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Synergistic inhibition of Influenza A virus replication by a plant polyphenol-rich extract and ε-aminocaproic acid *in vitro* and *in vivo*

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Summary. – A combined antiviral effect of a polyphenol-rich extract of the medicinal plant *Geranium sanguineum* L. (PC) and a protease inhibitor, ε-aminocaproic acid (ACA) was examined in Influenza A virus (IAV)-infected MDCK cell cultures and mice. Synergistic, antagonistic, or indifferent antiviral effects were distinguished on the basis of virus yields, namely fractional yields of individual compounds and yields of both compounds in combination. Combinations of PC and ACA in particular concentrations proved synergistic in the inhibition of virus replication in MDCK cells and in protection of mice against virus infection as determined by virus titers, lung weight, mean survival time (MST), mortality rate, and protection rate (PR). Following the application of a combination of PC and ACA to the virus-infected mice, the levels of the lung protease and protease-inhibitory activity, which were increased due to the virus infection, were brought to normal. These results demonstrate the rationale for a combined application of viral inhibitors with different modes of action to the treatment of IAV infection, in particular PC as a natural inhibitor of early viral transcription and translation and ACA as a synthetic inhibitor of cellular proteases.

Keywords: Influenza A virus; antiviral effect; synergism; plant polyphenol extract; ε-aminocaproic acid; protease inhibitors

Introduction

Influenza disease remains a major burden on mankind with annual epidemics**.** The infection caused by influenza viruses is usually self-limiting, culminating in a local and systemic reaction. However, a significant proportion of patients develop severe illness and complications such as the elderly, very young, and immunocompromised ones. Recently, a novel H1N1 influenza virus spread fast in the human population and the resulting pandemic has already proved to be a significant cause of mortality and morbidity in the human population. Obviously, even with the development of killed virus vaccine, the need for effective anti-influenza therapy still exists.

Current anti-influenza drugs (M2 ion channel blockers and neuraminidase inhibitors) target viral components. Recently, cellular proteins are emerging as the potential targets for new anti-viral drugs. The principal idea of this approach is to affect the mechanisms favoring virus replication in the infected cells. In the case of influenza viruses, the virulence of a particular influenza virus strain depends on the ability of its hemagglutinin precursor HA0 to be cleaved posttranslationally to HA1 and HA2 by the host trypsin-like proteases (Kido *et al*., 1996; Lozitski *et al*., 1987; Steinhauer, 1999; Zhirnov *et al*., 1985, 2002). Cleavage of hemagglutinin (HA) allows fusion of the viral and host cell membrane prior to the release of nucleocapsid into cytoplasm. The mammalian

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Abbreviations: A/Aichi = A/Aichi/2/68 (H3N2); ACA = ε-aminocaproic acid; A/Rostock = A/chicken/Germany/34, strain Rostock (H7N1); CH = control healthy; CPE = cytopathogenic effect; HA = hemagglutinin; IAV = Influenza A virus; i.n. = intranasally; MST = mean survival time; PC = polyphenol-rich extract; p.i. = post infection; PI = protection index; PR = protection rate; Rim = rimantadine hydrochloride; s.c. = subcutaneously**;** TIU = trypsin inhibitory units; VC = virus control, virus-infected mice

and non-pathogenic avian influenza virus strains contain HA with monobasic cleavage site that is usually cleaved only in a restricted number of cell types and therefore, these viruses cause local infection. In general, the glycoproteins are activated by secreted proteases like serum plasmin, kallikrein, urokinase, thrombin, acrosin, tryptase Clara and mini-plasmin in rat lungs, mast cell tryptase and tryptase TC30 found in porcine lungs (Steinhauer, 1999). Cleavage activation of the influenza monobasic HA by host proteases is generally thought to occur extracellularly on the surface and/or in the lumen of the respiratory tract. The induction of influenza virus infectivity by host cell proteases is strictly regulated by the inhibitors of serine proteases such as human mucus protease inhibitor in the upper respiratory tract (Kido *et al*., 1999) and pulmonary surfactant (Kido *et al*., 1993) in the lower respiratory tract.

