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Combined Antiinfluenza Virus Activity of *Flos verbasci* Infusion and Amantadine Derivatives

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The infusion prepared from flowers of *Verbascum thapsiforme* Schrad. (Scrophulariaceae) (FVI) reduced the infectious and haemagglutination yields of a range of influenza viruses in tissue cultures. Amantadine hydrochloride is an accepted and well studied selective inhibitor of influenza virus reproduction. The combined application of the plant preparation FVI and three amantadine derivatives resulted in a marked enhancement of the inhibitory effect of FVI on the reproduction of influenza virus A/chicken/Germany/27, strain Weybridge (H7N7) in cell cultures of chicken embryo fibroblasts. The antiviral activity was determined by the difference in the infectious titres of control and treated viruses. The combined effect was defined on the basis of infectious viral yields. The most pronounced enhancement was shown for the combination of FVI and adamantanamine glucuronide.

Keywords: viral inhibition; influenza virus; plant preparation; amantadine derivatives; combined effect.

INTRODUCTION

While the need for novel potent antiviral agents continues to exist, the strategy of combined antiviral therapy with available antiviral drugs has proved its usefulness for a number of viral infections. The combined use of antiviral agents enables the potentiation of viral inhibition, the reduction of toxicity and the prevention of antiviral resistance. The application of natural and synthetic viral inhibitors in appropriate combinations also offers possibilities for enhancing the antiviral effect of the individual compounds. The data on the combined inhibitory activity of natural and synthetic antiviral agents, though scarce, suggest that this could be a promising approach in the control of viral infections (Gegova *et al.*, 1993; Dzeguze *et al.*, 1982; Musci, 1984; Musci *et al.*, 1992; Kurokawa *et al.*, 1995; Weaver and Arou, 1998; Uzunov *et al.*, 1991).

This paper presents the results of the combined effect of the plant infusion FVI with three amantadine derivatives on the reproduction of influenza virus in cell cultures.

EXPERIMENTAL PROCEDURES

Plant raw material. The flowers of *Verbascum thapsiforme* Schrad. (Scrophulariaceae) were collected in summer. A specimen was deposited in the Herbarium of the Department of Pharmaceutical Botany, Medical Academy, Kraków.

Plant infusion. The preparation of the infusion is described in Zgorniak-Nowosielska *et al.*, (1991). In short, the air-dried plant material (200 g) was ground and extracted with hot distilled water to obtain an infusion. The infusion was lyophilized yielding 50.0 g of orange-yellow powder (FVI). FVI was kindly provided by Professor I. Zgorniak-Nowosielska from the Institute of Microbiology, Medical Academy, Kraków. It was stored at 4 °C in an air-tight jar.

Amantadine derivatives. Rimantadine hydrochloride was obtained from Hoffman—La Roche Inc., Nutley, NJ. Gludantane—adamantanamine glucuronide (Gl) and its derivative (dGl) were synthesized in the Institute of Organic Chemistry, Latvian Academy of Sciences, Riga and were kindly provided by Professor M. Lidaks.

1% stock solutions of the substances were prepared in sterile distilled water, further dilutions were made in cell culture medium *ex tempore* and pH was adjusted to 7.2.

Cells and medium. Primary chick embryo fibroblast (CEF) cell cultures were prepared according to a standard procedure and maintained in growth medium, containing 45% MEM (minimal Eagle's medium), 45% Hank's solution, 5% LAH (lactoalbumin hydrolysate) and supplemented with 5% calf serum and antibiotics (100 IU/mL benzylpenicillin and 100 μg/mL streptomycin).

Virus. Avian influenza virus A/chicken/Germany/27, strain Weybridge (H7N7) (A/Weybridge) was grown in 11-day-old fertile hen's eggs and allantoic fluid was used as virus inoculum. The virus stock was stored at −70 °C.

Cellular toxicity. This was studied in confluent monolayers of CEF and monitored as described in Serkedjieva and Ivancheva (1999). The 50% toxic concentration was determined (TC_{50}).

