Differential Antiviral Activity of Benzastatin C and Its Dechlorinated Derivative from *Streptomyces nitrosporeus*

Jong-Gyo Lee,[a] Ick-Dong Yoo,[b] and Won-Gon Kim*,[b]

[a] *Pharmacology Research Center, Korea Research Institute of Chemical Technology; Daejeon 305–600, Korea: and* [b] *Functional Metabolomics Research Center, Korea Research Institute of Bioscience and Biotechnology; Yusong, Daejeon 305–806, Korea.* Received October 30, 2006; accepted January 25, 2007

Benzastatin C, a 3-chloro-tetrahydroquinolone alkaloid from *Streptomyces nitrosporeus*, showed antiviral activity in a dose-dependant manner with EC_{50} values of 1.92, 0.53, and 1.99 μg/ml against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and vesicular stomatitis virus (VSV), respectively. In contrast, benzastatin D, the corresponding dechlorinated derivative, did not exhibit any antiviral activity. These results indicate that the antiviral activity of benzastatin C is mediated, in part, due to the chlorine moiety in its molecular structure.

Key words benzastatin C; antiviral activity; *Streptomyces nitrosporeus*; chloro-tetrahydroquinolone

Benzastatin C is a unique alkaloid that contains the 3-chloro-1,2,3,4,-tetrahydroquinolone unit in its molecule. Benzastatin C was originally isolated as a free radical-scavenger from *Streptomyces nitrosporeus* 30643.[1,2] Benzastatin D was co-isolated as the corresponding dechlorinated derivative. Benzastatin C is a carboxamide derivative of virantmycin with the same absolute configuration. Virantmycin, an antiviral metabolite from *Streptomyces nitrosporeus*, has been reported to show potent inhibitory activity against various DNA and RNA viruses.[3—5] Its total synthesis has been reported by several groups.[6—10] The active moiety of virantmycin, however, has not been reported. In this study, we report the differential antiviral activity of benzastatin C and the corresponding dechlorinated derivative, benzastatin D.

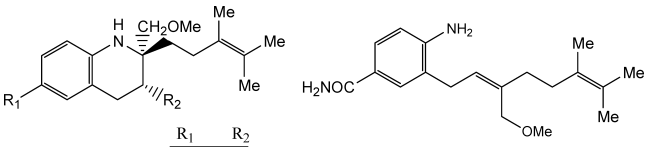
MATERIALS AND METHODS

Chemicals Benzastatins A, C, and D were isolated from *Streptomyces nitrosporeus* 30643 as previously reported.[1] Acyclovir (ACV), cytosine β-D-arabinofuranoside (Ara-C), and ribavirin were purchased from Chemicals Co., Ltd.

Viruses and Cell Lines The strains of viruses used in this experiment were as follows. (1) For the anti-HSV test, herpes simplex virus type 1 (HSV-1) strain F (ATCC VR-733) and type 2 (HSV-2) strain MS (ATCC VR-540), which were grown on Vero cells (African green monkey kidney cells; ATCC CCL81), were used. (2) For the anti-RNA virus test, Poliovirus type 1 (PV-1) strain Brunhile (ATCC VR-58), Coxsackie B virus type 3 (Cox-B3) strain Nancy (ATCC VR-30), and Vesicular stomatitis virus (VSV) strain Indiana (ATCC VR-158), all of which were grown on HeLa cells (human cervical carcinoma cells; ATCC H.H), were used.

Antiviral Assays Measurements of the antiviral activity were based on the inhibition of virus-induced cytopathic effects (CPE).[11] The growth of Vero and HeLa cells was performed in monolayer cultures using Dulbecco's minimal essential medium (DMEM) supplemented with 2% fetal bovine serum (FBS). Host cells in 96-well flat-bottomed tissue culture plates were infected with each virus at a multiplicity of infection (MOI) of 100 $CCID_{50}$ (50% cell culture infectious dose) per well. The inoculum size was verified by a simultaneous CPE assay. Following virus adsorption for 30 min (PV-1, Cox-B3, and VSV) or for 1 h (HSV-1 and HSV-2), the inoculum was aspirated, and 100 μl of DMEM plus 2% FBS medium that contained the test compound was applied to duplicate wells for each concentration. After incubation at 37 °C for 2 d (PV-1, Cox-B3, and VSV) or for 3 d (HSV-1 and HSV-2), viable cells were measured with a modified 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[11] The antiviral effects of the drugs were calculated as a percentage of viable cells in the presence of each drug concentration compared to viable cells observed in the absence of drug. The following formula was used: antiviral activity=[{A (compound/virus)−A (virus control)}/{A (cell control)−A (virus control)}]×100 (%), where A (compound/virus), A (virus control), and A (cell control) mean the absorbances at 550 nm of virus-infected cells in the presence of compound, virus-infected cells in the absence of compound, and virus-untreated cells, respectively. The effective antiviral concentration was expressed as the EC_{50}, which indicated the concentration of compound that was required to inhibit virus-induced CPE by 50%.