Numerous reports have proved that the strategy of combined antiviral therapy with available antiviral drugs is useful for a number of viral infections. The combined use of antiviral agents enables the amplification of viral inhibition, reduction of toxicity and prevention of antiviral resistance. The application of natural and synthetic viral inhibitors in appropriate combinations also offers a possibility to enhance the antiviral effect of individual compounds. The relevant data on the combined inhibitory activity of natural and synthetic antiviral agents suggest that their use could be a promising approach in the control of viral infections (Barquero and Villamil, 1997; Corina *et al*., 1999, Kahlon *et al*., 1991; Kurokawa *et al*., 1995; Musci *et al*., 1992; Weaver and Arou, 1998). Our group has a substantial experience in this field of research (Gegova *et al*., 1993, Serkedjieva, 2000, Serkedjieva and Zgorniak-Nowosielska, 1993, Serkedjieva *et al*., 1986, 2005). We have studied intensively the mode of the anti-IAV activity of the semi-standardized polyphenol-rich extract PC isolated from *Geranium sanguineum* L. It was shown that its virus-inhibitory effect *in vitro* was specific and selective. PC affected the virus-specific RNA and protein synthesis of A/chicken/Germany/34, strain Rostock (H7N1) (A/Rostock) virus (Serkedjieva and Hay 1998). We have demonstrated that the plant preparation markedly protected mice from death in experimental infection (Ivanova *et al*., 2001; Serkedjieva *et al*., 2007; Murzakhmetova *et al*., 2008). PC interfered with the infection alternatively through enhancement and restoration of the host immune response (Ivanova et al., 2001), regulation of the host lung protease activities (Serkedjieva *et al*., 2007), and exhibition of antioxidant and radical scavenging properties (Murzakhmetova *et al*., 2008). The variety of biological activities of PC was related to the presence of large quantities of potential bioactive compounds mainly polyphenols (Pantev *et al*., 2006).

The aim of the present study was to examine a possible synergism in the antiviral effects of PC, obtained from *Geranium sanguineum* L. and protease inhibitor ACA in IAV-infected MDCK cells and IAV-infected mice. At the same time the levels of proteases and protease inhibitors in the lungs of IAV-infected mice under the influence of both inhibitors were investigated.

Materials and Methods

Viruses, cells, and reagents. The avian influenza virus A/Rostock and the human influenza virus A/Aichi were grown in 11-day-old fertile eggs and the allantoic fluid was used as a virus inoculum. In animal experiments A/Aichi adapted to mice lungs and maintained by passages in mice lungs and fertile hen's eggs (A/Aichi-a) was used. The viral infectious titers 10^6–10^7 $TCID_{50}$/ml (50% TCID/ml) and hemagglutination titers 1024-2048 were determined. The viral stocks were stored at -80°C. Madin-Darby canine kidney (MDCK) cells were passaged in Dulbecco's Eagle medium (GibcoBRL) supplemented with 5% fetal calf serum (FCS) and antibiotics (benzylpenicillin – 100 IU/ml, streptomycin – 100 μg/ml) and cultivated until confluent. The medium containing 0.5% FCS and 2 μg/ml trypsin was used for IAV-infected cells. Bovine serum albumin (BSA), N-bensoil-D,L-arginine-*p*-nitroanilide (BAPNA), trypsin, ACA were obtained from Sigma-Aldrich and rimantadine hydrochloride (Rim) from Hoffman-La Roche.

Plant extract. The medicinal plant *Geranium sanguineum* L. was introduced into the experimental field of the Institute of Botany, Bulgarian Academy of Sciences, Sofia. The preparation of the extract was described in detail (Serkedjieva and Manolova, 1992). The polyphenol content of PC was checked by thin layer chromatography and by quantitative determination of tannins, flavonoids, and catechins (Ivancheva *et al*., 1996).

Lung extracts. Inbred ICR mice (16–18 g) were obtained from the Experimental Animal Station, Slivnitsa, and quarantined 24 hrs prior to the use. On days 6 and 9 post infection (p.i.), 3 mice of each group were anaesthetized and their lungs were removed, washed in cold PBS, disintegrated mechanically and by ultrasound disintegrator (MSE) for 3 mins. The homogenates were centrifuged (9,000 rpm, 30 mins, 4°C) and the supernatants were examined for protease and protease-inhibitory activity.

Protease and protease-inhibitory activity. Protease inhibition was determined by the inhibitory activities towards trypsin. The method based on the suppression of the cleavage of BAPNA by trypsin (Angelova *et al*., 2006) was used. Trypsin inhibitory units (TIU) were defined as the amount of inhibitor necessary to decrease the activity of 1 trypsin unit by 50%. One trypsin unit hydrolyses 1 μmol of BAPNA/min. The specific protease-inhibitory activity was evaluated (TIU/mg protein). Protease activity in the lungs was determined by the method that utilizes BAPNA as a substrate. One unit of proteolytic activity (U) was defined as the amount of the enzyme, producing 1 μmol *p*-nitroanilide for 1 min at 37°C. The specific proteolytic activity was evaluated (U/mg protein).

