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In vitro anti-influenza virus activity of a plant preparation from *Geranium sanguineum* L.

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Abstract

A polyphenolic complex (PC), isolated from the Bulgarian medicinal plant *Geranium sanguineum* L., was shown to have selective anti-influenza activity in vitro. Expression of HA on the surface of cells infected with A/chicken/Rostock/34, virus-induced cytopathic effect, infectious virus yield and plaque formation were all reduced at non-toxic concentrations of PC. Synthesis of virus proteins was also selectively inhibited. High concentrations of PC (>200 μg/ml) exhibited a strong virucidal effect. Although the action was directed against an early stage of infection (within 3 h of infection), the process directly affected was not identified. The selectivity of antiviral action was confirmed by the variation in sensitivity of different influenza viruses to PC and the selection of variants with reduced drug sensitivity.

Keywords: Plant preparation; Influenza virus; Inhibition

1. Introduction

One approach used in antiviral chemotherapy is the search for viral inhibitors of plant origin. A large number of extracts and pure substances have been tested and some have been shown to have selective antiviral activity (Vanden Berghe et al., 1986).

Geranium sanguineum L. is wide spread in Bulgaria. Aqueous and alcoholic extracts from its root are used in traditional medicine to treat gastrointestinal disorders and various infections and inflammatory conditions (Jordanov et al., 1973). A methanol extract from the aerial roots of the plant, characterised as a polyphenolic complex (PC), was shown to inhibit the reproduction of

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influenza A and B viruses (Manolova et al., 1987). PC administered intranasally or by aerosol, significantly reduced the mortality of white mice resulting from experimental infection with influenza A/Aichi/2/68 (H3N2) (Serkedjieva and Manolova, 1992). Phytochemical investigation revealed the presence of flavonoids, catechins, gallotannins and phenolic acids in PC preparation (Ivancheva et al., 1992, 1996). To investigate the active components the PC was fractionated and a *n*-butanol fraction was shown to contain the majority of the in vitro antiviral activity (Ivancheva et al., 1996).

The current study was undertaken to investigate the in vitro anti-influenza virus activity of the plant polyphenolic complex in cell culture with respect to the selectivity and specificity of inhibition.

2. Materials and methods

2.1. Compounds

The medicinal plant *Geranium sanguineum* L. (Geraniaceae) has been introduced into the experimental field of the Institute of Botany, Bulgarian Academy of Science, Sofia, and a voucher specimen was deposited in the Herbarium of the Institute. The preparation of the PC has been described previously (Serkedjieva and Manolova, 1992). In short, ground air-dried aerial roots, collected during the flowering period, were defatted with petroleum ether and treated with methanol to fully extract the polyphenolic components. The extract was lyophilised (yield 16%). The polyphenolic content of the preparation was controlled by thin layer chromatography and by quantitative determination of tannins, flavonoids and catechins (Ivancheva et al., 1992). PC was prepared by Dr Stefka Ivancheva at the Institute of Botany. A 1% stock solution was prepared in sterile distilled water. For the in vitro experiments, further dilutions were made in cell culture medium ex tempore. Rimantadine hydrochloride was obtained from Hoffman-La Roche, Nutley, NJ.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) cells were passaged in F15 medium containing 10% new-born calf serum (Gibco). Primary chick embryo fibroblast (CEF) cell cultures were prepared according to Portfield (1960) and maintained in Tris-buffered Gey's medium supplemented with 5% calf serum.

Human influenza viruses A/Victoria/36/88 (H1N1), A/Singapore/1/57 (H2N2), A/Beijing/352/89 (H3N2), A/Gabrovo/539/79 (H1N1), A/Krasnodar/101/59 (H2N2), A/Krasnodar (R) (rimantadine-resistant mutant) and A/Sofia/92/72 (H3N2), equine influenza viruses A/equine/Miami/63 (H3N8) and A/equine/Fontainbleu/78 (H3N8) and avian influenza viruses A/chicken/Rostock/34 (H7N1), A/Rostock (41R), a rimantadine-resistant mutant (Hay et al., 1985) and A/chicken/Weybridge/27 (H7N7) were grown in 11-day-old fertile hen's eggs and allantoic fluids were used as virus inoculum. The virus stocks were stored at $-70^{\circ}C$. For plaque assay, CEF were overlaid with 1% agarose in 199 medium supplemented with 10% bovine serum albumin (BSA) and 1% dextran sulphate. The haemagglutination (HA) assay was performed as described by Serkedjieva et al. (1992).