Cytotoxicity Assays Toxicity analyses were performed in order to assess whether any observed antiviral effects resulted from a general effect on cell viability. Cells for the toxicity analyses were cultured in 96-well plates and treated with test compounds with the same schedule as used for antiviral evaluations without the addition of virus. Each compound was tested at five concentrations, each in duplicate cultures. Viable cells were assayed with MTT dye. Toxic effects of the drugs were calculated as a percentage of the reduction of viable cells in the presence of each drug concentration compared to viable cells observed in the absence of drug. The following formula was used: cytotoxicity=[{A



	R_1	R_2
Benzastatin C	$CONH_2$	Cl
Benzastatin D	$CONH_2$	OH
Virantmycin	COOH	Cl

Fig. 1. Structures of Benzastatin C and Related Compounds

* To whom correspondence should be addressed. e-mail: wgkim@kribb.re.kr

(compound)−A (blank)}/{A (cell control)−A (blank)}]× 100 (%). The cytotoxic concentration was expressed as the CC_{50}, which indicated the concentration of the compound that killed 50% of the mock-infected cells. Values for antiviral and cytotoxic activities of the drugs are presented as the averages of two or three independent experiments.

RESULTS AND DISCUSSION

As shown in Fig. 2A, benzastatin C showed dose-dependent inhibitory activity with EC_{50} values of 1.92 and 0.53 μg/ml, respectively, against HSV-1 and HSV-2. The antiviral potency of benzastatin C against HSV-2 was two-fold higher than that of Ara-C as a positive control, since the SI index (CC_{50}/EC_{50}) of benzastatin C against HSV-2 was two-fold higher than that of Ara-C (Table 1). The antiviral activity of benzastatin C against HSV-2, however, seems to result from cytotoxicity, since benzastatin C showed a weak cytotoxicity of 8.9% on Vero host cells at 1.1 μg/ml although it exhibited antiviral activity of 74.7% at the concentration. On the other hand, benzastatin D, the dechlorinated derivative of benzastatin C, exhibited no antiviral activity against HSV-1 or HSV-2 at 10 μg/ml, the highest concentration used (Fig. 2B). Benzastatin A also showed no antiviral activity.

For RNA viruses, as shown in Fig. 3A, benzastatin C showed antiviral activity that was selective for VSV among PV-1, CoX-B3, and VSV. The growth of VSV was inhibited by benzastatin C in a dose-dependent manner, with an EC_{50} of 1.99 μg/ml. Moreover, at 10 μg/ml, benzastatin C did not show any cytotoxic effect on HeLa cells that were not treated with virus. This finding suggests that the antiviral activity of benzastatin C against VSV is well separated from cytotoxicity, and indicates that benzastatin C has specific antiviral activity. The SI index of benzastatin C against VSV was similar to that of ribavirin as a positive control, indicating that ben-

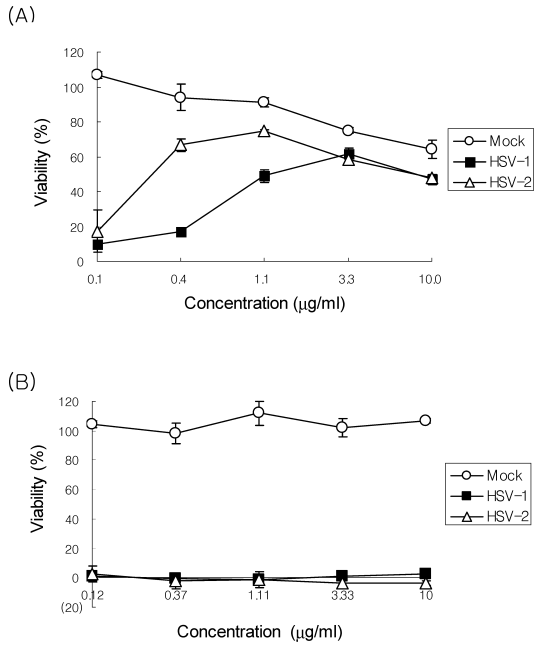


Fig. 2. Antiviral Activity and Cytotoxicity of Benzastatin C and Benzastatin D on HSV-1 and HSV-2

(A) HSV-1 and HSV-2 infection of Vero cells with benzastatin C. (B) HSV-1 and HSV-2 infection of Vero cells with benzastatin D. Antiviral activity was assessed using a virus-induced CPE assay. Cytotoxicity was assayed with virus-untreated cells (Mock). The results are expressed as the mean±S.D. of three independent experiments performed in duplicate.