Protein determination. Bio-Rad assay reagent (Bio-Rad) and BSA as a standard was used. The protein content was expressed in mg/ml.

Antiviral assays in cell cultures. The antiviral effect was studied in multicycle experiments of viral growth. The virus-induced cytopathogenic effect (CPE) and the production of infectious virus

were used as a measure of viral replication. CPE-reduction and infectious virus yield reduction assays were used for the detection of the antiviral effect (Serkedjieva and Hay 1998). The 50% concentration, toxic to the cells (TC_{50}) and the concentration reducing CPE by 50% (EC_{50}) with respect to virus control were estimated from the graphic plots. The selectivity index (SI) was determined from the ratio TC_{50}/EC_{50}. Virus titers were determined by 50% endpoint titration and expressed in log $TCID_{50}$/ml. The concentrations reducing virus infectivity by 90% (1 $\log_{10}$ $TCID_{50}$/ml) were determined (EC_{90}).

PC (0.15–10.0 μg/ml) and ACA (0.75–50.0 mg/ml) were applied simultaneously with the virus inoculation. The type of the combined antiviral effect was determined according to the method of Schinazi *et al*. (1982). Fractional yield of the compound A (Y_A) was defined as a viral titer in the presence of compound divided by the titer obtained in the absence of compound. The same was done for the compound B (Y_B) and their combination (Y_{AB}). Then, Y_C was calculated according to the formula: $Y_C = Y \times YB$, when $Y_C > Y_{AB}$ – the effect was synergistic, when $Y_C = Y_{AB}$ – the effect was indifferent, and when $Y_C < Y_{AB}$ – the effect was antagonistic. The character of the combined effects was presented also by the use of three-dimensional model MacSynergy II (Prichard and Shipman, 1990). The additivity assumption equations for both the single and different site inhibitors were used. The difference between the observed and expected combination effect was calculated. Positive values were indicative for synergism, while negative ones – for antagonism. DELTA GRAPH computer program was applied for the graphic representation of dose-response surface.

Mice infection and antiviral therapy. The infection of mice was performed under light ether anesthesia by intranasal (i.n.) inoculation of A/Aichi-a. This virus causes hemorrhagic pneumonia in mice. To produce lethal infection, the mice were infected with 10 x LD_{50} of the virus in volume of 0.05 ml PBS/mouse. PC was applied i.n. 3 hrs before viral infection in the doses 2.5–10 mg/kg. ACA was administered subcutaneously (s.c.) at 24, 2 hrs before infection and 24, 48, 72 hrs after infection in doses 12.5–100 mg/kg in the volume of 0.2 ml PBS/mouse. Rim was applied orally 24, 2 hrs before and 24, 48, 72 hrs after infection, in the dose 40 mg/kg and the treated mice were used as a positive control. Mice were divided in 5 groups, 12 animals each. Group 1 (CH) represented mock-infected and PBS-treated mice, group 2 (VC) virus-infected and PBS-treated, group 3 (PC) virus-infected and PC-treated, group 4 (ACA) virus-infected and ACA-treated, group 5 (PC+ACA) virus-infected and PC+ACA-treated. The mice were monitored daily for body weight and mortality during 14 days.

Toxicity control for the combinations was run in parallel. After completion of the experiments, the surviving mice were sacrificed by cervical dislocation under ether anesthesia.

Treatment of mice. the experimental groups included 10 mice each, except virus control groups (VC) that contained 12 mice. Additional groups of 3 animals from each experimental group were sacrificed on day 6 and 9. Their lungs were weighed and the lung consolidation was scored according the scale, e.g. 0 – normal, 1 – 25% consolidation, 2 – 50% consolidation, 3 – 75% consolidation, 4 – 100% consolidation. Mice lungs were homogenized to a 10% suspension in PBS and 10-fold dilutions were assayed for infectious virus in MDCK cells.

