THE KOREAN JOURNAL OF MYCOLOGY
Vol. 27, No 3, p237-241 June 1999
Printed in S. KOREA

Anti-Varicella Zoster Virus Activity of Water Soluble Components of *Elfvingia applanata* Alone and in Combinations with Interferons

Young-So Kim, Seong-Kug Eo, Young-Nam Lee[1] and Seong-Sun Han*

College of Pharmacy and [1]College of Natural Science, Chungbuk National University, Cheongju 361-763, Korea

잔나비걸상 수용성성분의 항-Varicella Zoster Virus 작용과 Interferon과의 병용효과

김영소 · 어성국 · 이영남[1] · 한성순*

충북대학교 약학대학, [1]자연과학대학

ABSTRACT: To search for less toxic antiviral agents from Basidiomycetes, the water soluble components (=EA), were isolated from the carpophores of *Elfvingia applanata* (Pers.) Karst. Anti-varicella zoster virus (Oka strain; anti-VZV/Oka) activity of EA was examined in MRC-5 cells by plaque reduction assay *in vitro*. And the combined antiviral effects of EA with interferon (IFN) alpha or IFN gamma were examined on the multiplication of VZV/Oka. EA exhibited a dose-dependent reduction in the plaque formation of VZV/Oka with 50% effective concentration (EC_{50}) of 464.14 μg/ml. The results of the combination assay were evaluated by the combination index (CI) that was calculated by the multiple drug effect analysis. The combination of EA with IFN alpha showed partially synergistic or additive effects with CI values of 0.83~1.09 for 50%, 70%, 90% effective levels, and those with IFN gamma showed antagonism with CI values of 1.20~1.24.

KEYWORDS: Combination index (CI), *Elfvingia applanata*, Interferon, Varicella zoster virus (Oka strain; VZV/Oka).

The carpophores of *Elfvingia applanata* (Pers.) Karst. (Polyporaceae) in Basidiomycetes have been used to treat various ailments including cancers in oriental folk medicine, as have been the carpophores of *Ganoderma lucidum* (Kim *et al.*, 1980). *E. applanata* was reported to contain some biologically active components such as bitter triterpenoids (Nishitoba *et al.*, 1989), alnusenone and friedelin (Protiva *et al.*, 1980), α-D-glucan and β-D-glucan (Mizuno *et al.*, 1981; Usui *et al.*, 1983). Recently, FDP isolated from *E. applanata* was reported to modulate humoral immune response (Kim *et al.*, 1994a), and the aqueous extract was also reported to show the antibacterial and antiviral activities on pathogenic microorganisms (Kim *et al.*, 1994b, Rym *et al.*, 1999), and not to display any toxicity in the acute toxicity test (Kim *et al.*, 1994c).

Varicella zoster virus (Oka strain; VZV/Oka), which belongs to herpesvirus, causes chickenpox in childhood, and as a latent virus, on reactivation it causes shingles. It is characterized by latent and prolonged infection and has become a problem with AIDS patients (Lapucci *et al.*, 1993). Drugs with a clinically relevant activity against VZV infections include interferons (IFNs), acyclovir, vidarabine, bromodeoxyuridine and desciclovir. However, they have resulted in some undesirable complications and also induced the emergence of drug-resistant viruses (Coen, 1986). Therefore, it is necessary to develop new anti-VZV agents and combination therapy with currently available drugs is attractive (Schinazi, 1991).

In this study, we investigated the anti-VZV/Oka activity of EA, the water soluble components isolated from the carpophores of *E. applanata*, and the combined anti-VZV effect of EA with protein antiviral agents, IFN alpha and IFN gamma.

Materials and Methods

Materials

The carpophores of *Elfvingia applanata* (Pers.) Karst. (Polyporaceae) were purchased from a commercial supplier of Cheongju, Chungbuk, Korea, July 1997, and authenticated by Dr. Wan Hee Park, Seoul National Industrial University. A voucher specimen (No. Cpm 319) has been deposited in the Herbarium of the Herbal Garden, College of Pharmacy, Chungbuk National University.

Cells and virus

*Corresponding author <e-mail: sshan@cbucc.chungbuk.ac.kr>

HEL299 cells (Lung, embryonic, Human diploid), ATCC CCL 137), MRC-5 cells (Lung, Human diploid) ATCC CCL 171, varicella zoster virus (Oka strain; VZV/Oka) ATCC VR 795 were obtained from the American Type Culture Collection (Rockville, USA). HEL299 and MRC-5 cells were cultured with DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 IU/m*l* penicillin, 100 μg/m*l* of streptomycin and 0.25 μg/m*l* of amphotericin B. The cell associated VZV/Oka was prepared from infected confluent HEL299 cells at an input multiplicity of infection (MOI) of 0.01 pfu/cell. The infected HEL299 cells were incubated for 3~4 days until 70~80% of cells were cytopathic by viruses, dissociated by trypsin-EDTA solution, collected by centrifugation, suspended in FBS containing 10% dimethyl sulfoxide (TEDIA, Fairfield, Ohio, USA), and aliquots of cell associated virus were stored at -70°C.

