Evaluation of Scopadulciol-Related Molecules for Their Stimulatory Effect on the Cytotoxicity of Acyclovir and Ganciclovir against Herpes Simplex Virus Type 1 Thymidine Kinase Gene-Transfected HeLa Cells

Kyoko Hayashi,[a] S. M. Abdur Rahman,[b] Hiroaki Ohno,[b] Tetsuaki Tanaka,[b] Naoki Toyooka,[c] Hideo Nemoto,[c] and Toshimitsu Hayashi*,[c]

[a] *School of Medicine, Toyama Medical and Pharmaceutical University; and* [c] *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan: and* [b] *Graduate School of Pharmaceutical Sciences, Osaka Univeristy; 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan.*

Received April 8, 2004; accepted May 11, 2004

Herpes simplex virus type 1 thymidine kinase (HSV TK) is involved in both antiherpetic therapy and cancer gene therapy with acyclovir (ACV) and ganciclovir (GCV). Enhanced sensitivity to these drugs is advantageous in their clinical use. In the present study, scopadulciol (SDC) and its related compounds were evaluated for their stimulatory effect on the cytotoxicity of ACV and GCV by determination of selective toxicities against HSV TK-expressing HeLa cells. Although SDC remarkably potenciated the cytotoxicity of ACV and GCV, the other tested compounds showed only weak selectivity, except for compound 34.

Key words herpes simplex virus (HSV) thymidine kinase stimulator; scopadulciol; acyclovir; ganciclovir; cytotoxicity

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), which are enveloped DNA viruses, have been recognized as two causative pathogens of infectious diseases developing mucocutaneous lesions of the mouth, face, eyes, or genitalia. After primary infection, they can establish latency in the host and are reactivated by the proper stimulus to cause recurrence. Recent progress in medical technology for organ transplantation has increased the number of recipients who are at a high risk of infection with or recurrence of HSV-1 and HSV-2.[1)] In addition, clinical and experimental studies on infectious viral diseases suggest that HSV-1 and HSV-2 can act as cofactors in spreading human immunodeficiency virus type 1 (HIV-1)[2)] and are major opportunistic agents involved in the pathogenesis of acquired immunodeficiency syndrome (AIDS), which is caused by HIV-1.[3)]

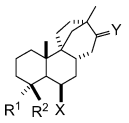
Acyclovir (ACV), a synthesized nucleoside analogue, has been widely used for the treatment of HSV infections because it was shown to have remarkable efficacy and safety with systemic administration. It acts as a prodrug that is preferentially phosphorylated by viral thymidine kinase (TK) in infected cells. Although it is a highly selective antiherpes agent, the emergence of ACV-resistant HSV strains has been observed in immunocompromised patients such as transplant recipients and patients with AIDS.[4)] On the other hand, ganciclovir (GCV), which is clinically used to treat patients infected with cytomegalovirus, has also been used for cancer gene therapy.[5,6)] Therefore potentiators of the viral TK could be useful in solving such problems as the side effects of GCV as well as the emergence of ACV-resistant HSV strains.

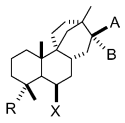
In the course of our search for novel antiviral molecules from natural resources, we succeeded in the isolation of tetracyclic diterpenes such as scopadulciol (SDC, **1**), scopadulcic acid B (**2**), and scopadulin (SD, **3**) as anti-HSV-1 molecules from the tropical medicinal plant *Scoparia dulcis* L. (Scrophulariaceae).[7—9)] Among these diterpenes, SDC was found to potentiate the anti-HSV-1 effect of ACV. The syner-

Table 1. Derivatives of SDC (**1**) Evalutated for Stimulatory Effects on the Cytotoxicity of ACV and GCV

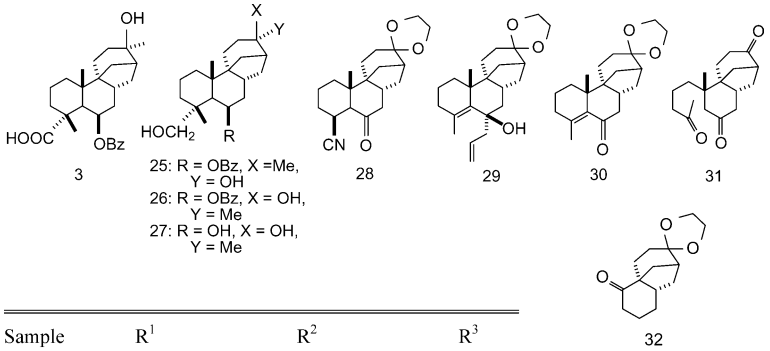
Sample	R^1	R^2	X	Y	Sample	R	X	A	B
1	CH_2OH	Me	OBz	O	**15**	COOH	OBz	H	OH
2	COOH	Me	OBz	O	**16**	COOH	OBz	OH	H
4	CH_2OH	COOH	OBz	O	**17**	COOH	OBz	H	OAc
5	CH_2OAc	Me	OBz	O	**18**	COOH	OBz	OAc	H
6	COOMe	Me	OBz	O	**19**	COOMe	OBz	OH	H
7	COONa	Me	OBz	O	**20**	COOMe	OBz	H	OAc
8	COOH	Me	OBz	NOH	**21**	COOH	OBz	OH	H
9	COOMe	Me	OBz	NOH	**22**	COOMe	OBz	OH	H
10	COOH	Me	OH	O	**23**	CH_2OAc	OH	H	OAc
11	COOMe	Me	OH	O	**24**	CH_2OAc	OAc	OAc	H
12	COOH	Me	OAc	O					
13	COOMe	Me	OAc	O					
14	COOH	Me	O	O					