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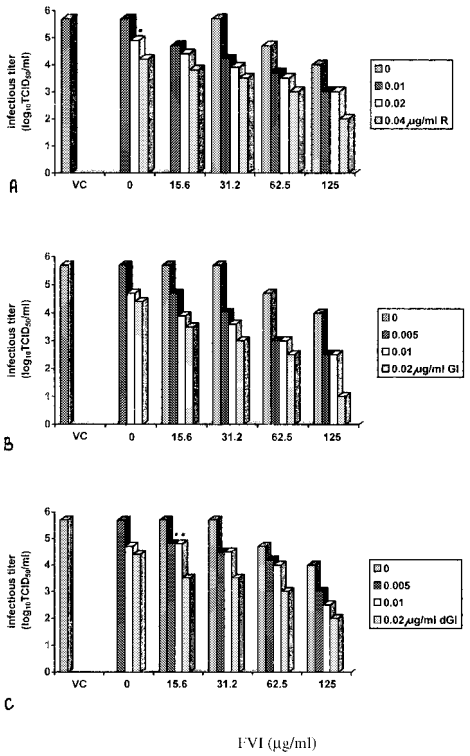

Figure 1. Inhibition of A/Weybridge virus reproduction in CEF by FVI and its combinations with R (A), GI (B) and dGI (C), VC, virus control*. Difference between control and treated virus is not significant ($P < 0.05$).

Virucidal activity. This was tested by contact assay as described in Serkedjieva *et al.* (1992).

Haemagglutination assay. This was performed as described in Serkedjieva *et al.* (1992).

Antiviral assays. The antiviral effect was studied in multicycle experiments of viral growth. The virus-induced cytopathogenic effect (CPE), the production of infectious virus and HA were used as measures for viral growth.

Cytopathogenic effect (CPE) reduction assay. Quadruplicate confluent monolayers in 96-well plates were overlaid with 2x drug-containing medium (0.1 mL) and an equal volume of virus suspension (100 $TCID_{50}$/0.1 mL). The virus-induced CPE was scored after 48–72 h of incubation at 37 °C as described in Serkedjieva and Ivancheva (1999). The concentration reducing CPE by 50% (EC_{50}) with respect to virus control was estimated from graphic plots. The selectivity index was determined by the ratio TC_{50}/EC_{50}.

Infectious virus yield (IVY) reduction assay. Triplicate monolayers in 24-well plastic plates were inoculated with 2x drug-containing medium (0.5 mL) and an equal volume of virus suspension (100 $TCID_{50}$/0.1 mL). The monolayers were incubated at 37 °C for 24 h. Cells and supernatants were pooled after one freeze–thaw cycle and titrated by HA and CPE assays. Virus titres were determined by endpoint titration (Reed and Muench, 1938) and expressed accordingly in HA units (HAU) and 50% tissue culture infectious doses ($TCID_{50}$/mL). The significance of differences in infectious virus titres was estimated using Student's *t*-test. The concentrations that reduced virus infectivity by 90% (1 $\log_{10}TCID_{50}$/mL; EC_{90}) and reduced HA titres >2 $\log_2$ were determined.

The type of the combined antiviral effect was determined according to Schinazi *et al.* (1982) based on virus yields. The fractional yield of the compound A (Y_A) was defined as the virus titre in the presence of the compound divided by the titre obtained in the absence of the compound. The same was done for the second compound (Y_B) and the combination (Y_{AB}). Then Y_C was calculated:

$$Y_C = Y_A \times Y_B$$

if $Y_C > Y_{AB}$ the effect was synergistic
$Y_C = Y_{AB}$ the effect was additive
$Y_C < Y_{AB}$ the effect was antagonistic.

RESULTS AND DISCUSSION

In concentrations above 1500 μg/mL FVI was toxic for CEF. From dose-response dependence curves the TC_{50}

Table 1. Virus-inhibitory effect of selected combinations of FVI with either R, GI or dGI

FVI (μg/mL)	+R (μg/mL)	+GI (μg/mL)	+dGI (μg/mL)	Y_A[a]	Y_B[b]	Y_C[c]	Y_{AB}[d]	Effect
62.5	0.02			0.82	0.86	0.7	0.61	+
–	0.04			0.82	0.74	0.6	0.53	+
		0.01		0.82	0.82	0.67	0.53	+
		0.02		0.82	0.77	0.63	0.44	+
			0.01	0.82	0.88	0.72	0.7	+
			0.02	0.82	0.79	0.65	0.53	+
125	0.02			0.7	0.86	0.6	0.53	+
	0.04			0.7	0.74	0.52	0.35	+
		0.01		0.7	0.82	0.57	0.44	+
		0.02		0.7	0.77	0.54	0.18	+
			0.01	0.7	0.88	0.62	0.44	+
			0.02	0.7	0.79	0.55	0.35	+