Extraction of water soluble components

The carpophores of *E. applanata* (500 g) were extracted with hot water for 8 h. The extract was concentrated, lyophilized, and EA (21.1 g) of a dark brownish powder was obtained.

Cytotoxicity assay

For cytotoxicity assay, the cells were seeded in 96 well plates (Falcon, NJ, USA) at an initial density of 3.5×10^4 cells per well. After the cells had been incubated for 16~18 h at 37°C, various concentrations of EA were added, and the incubation was continued for 48 h. Viable cell yield was determined by MTT reduction assay which was previously described (Scubiero *et al.*, 1988). The cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}) which is the concentration of samples to inhibit the growth of cells up to 50% by regression analysis.

Anti-VZV activity assay

Anti-VZV/Oka activity was evaluated by plaque reduction assay previously described (Hondo *et al.*, 1976). Host cell monolayers grown in 6 well plates (Falcon, NJ, USA) were infected with about 150 pfu of cell associated VZV/Oka per well. After 1 h adsorption, agar overlay medium containing EA of various concentrations was overlaid. After incubation at 37°C until plaques formed, virus plaques were counted. The degree of inhibition was expressed as the 50% effective concentration (EC_{50}) which was calculated as the concentration of EA required to reduce virus plaque by 50% using regression analysis. Anti-VZV/Oka activity for EA was evaluated by selectivity index (SI) which was calculated by dividing the CC_{50} by EC_{50}. Recombinant human interferon alpha (IFN alpha; Boehringer Mannheim Co.) and recombinant interferon gamma (IFN gamma; Genzyme), which are clinically used for the treatment of VZV diseases, were used as a positive control in this assay system.

Combination assay

The combination assay was essentially performed according to the published method (Tachedjian *et al.*, 1992) with some modifications. Confluent MRC-5 cells in 6 well culture plates were preincubated with IFN alpha or IFN gamma in a range of concentrations of 10~300 IU/m*l* at 5% CO_2, 37°C for 1 h. A cell monolayer was infected with about 150 pfu of cell associated VZV per well, and the plates were incubated at 37°C for 1 h with intermittent rocking at 15 min intervals. Then the plates were overlaid with agar overlay medium containing EA of 80~2,400 μg/m*l* in a constant ratio (8 : 1) compared with concentrations of IFN alpha or IFN gamma. The above plates were incubated at 37°C until the formation of plaques. The formed virus plaques were counted.

Calculation and analysis of drug interactions

To enable the determination of synergistic or antagonistic drug interactions using the multiple drug effect analysis procedure (Chou and Talalay, 1984), data was expressed as the fraction affected relative to the untreated control cultures. Multiple drug effect analysis involves plotting the results obtained for each drug alone, or when combined at multiple fixed-ratio drug concentrations, in the form of a dose-effect curve defined by the median effect equation $f(a)/f(u) = (C/C_m)^m$. F(a) and f(u) are the affected and unaffected fractions, respectively, by the concentration C. C_m is the concentration required to produce the median effect (i.e. 50% effective dose), and m represents the sigmoidicity of the curve. If the slopes (m) of the dose-effect curves for each drug alone and in combination are all parallel, the drugs are said to be mutually exclusive (similar mode of action). Conversely, a mutually nonexclusive case (different mode of action) is defined by parallel gradient for each drug alone, with the gradient for the drug combinations being non-parallel. A combination index (CI) can be calculated for either mutually exclusive or nonexclusive assumption. CI values less than 1.0 indicate synergism, CI values greater than 1.0 represent antagonism, and CI values equal to 1.0 indicate additive effect.

For the analysis of combinations of EA with IFN alpha or IFN gamma by multiple drug effect analysis, only data with high linear correlation coefficients ($r>0.8$) as determined by the median effect plot were used in this analysis.