Processing of results. The protective effects of PC, ACA, Rim and the combinations PC+ACA were estimated by the reduction of lung virus infectious titers, lung consolidation, lung weights and rates of mortality, increase of indices of protection and prolongation of MST as described (Serkedjieva and Ivanova, 1997). The protection index (PI) was determined from the equation PI = (PR-1)/PR x 100, where $PR = M_{control}/M_{experiment}$ and M is mortality. The combined effect was evaluated according to Webb (1966). The effect of the combination ($E_{1,2} = PI_{1,2}/100$) and the effects of the individual substances ($E_1 = PI_1/100$ and $E_2 = PI_2/100$) are related in the equation $E_{1,2} = E_1 + E_2 - E_1 x\ E_2$; the combined effect is synergistic if $E_{1,2}$ is >, additive if $E_{1,2}$ is = and antagonistic if $E_{1,2}$ is $< E_1 + E_2 - E_1 x\ E_2$.

Statistical methods. Results from *in vivo* experiments are given either as arithmetic mean values or their ratios from 2–4 experiments. For the biochemical parameters 5–7 measurements were made for every sample. Every experimental group was compared with the mean value of the group of healthy animals on the respective day of investigation. The results were analyzed statistically by the one-way analysis of variance (ANOVA). Two tailed Student's *t*-test was used to evaluate the differences in lung weights, virus titers, and scores. Fisher's exact test was used for comparison of the mortality rates. $P \leq 0.05$ was accepted for statistical significance.

Results

Inhibition of IAV replication in cell cultures

Initially, we tested the combined inhibitory effect of PC and ACA on the replication of A/Rostock and A/Aichi viruses in MDCK cells. We determined the EC_{90} values in the virus yield reduction assay for both substances and for both viruses: 5.2 μg/ml for PC, 25.0 mg/ml for ACA for A/Aichi and 3.0 μg/ml for PC, 20.5 mg/ml for ACA for A/Rostock. TC_{50} values of PC and ACA for MDCK cells were estimated from dose-response curves – 75.0 μg/ml and 60.5 mg/ml, respectively. EC_{50} values for A/Aichi were assessed – 2.2 μg/ml and 11.2 mg/ml, respectively, and accordingly the selectivity indices were 34.1 and 5.4, respectively. EC_{50} values for A/Rostock were 1.1 μg/ml and 6.25 mg/ml, respectively, and the selectivity indices were 68.2 and 7.2, respectively. These data showed that the antiviral effect of both substances was selective, dose-related, and strain-specific.

In the combination experiments, we applied PC and ACA in doses that do not suppress significantly the replication of influenza viruses A/Rostock and A/Aichi in cell cultures (1/2–1/32 EC_{90}). The results of these experiments are shown in Table 1 and Fig. 1. In most cases, the combined use of PC with ACA resulted in a synergistic enhancement of the inhibition of A/Rostock and A/Aichi viruses in MDCK cells. There were found also 2 indifferent combinations for A/Rostock – PC 1.25 μg/ml + ACA 6.2 mg/ml, PC 1.25 μg/ml + ACA 3.1 mg/ml (Table 1) and some antagonistic combinations for A/Aichi – PC 2.5 μg/ml + ACA 0.7 mg/ml, PC 0.31 μg/ml + ACA 12.5 mg/ml, PC 0.31 μg/ml + ACA

Table 1. Combined inhibitory effect of PC and ACA on replication of IAVs in MDCK cells