The infectious titres are presented in Fig. 1.
[a] Fractional yield of FVI.
[b] Fractional yield of R, GI, dGL.
[c] Fractional yield the combination.
[d] $Y_C = Y_A \times Y_B$.

was found to be 1200 μg/mL. The EC_{50} of the preparation for A/Weybridge in CEF was 64 μg/mL; the selectivity index was 18.8.

The results from the combined application of FVI with the three amantadine derivatives are presented in Fig. 1 and Table 1.

FVI was used in doses two and four times lower than its EC_{50} and two times greater than EC_{50} (Fig. 1). R, Gl and dGl were also applied in concentrations close to their EC_{50}. The combinations did not exhibit any virucidal effect and the cellular toxicity was not enhanced. As a rule the combinations showed an increased virus—inhibitory effect with respect to the individual compounds. Most of the combinations proved to be synergistic (Table 1.). As the EC_{50} of the plant preparation in some of the effective combinations could be reduced four fold the selectivity index was respectively raised. The most pronounced enhancement was shown for the combination of FVI and adamantanamine glucuronide.

Earlier we found that the lyophilized infusion from flowers of *Verbascum thapsiforme* Schrad. showed antiviral activity in *in vitro* studies against several influenza viruses type A and B as well as herpes simplex virus type 1 (Zgorniak-Nowosielska *et al.*, 1991). Both infectious and haemagglutination yields of influenza viruses were reduced. Virus titres decreased by 1–3 log $TCID_{50}$/mL and the effect was most pronounced when the preparation was inoculated simultaneously with or after viral infection. FVI inhibited some intracellular phase of viral reproduction. The infusion did not show any virucidal activity.

The phytochemical investigation of FVI, performed by chromatographic methods revealed the presence of the following substances: flavonoids, iridoids, phenolic acids, saponins, amino acids and free sugars (Zgorniak-Nowosielska *et al.*, 1991). We presume that the antiviral effect of the preparation could not be attributed to one or few separate ingredients. The presence of a variety of biologically active compounds as well as the possible synergistic interactions between the constituents seemed to be more significant for the overall virus-inhibitory effect.

Rimantadine is a highly effective drug in the prophylaxis and treatment of influenza A virus infection. With many influenza virus strains inhibition occurs at an early stage of virus reproduction, preventing virus uncoating (Bukrinskaya *et al.*, 1982). For certain influenza H7 infections inhibition takes place at a later stage during replication and prevents virus release by a specific interaction with the viral M2 protein (Hay, 1989).

The antiinfluenza action of adamantanamine glucuronide was studied by Indulen *et al.* (1973). The substance was less toxic for cell cultures than amantadine and inhibited the reproduction of several influenza A viruses. No published data are available on the properties or biological activities of the derivative of gludantane, used in our experiments.

Previously we have studied the combined antiviral effects of amantadine derivatives with two other viral inhibitors of plant origin—a polyphenolic complex (PC), isolated from *Geranium sanguineum* L. (Uzunov *et al.*, 1991) and the preparation SHS-174, obtained from three higher plants (Serkedjieva and Zgorniak-Nowosielska, 1993). The application of the combinations augmented the inhibition of several influenza A viruses in tissue cultures with respect to the individual substances. The mortality of white mice in experimental influenza infection was reduced synergistically by the simultaneous use of PC and rimantadine (Gegova *et al.*, 1993).

Plant extracts, decoctions and infusions have been used traditionally for the treatment of various human diseases. The presented results together with the data from others (Dzeguze *et al.*, 1982; Musci, 1984; Musci *et al.*, 1992; Kurokawa *et al.*, 1995; Weaver and Arou, 1998) suggest that the combined application of natural and synthetic viral inhibitors may be used successfully to potentiate the antiviral efficacy of the plant preparations and may enable dose reduction of their toxic components.

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