Results and Discussion

In an attempt to find anti-VZV substances to reduce the

Table 1. Antiviral activity of EA[a], IFN alpha and IFN gamma on varicella zoster virus (Oka strain) in MRC-5 cells by plaque reduction assay

Antiviral substances	CC_{50}[b]	EC[c]			SI[d]		
		EC_{50}	EC_{70}	EC_{90}	SI_{50}	SI_{70}	SI_{90}
EA (μg/ml)	5,874.43	464.14	1,274.21	3,499.28	12.66	4.61	1.68
IFN alpha (IU/ml)	-	249.11	359.07	469.02	-	-	-
IFN gamma (IU/ml)	-	179.01	282.80	386.59	-	-	-

[a]Water soluble components isolated from the carpophores of *Elfvingia applanata*.
[b]50% cytotoxic concentration (CC_{50}) is the concentration of the 50% cytotoxic effect.
[c]Effective concentration (EC) is the concentration of antiviral substance required to reduce plaque formation of virus by 50%, 70%, and 90%.
[d]Selectivity Index (SI) = CC_{50}/EC_{50}.

adverse side effects of in long term therapy and to limit the emergence of resistant virus, water soluble components were isolated from the carpophores of *E. applanata*. Antiviral activity of EA on VZV/Oka was evaluated by plaque reduction assay in MRC-5 cells. The inhibitory effects of EA on plaque formation of VZV in MRC-5 cells is shown in Table 1. EA inhibited plaque formation of VZV with EC_{50} of 464.14 μg/ml and had the cytotoxicity on MRC-5 cells with CC_{50} of 5,874.43 μg/ml. Therefore, EA exhibited anti-VZV activity with SI of 12.66. *E. applanata* in Basidiomycetes was reported to contain some biologically active components such as polysaccharides, proteins and nucleoside analogue substances (Mizuno *et al.*, 1981; Usui *et al.*, 1983; Kim *et al.*, 1994a), and nucleic acids isolated from *E. applanata* showed to induce the secretion of interferon-like materials in spleen cells of mice (Kandefer *et al.*, 1979). Therefore, these water soluble components included in EA may be responsible for inhibition of the replication of VZV/Oka, although it remains to be clarified.

Reasons for combination chemotherapy for viral infections include synergy of antiviral effects, antagonism of toxicities, distribution of toxicities among organ systems, prevention of emergence of resistant variants, enhancement of immune functions (Schinazi, 1991). To determine if there was antiviral synergism between EA and nucleoside anti-VZV agents, combined effects with IFN alpha or IFN gamma were examined on the plaque formation of VZV/Oka in MRC-5 cells. Combinations of EA with IFN alpha or IFN gamma showed a greater inhibition on the plaque formation of VZV/Oka than when tested individually as shown in Figs. 1 and 2. Analysis of combinations of EA with IFN alpha or IFN gamma by multiple drug effect analysis gave the following parameters: for EA, m = 1.09, r = 0.95; for IFN alpha, m = 1.27, r = 0.95; for IFN gamma, m = 1.03, r = 0.99 (Table 2). EA showed the inhibitory effect on VZV with EC_{50} of 554.50 μg/ml, and when combined, the parameters were m = 0.99 and r = 0.96 for IFN alpha, and m = 1.09 and r = 0.97 for IFN gamma (Table 2). CI values were calculated as assumptions of mutually exclusive case and plotted with respect to f(a) ranging from 0.00 to 1.00 as indicated in Fig. 3. We could infer that EA and protein antiviral agents, IFN alpha and IFN gamma, exhibited similar mode of action because the slopes (m) of the dose-effect curves for each drug alone and in combination were all parallel. When CI values for 50%, 70% and 90% effect levels were

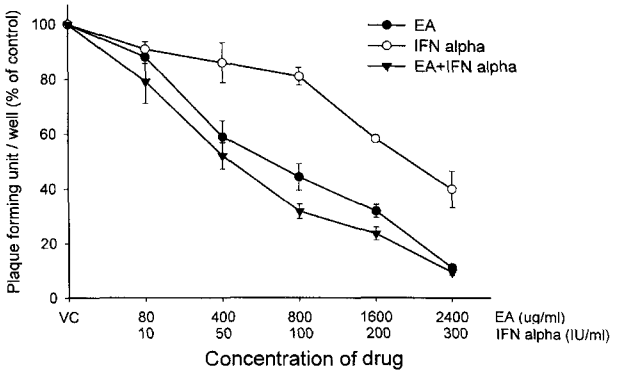

Fig. 1. Inhibitory effect of plaque formation of varicella zoster virus (Oka strain) in MRC-5 cells by EA, IFN alpha, and their combination at a fixed ratio. Results are expressed as percent with respect to virus control (VC) group. Each value is the mean±S.D. of quadruplicate determinations.