Virus	Inhibitors		Virus titer log $TCID_{50}$/ml	Y_A, Y_B	Y_{AB}	Y_C	Effect
	PC (μg/ml)	ACA (mg/ml)					
Rostock	0	0	5.6				
	2.5	0	4.5	0.8			
	1.25	0	5.2	0.93			
	0.62	0	5.5	0.98			
	0	12.5	4.8	0.86			
	0	6.2	5.0	0.89			
	0	3.1	5.0	0.89			
	2.5	6.2	4.0		0.71	0.71	I
		3.1	4.0		0.71	0.71	I
	1.25	**6.2**	**3.8**		**0.68**	**0.83**	**S**
		3.1	**4.0**		**0.71**	**0.83**	**S**
	0.62	**6.2**	**4.0**		**0.71**	**0.87**	**S**
		3.1	**4.0**		**0.71**	**0.87**	**S**
	0	0	7.2				
	5.0	0	5.9	0.82			
	2.5	0	6.2	0.86			
	1.25	0	6.7	0.93			
	0.62	0	6.8	0.94			
	0.31	0	6.7	0.93			
	0.15	0	7.0	0.97			
A/Aichi	0	25	6.1	0.85			
	0	12.5	5.5	0.76			
	0	6.2	6.5	0.90			
	0	3.1	7.0	0.97			
	0	1.5	7.0	0.97			
	0	0.7	7.0	0.97			
	2.5	**25**	**6.7**		**0.93**	**0.73**	**S**
		12.5	**4.5**		**0.62**	**0.65**	**S**
		6.2	**5.5**		**0.76**	**0.77**	**S**
		3.1	**5.8**		**0.81**	**0.83**	**S**
		1.5	6.4		0.89	0.83	A
		0.7	6.7		0.93	0.83	A
	1.25	12.5	5.1		0.71	0.71	I
		6.25	**4.5**		**0.67**	**0.84**	**S**
		3.1	**4.9**		**0.73**	**0.90**	**S**
		1.5	**4.9**		**0.73**	**0.90**	**S**
		0.7	**5.4**		**0.81**	**0.90**	**S**
	0.62	**25.0**	**3.2**		**0.48**	**0.80**	**S**
		12.5	**4.0**		**0.59**	**0.71**	**S**
		6.25	**4.9**		**0.73**	**0.85**	**S**
		3.1	**5.2**		**0.78**	**0.91**	**S**
		0.15	**5.6**		**0.83**	**0.91**	**S**
		0.07	**5.7**		**0.85**	**0.91**	**S**
	0.31	**25.0**	**3.0**		**0.45**	**0.96**	**S**
		12.5	5.0		0.75	0.71	A
		6.25	**5.4**		**0.75**	**0.84**	**S**
		3.1	**5.6**		**0.83**	**0.96**	**S**
		0.15	6.6		0.98	0.96	A
		0.07	6.7		1.0	0.96	A
	0.15	12.5	6.0		0.90	0.74	A
		6.25	**6.2**		**0.87**	**0.94**	**S**
		3.1	**6.5**		**0.90**	**0.94**	**S**
		0.15	**6.7**		**0.93**	**0.94**	**S**
		0.07	7.0		0.97	0.94	A

S = synergistic effect (boldface), I = indifferent, A = antagonistic.

1.5 mg/ml, PC 0.31 μg/ml + ACA 0.7 mg/ml, PC 0.15 μg/ml + ACA 12.5 mg/ml (Table 1).

The character of the combined antiviral effect of PC and ACA on A/Aichi virus replication was estimated also by the three-dimensional model of Prichard and Shipman (1990, Fig. 1). A properly reproducible transition from synergistic to the antagonistic effect was demonstrated. The synergistic peaks were achieved, when the combinations PC 0.31 μg/ml + ACA 25 mg/ml and PC 0.62 μg/ml + ACA 3.1 mg/ml were applied. The combinations PC 2.5 μg/ml + ACA 0.7 mg/ml, PC 1.25 μg/ml + ACA 25 mg/ml, PC 1.25 μg/ml + ACA 1.5 mg/ml were antagonistic. Obviously the method of Schinazi *et a.* (1982) is more restrictive. The combinations did not exhibit any virucidal effect and the cellular toxicity was not enhanced.

A determination of the protease-inhibitory activity of the combination assayed in a model system showed that it was not increased in correspondence to the synergistic enhancement of virus-inhibitory effect (data not shown).

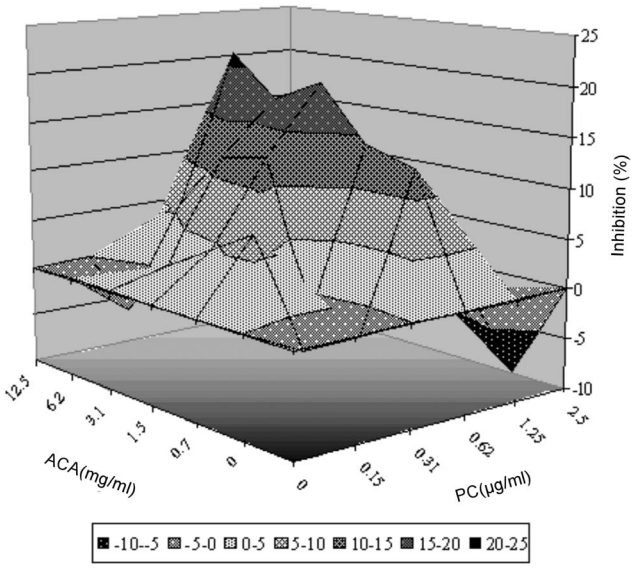

Fig. 1

Inhibition of IAV replication in cell cultures by PC and ACA

Data from Table 1 were used for drawing up of 3D-model of Prichard and Shipman (1990). Different pattern represent different extent of inhibition.