Bz, benzoyl; Ac, acetyl.





* To whom correspondence should be addressed. e-mail: hayashi9@ms.toyama-mpu.ac.jp



Sample	R^1	R^2	R^3
33	Me	CH_2OH	OH
34	Me	COOH	OBz
35	Me	CH_2OH	OBz
36	CH_2OH	CH_2OH	OH
37	CH_2OH	CH_2OCH_2Ph	OH
38	CN	CH_2OCH_2Ph	OH

Ph, phenyl; Bz, benzoyl.

Fig. 1. Structures of Scopadulin **3** and Its Related Compounds Evaluated in This Study

Table 2. Cell Growth Inhibition of ACV and GCV against HeLa Cells in the Presence or Absence of Each Sample

Sample	Dose μg/ml	ACV			GCV		
		HeLa-TK$^-$ CC_{50} (μM)[a]	HeLa-TK$^+$ CC_{50} (μM)[a]	Selective cytotoxicity[b]	HeLa-TK$^-$ CC_{50} (μM)[a]	HeLa-TK$^+$ CC_{50} (μM)[a]	Selective cytotoxicity[b]
Experiment I							
None	—	3100	170	18	490	3.4	140
1	0.01	3300	11	300	490	0.15	3300
Experiment II							
None	—	2100	230	9.1	230	8.1	28
5	0.025	2400	270	8.9	240	14	17
9	0.5	2300	190	12	220	6.8	32
	2	2000	270	7.4	190	13	15
12	2	2300	190	12	240	6.8	35
17	2	1400	83	17	220	5.0	44
25	2	1800	140	13	170	6.0	28
32	25	2300	230	10	230	7.7	30
	50	2000	210	9.5	240	8.6	28
34	2	2200	250	8.8	230	11	21
	10	1800	21	86	230	8.1	28
35	2	2400	250	9.6	220	8.3	27
	10	1300	88	15	120	5.4	22

a) 50% inhibitory concentration of ACV or GCV for cell growth. HeLa-TK$^-$, wild-type HeLa 229 cells; HeLa-TK$^+$, HeLa cells expressing HSV TK. *b*) Ratio of the CC_{50} of ACV or GCV for HeLa-TK$^-$ cells to that for HeLa-TK$^+$ cells. Samples not listed here showed selective cytotoxicity of less than 10.

gistic effect of SDC with ACV was suggested to be due to the activation of HSV TK by SDC.[9)]

In the present study, several derivatives of SDC as well as some reaction intermediates obtained during the total synthesis of SD were evaluated for their stimulatory effect on the cytotoxicity of ACV and GCV against HSV TK gene-transfected HeLa cells.

Results and Discussion

Monophosphorylation of ACV and GCV by HSV TK is a crucial step for their activation.[10—14)] Thus the cytotoxic efficacy of these prodrugs is dependent on HSV TK activity. In the present study, we evaluated the stimulation of the cytotoxicity of ACV and GCV by compounds using HSV TK-expressing HeLa cells. The chemical structures of SDC and its related compounds tested are illustrated in Table 1 and Fig. 1. The levels of TK activity in these cell cultures were measured at passage 5 (Table 2, experiment I) and 8 (Table 2, experiment II) by determining the radioactivity of [^{3}H]-ACV incorporated into the phosphorylates, showing 10700—11400 dpm or 8600—9600 dpm/10^7 cells at passage 5 or 8, respectively. Immunoblotting analysis of HSV TK-expressing cells revealed the presence of HSV TK protein in the cell cultures.

As shown in Table 2, SDC potentiated the toxicities of ACV and GCV by 300 and 3300 times in HSV TK-expressing cells as compared with those in wild-type HeLa cells, respectively. Derivatives of SDC and related compounds were

tested for the stimulation of the cytotoxic effects of the prodrugs at concentrations lower than the 50% cytotoxic concentration (CC_{50}) against HeLa 229 cells (Table 2, experiment II). The other compounds listed in Table 1 showed no effect on the cytotoxicities of ACV and GCV. Only compound **34** at 10 μg/ml exerted a relatively high selective cytotoxicity in ACV-treated cells, being approximately 10 times more potent than the no-drug control. This compound, however, was less potent than SDC.