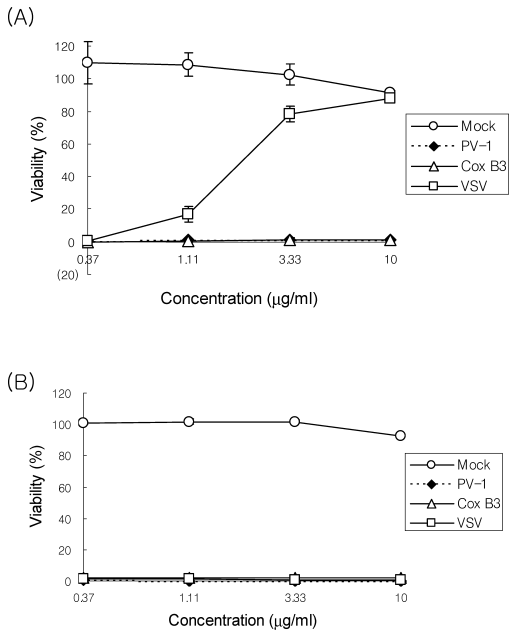


Fig. 3. Antiviral Activity and Cytotoxicity of Benzastatin C and Benzastatin D on RNA Viruses

(A) PV-1, Cox-B3, and VSV infection of HeLa cells with benzastatin C. (B) PV-1, Cox-B3, and VSV infection of HeLa cells with benzastatin D. Antiviral activity was assessed using a virus-induced CPE assay. Cytotoxicity was assayed with virus-untreated cells (Mock). The results are expressed as the mean±S.D. of three independent experiments performed in duplicate.

Table 1. Summary on Antiviral Activities and Cytotoxicities of Benzastatins C and D, and Reference Compounds

	50% inhibitory concentration (μg/ml)						
	Antiviral activity (EC_{50}, μg/ml)[a]					Cytotoxicity (CC_{50}, μg/ml)[b]	
	HSV-1	HSV-2	VSV	PV-1	CoxB3	Vero	HeLa
Benzastatin A	>10	>10	>10	>10	>10	>10	>10
Benzastatin C	1.92±0.08	0.53±0.07	1.99±0.13	>10	>10	>10	>10
Benzastatin D	>10	>10	>10	>10	>10	>10	>10
ACV	0.5±0.08	1.0±0.02	NT	NT	NT	>250	NT
Ara-C	0.43±0.04	0.92±0.05	NT	NT	NT	>10	NT
Ribavirin	NT[c]	NT	22.5±0.14	69.6±0.78	>100	NT	>100

a) Antiviral activity was evaluvated by virus-induced CPE assay. EC_{50} is the concentration of the compound that is required to inhibit virus-induced CPE by 50%. *b*) Cytotoxicity was assayed in virus-untreated cells. CC_{50} is the concentration of the compound that kills 50% of the virus-untreated cells. The results are presented as the mean of two or three independent experiments performed in duplicate. *c*) Not tested.

zastatin C has the similar antiviral potency against VSV with ribavirin (Table 1). Interestingly, benzastatin C was inactive against other RNA viruses such as PV-1 and Cox-B3 at 10 μg/ml, the highest concentration used. In contrast, benzastatin D was inactive against all of the RNA viruses tested, like the case of HSV-1 and HSV-2 (Fig. 3B). The antiviral activity and cytotoxicity of benzastatins C and D are summarized in Table 1. The results of the present study indicate that the active moiety responsible for the antiviral activity of benzasatin C might be the chlorine that is linked to the tetraquinoline unit in the molecule. The results also suggest that the active moiety of virantmycin[3] could be the chlorine.

Virantmycin, a carboxylic acid derivative of benzastatin C, has been reported to exert an effect on cell membranes that contain specific virus receptor sites and suppress replication at a very early stage.[3,4] Some halogenated compounds have been reported as potent antiviral agents. For example, chlorinated benzimidazole ribonucleosides have been known to exhibit potent activity against human cytomegalovirus (HCMV), with low cellular toxicity at concentrations that inhibit viral replication, while displaying weak antiviral activity against HSV-1, HSV-2, and RNA viruses.[12] The ribose in the chlorinated benzimidazole ribonucleosides was reported to be critical for their antiviral activity, and the position of the chloride has been reported to be important for selectivity. Chlorinated indole ribonucleosides, however, have very weak antiviral activity against HCVM, with no antiviral activity against HSV-1.[13] This suggests that the role of chloride as an active moiety for antiviral activity in chlorinated compounds is dependent on the skeleton in which the chloride resides.

In conclusion, our results indicate that benzastatin C shows potent antiviral activity against VSV, and its activity is mediated, in part, due to the chlorine moiety of the 3-chloro-1,2,3,4,-tetrahydroquinolone unit in its molecule. The antiviral mode of action of benzastatin C should be elucidated further, and benzastatins C and D will be useful in investigating the precise mode of action of antiviral activity.

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