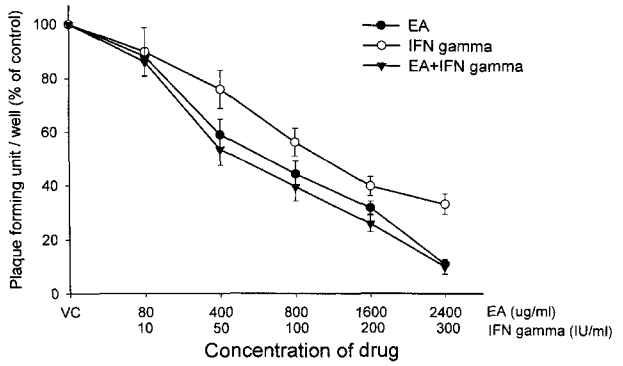

Fig. 2. Inhibitory effect of plaque formation of varicella zoster virus (Oka strain) in MRC-5 cells by EA, IFN gamma, and their combination at a fixed ratio. Results are expressed as percent with respect to virus control (VC) group. Each value is the mean±S.D. of quadruplicate determinations.

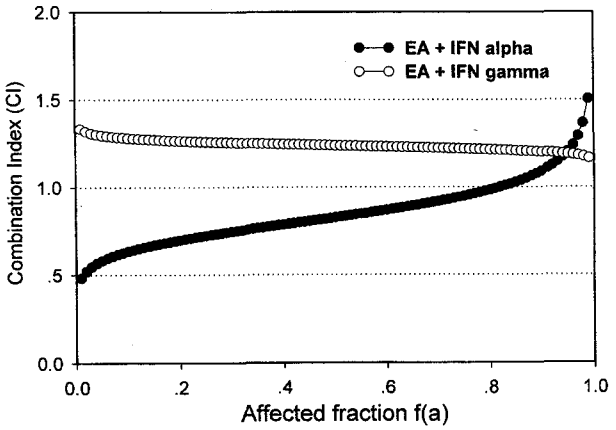

Fig. 3. CI values for combinations of EA with IFN alpha (●) or IFN gamma (○) on varicella zoster virus (Oka strain) corresponding to affected fraction f(a) ranging from 0.00 to 1.00. CI values were calculated under mutually exclusive assumptions.

tabulated in mutually exclusive assumptions, CI values were in a range of 0.83~1.09 for the combination with IFN alpha, and in a range of 1.20~1.24 for the combination with IFN gamma (Table 2). As a whole, combination with IFN alpha exhibited better synergistic or additive effects than that with IFN gamma.

In conclusion, EA was reported to have no toxicity when administered *in vivo* (Kim *et al.*, 1994c), and combination of EA with IFN alpha showed synergistic or additive effect on the replication of VZV/Oka in this study, but combination with IFN gamma showed antagonism. This discovery in Basidiomycetes is the first report as far as we know. Therefore, EA may be able to be used as a potential anti-VZV agent that can permit a significant reduction in the dosage of the toxic anti-VZV agents without compromising antiviral activity.

Table 2. Median effective concentrations and CI values of combinations of EA[a] with IFN alpha or IFN gamma on the plaque formation of varicella zoster virus (Oka strain) in MRC-5 cells

Antiviral substances	Parameters[b]			CI at f(a) of:[c]		
	m	EC_{50}	*r*	0.50	0.70	0.90
EA (μg/m*l*)	1.09	554.50	0.95			
IFN alpha (IU/m*l*)	1.27	248.10	0.95			
IFN gamma (IU/m*l*)	1.03	141.42	0.99			
EA/IFN alpha (8 : 1)	0.99	358.66/44.83	0.96	0.83	0.92	1.09
EA/IFN gamma (8 : 1)	1.09	461.08/57.63	0.97	1.24	1.22	1.20

[a]Water soluble components isolated from the carpophores of *Elfvingia applanata*.

[b]*m* is the slope, EC_{50} is the median effective concentration, and *r* is the correlation coefficient as determined from the median-effect plot.

[c]CI<1, synergism; CI = 1, additive effect; CI>1, antagonism. f(a) is a component of the median-effect equation referring to the fraction of the system affected (e.g., 0.5 means the CI at a 50% reduction of activity). CI values were determined under mutually exclusive assumptions.