TK derived from HSV-1 plays a key role in both antiviral and cancer gene therapies using ACV and GCV. In the present study, we evaluated the selective cytotoxicity between HSV TK-negative and -positive cell lines. The assay system reported here might be convenient in searching for compounds that would be expected to show synergistic action with ACV and GCV.

Experimental

SDC and Related Compounds Compounds **1**—**4** were isolated from *S. dulcis*. Compounds **5**—**24** were obtained by transformation from **2**.[15] The other compounds were reaction intermediates obtained during the total synthesis of **3**.[16]

Cells Human cervical carcinoma (HeLa 229) cells were obtained from the Human Science Research Resources Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum.

Plasmid The plasmid pSV2-HSTK-1 was constructed by inserting the *Bam*HI Q fragment of HSV-1 DNA into the expression vector pSV2-neo.[17]

Transfection HeLa 229 cells (1×10^5) were placed in 35-mm dishes and cultured for 24 h at 37 °C. Plasmid DNA was transfected with LipofectAMINE (Life Technologies, Gaithersburg, MD, U.S.A.) according to the manufacturer's directions. At 72 h after transfection, the medium was changed to selective medium containing geneticin (400 μg/ml). The transfected cells were cloned three times under selection in geneticin (800 μg/ml) to obtain stable HSV TK-expressing cell lines. HSV TK protein was detected by immunoblotting using polyclonal anti-HSV TK antibody and luminol reagent (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). HSV TK enzymatic activities of the transfectants were measured with [^{3}H]-ACV (Moravek Biochemicals, Brea, CA, U.S.A.) as a substrate, as reported previously.[18]

Growth of Cells Expressing HSV TK Wild-type (HSV TK$^-$) and HSV TK-expressing (HSV-TK$^+$) HeLa 229 cells were inoculated in 48-well plates at a density of 1×10^4 cells/well. After 1 d, the cells were replenished with medium containing varying concentrations of test compounds. The cells were allowed to proliferate for 7 d, and the viable cell numbers were counted using the trypan blue dye-exclusion method. The CC_{50} was obtained from the dose-response curves.

References

1) Kuo Y.-C., Lin C.-Y., *J. Med. Virol.*, **31**, 183—189 (1990).
2) Palu G., Benetti L., Calistri A., *HERPES*, **8**, 50—56 (2001).
3) Schacker T., *HERPES*, **8**, 46—49 (2001).
4) Elion G. B., Furman P. A., Fyfe J. A., de Miranda P., Beauchamp L., Schaeffer H. J., *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5716—5720 (1977).
5) Caruso M., Panis Y., Gagandeep S., Houssin D., Salzmann J.-L., Klatzmann D., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 7024—7028 (1993).
6) Chen S.-H., Shine H. D., Goodman J. C., Grossman G. R., Woo S. L. C., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3054—3057 (1994).
7) Hayashi K., Niwayama S., Hayashi T., Nago R., Ochiai H., Morita N., *Antiviral Res.*, **9**, 345—354 (1988).
8) Hayashi T., Hayashi K., Uchida K., Niwayama S., Morita N., *Chem. Pharm. Bull.*, **38**, 239—242 (1990).
9) Hayashi K., Hayashi T., *Antiviral Chem. Chemother.*, **7**, 79—85 (1996).
10) Ashton W. T., Karkas J. D., Field A. K., Tolman R. L., *Biochem. Biophys. Res. Commun.*, **108**, 1716—1721 (1982).
11) Cheng Y.-C., Grill S. P., Dutschman G. E., Nakayama K., Bastow K. F., *J. Biol. Chem.*, **258**, 12460—12464 (1983).
12) Cheng Y.-C., Huang E.-S., Lin J.-C., Mar E.-C., Pagano J. S., Dutschman G. E., Grill S. P., *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 2767—2770 (1983).
13) Field A. K., Davies M. E., DeWitt C., Perry H. C., Liou R., Germershausen J., Karkas J. D., Ashton W. T., Johnston D. B. R., Tolman R. L., *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4139—4143 (1983).
14) Smee D. F., Martin J. C., Verheyden J. P. H., Matthews T. R., *Antimicrob. Agents Chemother.*, **23**, 676—682 (1983).
15) Hayashi T., Kawasaki M., Miwa Y., Taga T., Morita N., *Chem. Pharm. Bull.*, **38**, 945—947 (1990).
16) Rahman S. M. A., Ohno H., Murata T., Yoshino H., Satoh N., Murakami K., Patra D., Iwata C., Maezaki N., Tanaka T., *J. Org. Chem.*, **66**, 4831—4840 (2001).
17) Southern P. J., Berg P., *J. Mol. Appl. Genet.*, **1**, 327—341 (1982).
18) Hayashi K., Hayashi T., Sun H.-D., Takeda Y., *Cancer Gene Ther.*, **7**, 45—52 (2000).