growth by interference with the binding of virions to cells and with a step in the viral replication cycle subsequent to virus-cell binding.

Andrographolide and dehydroandrographolide are not inhibitory to the HIV. It is only after conversion into a succinyl ester that the compound (DASM) becomes an HIV inhibitor. This is an important finding because it shows that a naturally occurring compound may not be an HIV inhibitor until it has been appropriately modified chemically.

Many species of Chinese medicinal herbs have been shown to contain HIV inhibitors (18). Some of the inhibitors have also been chemically characterized. For example, the HIV inhibitors in *Glycyrrhiza radix*, *Viola yedoensis*, *Trichosanthes kirilowii*, and *Prunella vulgaris* are, respectively, a triterpene diglucuronide (19), a sulfonated polysaccharide rich in galactose (20), a protein (21), and a sulfated polysaccharide containing a wide variety of monosaccharides (22). We now show that the diterpene lactone, andrographolide, extracted from *A. paniculata* can be converted to an HIV inhibitor by succination. Because our knowledge of the structure and purity of the DASM preparations is rather imprecise, we cannot exclude the possibility that the HIV inhibitory activity in these preparations is due to minute amounts of unidentified compound(s) with high anti-HIV activity. Needless to say, many more preclinical studies must be done before DASM can be considered for clinical trials.

Of interest is that the DASM prepared at the University of California, Davis and at Tian Xin Pharmaceutical Factory in Guangzhou, China had similar subtoxic concentrations to the H9 cell (108 vs 100 μg/ml) but different MIC against the HIV-1. The MIC of the Davis and Chinese preparations were 2.0 ± 0.9 and 6.9 ± 3.0, respectively. This difference is significant ($P = 0.0016$ by Student's t test). Possible explanations for this difference in HIV inhibitory potency are: (i) the Chinese preparations had been solubilized and stored at room temperature for at least 6 years before testing, whereas the Davis preparation was solubilized, stored at 0°C, and tested within 4 months; and (ii) the methods for extraction of andrographolide and its succination have not been sufficiently standardized to give uniform results.

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1. Huang X. Immunopharmacology. Shanghai: Shanghai Science and Technology Press, p206, 1986.
2. Duke JA, Ayensu ES. Medicinal Plants of China. Algonac, MI: Reference Publications, Inc., p53, 1985.
3. Medforth CJ, Chang RS, Chen GC, Olmstead MM, Smith KM. A conformational study of diterpenoid lactones isolated from the Chinese medicinal herb *Andrographis paniculata*. J Chem Soc Perkin Trans **2**:1011–1016, 1990.
4. Pan QC, Liu ZC, Xe BF, Zhang AL, Lin XS, He FY, Liu GC, Huang JG, Guo DL, Zong HJ, Huang HF, Zhao JL, He RQ,

Chen WC. The experimental research of anti-cancer and antitrophoblastic effect of *Andrographis paniculata.* Acta Acad Med (Zhong Shan) **2**:423–428, 1981 (in Chinese).
5. Popovic M, Sarngadharan MG, Red E, Gallo RC. Detection, isolation and continuous production of cytopathic retroviruses from patients with AIDS and pre-AIDS. Science **24**:497–499, 1984.
6. Harada S, Koyanagi Y, Yamamoto N. Infection of HTLV-III/LAV in HTLV-1 carrying MT2 and MT4 and application in a plaque assay. Science **299**:563–566, 1985.
7. Chang RS, Tabba HD, He Y, Smith KM. Dextran sulfate as an inhibitor against the human immunodeficiency virus. Proc Soc Exp Biol Med **189**:304–311, 1988.
8. Chang RS, Thompson H, Pomerantz S. EB virus infections in homosexual men with chronic persistent generalized lymphadenopathy. J Infect Dis **151**:459–463, 1985.
9. Yamamoto JK, Barre-Sinoussi F, Bolton V, Pedersen NC, Gardner MB. Human alpha- and beta-interferon but not gamma-suppress the *in vitro* replication of LAV, HTLV-III and ARV-2. J Interferon Res **6**:143–152, 1986.
10. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg **27**:493–497, 1983.
11. Pizzi M. Sampling variation of 50% endpoint determined by the Reed-Muench method. Hum Biol **22**:151–190, 1950.
12. Nakashima H, Tochikura T, Kobayashi N, Matsuda A, Veda T, Yamamoto N. Effect of AZT and neutralizing antibody on HIV induced CPE: Implication of giant cell formation for the spread of virus *in vivo.* Virology **159**:169–173, 1987.
13. Merigan TC. A plaque inhibition assay for human interferon employing human neonate skin fibroblast monolayers and bovine vesicular stomatitis virus. In: Brown BR, Glad PR, Eds. *In vitro* Methods in Cell-Mediated Immunity. New York: Academic Press, p489, 1971.
14. Rey MA, Spire B, Dormont D, Barre-Sinoussi F, Montagnier L, Chermann JC. Characterization of the RNA dependent DNA polymerase of a human T lymphotropic retrovirus (lymphadenopathy associated virus). Biochem Biophys Res Commun **121**:126–133, 1984.
15. Worcester J. The statistical method. N Engl J Med **274**:27–36, 1966.
16. Stewart WE. The Interferon System. 2nd Ed. New York: Springer-Verlag, pp27–57, 1981.
17. Hou YT. Studies on effects of *Astragalus membranaceus* on virus infection and interferon system. In: Laver WG, Ed. The Origin of Pandemic Influenza. New York: Elsevier, p277, 1983.
18. Chang RS, Yeung HW. Inhibition of growth of human immunodeficiency virus *in vitro* by crude extracts of Chinese medicinal herbs. Antiviral Res **9**:163–176, 1988.
19. Ito M, Stat A, Kirabayashi K, Tanabe F, Shigeta S, Bab M, DeClercq E, Nakashima H, Yamamoto N. Mechanism of inhibitory effect of glycyrrhizin on replication of HIV. Antiviral Res **10**:289–298, 1988.
20. Ngan F, Chang RS, Tabba HD, Smith KM. Isolation, purification and partial characterization of an active anti-HIV compound from *Viola yedoensis.* Antiviral Res **10**:107–113, 1988.
21. McGrath MS, Hwang KM, Caldwell SE, Gaston I, Luk KC, Wu P, Ng VL, Crowe S, Daniels J, Marsh J, Dienhart T, Lekas PV, Vennari JC, Yeung HW, Lifson JD. GLO 223: An inhibitor of HIV replication in acutely and chronically infected cells of lymphocyte and mononuclear phagocyte lineage. Proc Natl Acad Sci USA **86**:2844–2848, 1989.
22. Tabba HD, Chang RS, Smith KM. Isolation, purification and partial characterization of Prunellin, an anti-HIV component from aqueous extracts of *Prunella vulgaris.* Antiviral Res **11**:263–274, 1989.