Protection of mice from IAV infection

Further, we tested the combined treatment with PC and ACA in the A/Aichi-a-infected mice (Table 2). The experiments were carried out using 5 LD_{50} that caused 70–80% mortality of the control mice. Administration of PC in combination with ACA in the course of the experiment produced predominantly synergistic rise of protection. The synergistic decrease in mortality rate (PI = 91.3%) and distinct prolongation of mean survival time (+5.2 days) was observed. Two antagonistic combinations were found, e.g. PC 5 mg/kg + ACA 12.5 mg/kg and PC 2.5 mg/kg + ACA 12.5 mg/kg. The mice receiving combined treatment showed minimal pathological lesions in the lungs, whereas control untreated animals had total hemorrhagic pneumonia (Table 2). On day 6 p.i. when the infection peaked, all infectious parameters as the lung consolidation, lung virus titers, lung weights, and mortality rates of infected animals were significantly reduced. In addition, the lung indices were raised and the survival time was markedly increased by drug combinations (Table 2). This tendency was observed also on day 9 p.i., the convalescent stage of infection. The combinations were well-tolerated by the experimental animals and the improved protection was not associated with the increased toxicity.

Protease and protease-inhibitory activities in the lungs of IAV-infected mice

Next, we inspected the effect of PC, ACA, and their combination on the lung protease and protease inhibitory activities (Fig. 2). During the influenza A/Aichi infection the protease activity in the lungs of virus-infected mice was markedly increased on day 6 p.i. (177.1%, CH being 2,262 U/mg protein). Neither PC nor ACA affected the protease activity in intact mice compared to CH and did not induce significant changes in virus-infected mice (VC, data not shown). The combined treatment of VC with PC and ACA led to the normalization of this activity. On day 9 p.i. the protease activity in VC was slightly increased (112.4%, CH being 2,199 U/mg protein); all other measured values were close to control levels. Influenza virus infection triggered also an increase in the lung protease-inhibitory activity on days 6 and 9 p.i. (126.8 and 148.5%, respectively). CH represented 5,760 and 6,045 TIU/mg protein, respectively. While the individual treatment with PC or ACA did not induce significant changes in VC, their combination provoked a decrease in the protease-inhibitory activity on days 6 and 9 p.i. (101.8 and 110.2%, respectively).

Discussion

Taking into account the important role of proteolytic processing for viral reproduction (Kido *et al.*, 1996; Lozitski *et al.*, 1987; Steinhauer, 1999; Zhirnov *et al.*, 1985, 2002), one of the possible targets for chemotherapy of influenza virus infection is the blocking of proteolytic cleavage of viral proteins. It is believed that this action would result in the inhibition of subsequent rounds of viral replication and virus spread in the respiratory tract. For influenza viruses with monobasic HA, exogenous inhibitors of serine proteases including ACA

Table 2. Combined protective effect of PC and ACA on IAV infection in mice

Inhibitors		Mortality rate (%)	PI[a] (%)	MST[b](days)	Effect[c]	Lung parameters				
PC[$] (μg/ml)	ACA[*] (mg/ml)					Day (p.i.)	Weight (g)	Lung index[d]	Score[e]	Virus titer (log $TCID_{50}$/ml)
0 (virus control)		75.2		8.8		6	0.25	1.18	3.5	6.7
						9	0.20	1.08	3.5	2.5
10		9.5	87.3	13.5		6	0.22	1.0	2.0	2.1
						9	0.21	0.98	1.0	1.3
5.0		25.4	66.2	11.9		6	0.22	1.1	2.0	3.5
	0					9	0.21	1.0	1.0	1.5
2.5		58.7	21.7#	12.4		6	0.24	1.12	2.5	4.3
						9	0.22	1.02	2.0	2.5#
1.25		70.0	6.7#	8.5		6	0.21	1.2	4.0	6.7#
						9	0.2	1.2	0.5	2.5#
	100	10.0	86.7	13.2		6	0.21	0.95	2.3	5.1
						9	0.21	1.1	0.5	2.3#
	50	33.0	56.1	10.6		6	0.22	1.12	3.0	4.5
0						9	0.22	1.2	3.5	3.0#
	25	63.7	15.2#	11.5		6	0.24	1.2	3.5	5.1#
						9	0.23	1.12	3.0	2.3#
	12.5	70.0	6.7#	9.3		6	0.25	1.15#	3.0	6.5#
						9	0.23	1.12	2.5	2.5#
	50	6.7	90.3	12.2	**S**	6	0.21	0.96	0	0.5
						9	0.2	1.0	0	0
5.0	**25**	25.0	66.7	11.6	**S**	6	0.21	0.98	1.0	2.0
						9	0.21	1.0	0.25	1.3
	12.5	56.0	25.3	7.3	A	6	0.22	1.05	3.0	6.5#
						9	0.22	0.9	2.0	2.8#
	50	12.5	83.3	13.5	**S**	6	0.22	1.02	3.0	4.5
						9	0.22	1.0	2.0	2.2#
2.5	**25**	40.0	46.8	12.1	**S**	6	0.22	0.96	1.5	3.3
						9	0.21	0.95	1.0	1.3
	12.5	41.4	46.1	9.0	A	6	0.24	1.14	3.5	6.5#
						9	0.22	1.1	2.0	2.8#
	50	44.4	67.9	12.8	**S**	6	0.2	0.92	1.5	3.0
						9	0.22	1.0	1.0	1.2
1.25	**25**	56.0	25.3	11.6	**S**	6	0.22	0.96	2.5	3.0
						9	0.22	1.02	1.25	2.3
	12.5	48.1	35.8	11.9	**S**	6	0.2	0.95	1.0	3.1
						9	0.22	0.9	1.0	2.8#
Rimantadine (40 mg/ml)		10.0	86.7	13.5		6	0.24	1.02	0.5	0.5
Healthy control		0.0	0.0	>14.0			0.21	1.0	0.21	1.0