적 요

잔나비걸상 *Elfvingia applananta* 자실체의 수용성성분 EA의 varicella zoster virus(Oka strain; VZV/Oka)에 대한 항바이러스효과를 plaque reduction assay에 따라 실험한 결과 EA는 용량의존적으로 plaque 형성을 억제하였으며 EC_{50}는 464.14 μg/m*l*이었다. EA와 단백질성 항바이러스제인 interferon(IFN) alpha 및 IFN gamma와의 병용시험 결과, 단독처리시와 병용처리시에 m(slope) value가 서로 유사한 값을 보였으므로 이들은 상호 배타적인 약물(mutually exclusive drug)임을 알 수 있었고, IFN alpha와의 병용시 combination index(CI)는 f(a)가 0.50에서 0.90인 유효농도 범위내에서 0.83~1.09의 값을 나타내어 부분적 상승 또는 상가효과를 보였으며, IFN gamma와의 병용시에는 1.20~1.24를 나타내어 길항효과를 보였으므로, IFN alpha와의 병용이 IFN gamma와의 병용보다 더 우수한 병용효과를 나타내었다.

References

Chou, T. C. and Talalay, P. 1984. Quantitative analysis of dose-effect relationships; The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**: 27-55.

Coen, E. M. 1986. General aspects of virus resistance with special reference to herpes simplex virus. *J. Antimicrob. Chemother.* **18**(Suppl. B): 1-10.

Hondo, R., Shibuta, H. and Matsumoto, M. 1976. An improved plaque-assay for varicella virus. *Arch. Virology.* **51**: 355-359

Kandefer, S. M., Kawecki, Z. and Guz, M. 1979. Fungal nucleic acids as interferon inducers. *Acta Microbiol. Pol.* **28**: 277-291.

Kim, B. K., Chung, H. S., Chung, K. S. and Yang, M. S. 1980. Studies on the antineoplastic components of Korean Basidiomycetes. *Kor. J. Mycol.* **8**: 107-113.

Kim, Y. S., Ryu, K. H., Mo, Y. K., Lee, C. K. and Han, S. S. 1994a. Effects of the antitumor component, F-D-P, isolated from *Elfvingia applanata* on the immune response. *Kor. J. Pharmacogn.* **25**: 348-355.

Kim, Y. S., Ryu, K. H., Lee, C. K. and Han, S. S. 1994b. Antimicrobial activity of *Elfvingia applanata* extract alone and in combination with some antibiotics. *Yakhak Hoeiji* **38**: 742-748.

Kim, Y. S., Kang, J. K., Lee, C. K. and Han, S. S. 1994c. Effect of *Elfvingia applanata* extract on the acute toxicity in mice. *Yakhak Hoeji* **38**: 756-762.

Lapucci, A., Macchia, M. and Parkin, A. 1993. Antiherpes virus agents: A review. *Il Farmaco,* **48**(7): 871-895.

Mizuno, T., Hayashi, K., Arakawa, M., Shinkai, K., Shimizu, M. and Tanaka, M. 1981. Host-mediated antitumor polysaccharides. III. Fractionation, chemical structure, and antitumor activity of water-soluble homoglucans isolated from kofukisarunokoshikake, the fruit body of *Ganoderma applanatum. Shizuoka Daigaku Nogakubu Kenkyu Hokoku*

31: 49-64.

Nishitoba, T., Goto, S., Sato, H. and Sakamura, S. 1989. Bitter triterpenoids from the fungus *Ganoderma applanàtum. Phytochemistry* **28**: 193-197.

Protiva, J., Skorkovska, H., Urban, J. and Vystrcil, A. 1980. Triterpenes LXIII. Triterpenes and steroids from *Ganoderma applanatum. Coll Czech Chem. Commun.* **45**: 2710-2713.

Rym, K. H., Eo, S. K., Kim, Y. S., Lee, C. K. and Han, S. S. 1999. Antiviral activity of water soluble substance from *Elfvingia applanata. Kor. J. Pharmacogn.* **30**(1): 25-33.

Schinazi, R. F. 1991. Combined therapeutic modalities for viruses-rationale and clinical potential. Pp. 110-181. *In*: Chou, T. C. and Rideout, D. C., Eds. Molecular mechanism of chemotherapeutic synergism, potentiation and antagonism, Academic Press, Orlando, FL.

Scubiero, D. A., Shoemaker, R. H. and Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D. and Boyd, M. R. 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in cutlure using human and other tumor cell lines. *Cancer Res.* **48**: 4827-4833.

Tachedjian, G., Tyssen, D., Jardine, D., Locarnini, S. and Birch, C. 1992. Synergistic inhibition of human immunodeficiency virus type I *in vitro* by interferon alpha and coumermycin A1. *Antiviral Chem. Chemother.* **3**: 183-188.

Usui, T., Iwasaki, Y., Mizuno, T., Tanaka, M., Shinkai, K. and Arakawa, M. 1983. Isolation and characterization of antitumor active β-D-glucans from the fruit bodies of *Ganoderma applanatum. Carbohydr. Res.* **115**: 273-280.