[a,c]Protective index and effect calculated according to the formulas in Materials and Methods; [b]mean survival time; [d]scores 0–4, assigned to % visible consolidation; [$]administered i.n. 3 hrs before viral infection; [*]administered s.c. 24, 2 hrs before and 24, 48, 72 hrs after viral infection; [#]the difference with VC is not significant $P < 0.05$; S = synergistic effect (boldface); A = antagonistic effect.

(Lozitsky *et al.*, 1997), aprotonin (Ovcharenko *et al.*, 1994) and ambroxol (Yang *et al.*, 2002) have been shown to reduce HA-cleavage and virus activation in the cultured cells, chick embryos, and lungs of infected mice.

We have established that the plant extract PC interfered with IAV infection by regulation of the host lung protease activity in addition to its selective virus-inhibitory activity (Serkedjieva *et al.*, 2007). It restored the balance between the protease and the protease-inhibitory activities. This favorable capacity of the plant extract in virus-infected mice was in concert with the reduction of mortality and infectious virus load in the lungs. In addition, the severity of the

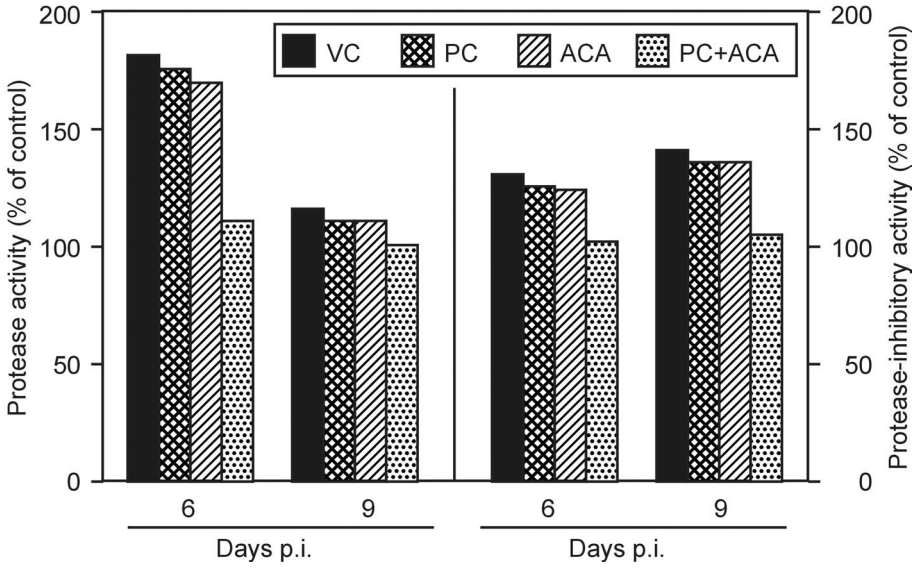

Fig. 2

Effects of PC and ACA on the lung protease and protease-inhibitory activities of IAV-infected mice

PC = 2.5 μg/ml and ACA = 25 mg/ml.

macroscopic lung lesions was also markedly decreased. The restoring effect of the extract on the lung proteolytic balance contributed substantially to its overall protective effect in IAV infection of mice.

Plant polyphenols, particularly flavonoids, are known for their strong trypsin-inhibitory ability (Maliar *et al.*, 2004). Moreover Rajbhandari *et al.* (2001) and Wegner *et al.* (2000) demonstrated that the antiviral effect of plant polyphenols was often associated with strong protease inhibition.

The antiviral effect of ACA, an inhibitor of plasminogen activation, has been intensively studied by Lozitski *et al.* (1987) in cell cultures and in animal models. It was shown that ACA reduced the HA-cleavage and virus proteolytic activation in cultured cells, chick embryos, and lungs of infected mice.

In the present paper we tested the hypothesis of the advantageous combined treatment of the A/Aichi-a infection in mice with a plant polyphenol-rich preparation and an established protease inhibitor. As a first approach we examined the effect of both substances on the replication of A/Aichi virus and showed that the virus-inhibitory effect of both substances was strain-specific and consistent with antiviral selectivity. The combined simultaneous application of PC and ACA in doses that by themselves did not suppress significantly the replication of influenza virus A/Aichi in cell cultures, resulted in most cases in a synergistic enhancement of viral inhibition. These results were evidenced also by the model of Prichard and Shipman (1990) for evaluation of drug-drug interactions. It should be stressed that the combinations did not exhibit any virucidal effect and moreover the cellular toxicity was not enhanced.

It is obvious that the assays *in vitro* could not be completely reliable to predict the effectiveness of combination therapy *in vivo*. Only the results from animal experiments could help to provide consistent evidence about the appropriate use of combined treatment. Therefore, the combined treatment with PC and ACA was tested in the IAV-infected mice.

The i.n. inoculation of A/Aichi-a virus causes viral pneumonitis in the lungs of infected mice. Administration of PC in combination with ACA produced an additive to synergistic rise of protection leading to a synergistic decrease in mortality rate (IP = 91.3%) and to a distinct prolongation of MST (+5.2 days). The mice receiving combined treatment showed minimal pathological lesions in the lungs, whereas control untreated animals experienced a total hemorrhagic pneumonia. Thus, the combined use of PC and ACA had a synergistic therapeutic effect in the animals, inoculated with a high dose of influenza A/Aichi-a virus.

The combined treatment with PC and ACA brought to the normal level both the lung protease and protease-inhibitory activities. It should be noted that while 2.5 mg/kg of PC did not affect the lung protease activities, the preventive treatment with 10 mg/kg of the extract modulated the excessive increase of both activities (Serkedjieva *et al.*, 2007).

Earlier we have found that the combined virus-inhibitory effects of PC and a protease inhibitor produced by *Streptomyces* sp. 225b on the replication of influenza virus A/Rostock in MDCK cells resulted predominantly in an antagonistic

virus-inhibitory effects. However, some synergistic combinations were found as well (Serkedjieva *et al.*, 2005).

These results together with our previous observations showed the need of a precise selection of individual component dose used in combination (Serkedjieva, 2000; Serkedjieva and Zgorniak-Nowosielska, 1993; Serkedjieva *et al.*, 2005). For a meaningful evaluation and interpretation of the effects of a drug combination, it is necessary to consider the biological significance of the combination, what means the therapeutic effect, lack of cytotoxicity, and mechanisms of action of individual components.

The present results on the combined protective effect of PC and ACA indicated that tissue damage during IAV infection could be reduced greatly by combination therapy with antiviral agents targeting cellular proteins. IAV like other viruses depends on its host cell and therefore, cellular functions and mechanisms essential for viral replication might be suitable targets for antiviral therapy. As a result, the viral growth could be affected independently of the type, strain and antigenic properties of the invading virus.

Currently, we are not convinced that the presented results could be of a clinical importance, when used for the treatment of the influenza infection. Nevertheless, the obtained results support the idea that the appropriate use of antiviral agents with alternative modes of action, protease inhibitors among them, is a promising approach for the control of the disease.

In conclusion, the present paper clearly indicates that combination therapy provides an advantage over single-agent therapy and suggests that the combined treatment may provide an effective basis for prophylactics and therapy of influenza virus infection. Our results underline the important role of proteolytic mechanism in the infectious process. In addition, they support the view that blocking of the specific host-cell functions that are required for viral replication might be an useful approach in antiviral therapy.

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