2.3. Cellular toxicity

Confluent CEF cell monolayers in 24-well plastic plates (Falcon, Beckton Dickinson Labware, Franklin Lakes, NJ, USA) were incubated with 2-fold dilutions of PC in growth medium and were observed microscopically for morphological changes at 24, 48 and 72 h of incubation.

The effect of PC on the proliferation of MDCK and CEF cells was determined in 96-well plastic microtiter plates (Falcon) by the tetrazolium-based colorimetric-MTT assay (Pauwels et al., 1988). Approximately 10^4 cells/well were seeded and incubated with drug-containing medium for 72 h before assay. The 50% cell-inhibitory concentration (IC_{50}) reduced by 50% the optical density values ($OD_{540,\ 690}$) with respect to control non drug-treated cells.

2.4. Virucidal activity

Allantoic fluid containing A/Rostock was diluted 5-fold and treated with equal volumes of increasing 2-fold dilutions of PC in PBS for 1 h at 37°C. The final concentrations ranged from 12.5 to 400 μg/ml. The HA titre and virus infectivity by plaque assay were determined. The minimum concentrations that reduced the HA titre by 2 $\log_2$ and infectious titre by 1 $\log_{10}$ PFU/0.5 ml were determined.

2.5. Antiviral assays

The inhibitory effect of PC on the replication of A/Rostock in CEF was studied by a number of antiviral assays. PC was diluted (2-fold dilutions) in serum-free medium.

2.5.1. Cytopathogenic effect reduction assay

Quadruplicate confluent monolayers in 24-well plates were overlaid with 2 × drug-containing medium (0.5 ml) and an equal volume of virus suspension (100 $TCID_{10}$/0.1 ml). The virus-induced cytopathogenic effect (CPE) was scored after 72 h under an inverted microscope (score 0 = 0% CPE, score 1 = 0–25% CPE, score 2 = 25–50% CPE, score 3 = 50–75% CPE and score 4 = 75–100% CPE). The concentration reducing CPE by 50% (EC_{50}) with respect to virus control was estimated from plots of the data.

2.5.2. Infectious virus yield reduction assay

Triplicate monolayers in 24-well plastic plates were inoculated with drug-containing medium (pre-treatment of cells), washed twice with PBS and challenged with 100 $TCID_{50}$/0.1 ml of the virus in the presence of PC. After 60 min adsorption at room temperature the monolayers were washed twice with PBS and were incubated at 37°C in drug-containing medium for 24 h. Cells and supernatants were pooled after one freeze–thaw cycle and titrated by HA, CPE and plaque assays. The concentrations that reduced virus infectivity by 90% (1 $\log_{10}$PFU/0.5 ml, 1 $\log_{10}TCID_{50}$/0.1 ml; EC_{90}) and reduced HA titres > 2 $\log_2$ were determined.

2.5.3. Plaque reduction assay

Confluent monolayers in 5 cm dishes were inoculated with 100 PFU/0.5 ml of virus and after adsorption for 30 min were washed twice with PBS and overlaid with drug-containing agarose. After 48 h at 37°C monolayers were stained with 0.01% neutral red and the plaques counted. The concentration reducing plaque number by 50% (EC_{50}) was evaluated.

2.5.4. Enzyme linked immunosorbent assay of HA expression

The enzyme linked immunosorbent assay (ELISA) method was as described by Belshe et al., 1988. Quadruplicate monolayers in 96-well microtiter plates were overlaid with 2 × drug-containing medium (50 μl) 30 min prior to inoculation with an equal volume of serial $\log_{10}$ dilutions of virus. The final drug concentration ranged from 0.4 to 50 μg/ml. After 16–20 h incubation at 37°C monolayers were fixed with 0.05% glutaraldehyde in PBS and assayed for HA on the cell surface. ELISA was performed with monoclonal antibodies (MAb) to HA of the corresponding virus strains and protein A-horseradish peroxidase conjugate (Bio Rad, Hercules, CA). The optical densities (OD_{450}) were measured and expressed as a percentage of non-drug-treated virus infected cells (virus control). MAbs were kindly provided by Alan Douglas of the WHO Influenza Centre, Mill Hill, London. The concentration causing 50% reduction in optical density values (EC_{50}) was evaluated from graphic plots. The selectivity index (SI) was determined from the ratio of IC_{50}/EC_{50}.

2.6. Time of addition studies

Time of addition studies used single cycle virus growth conditions. Confluent monolayers in 24-well plates were incubated with PC-containing medium (20 μg/ml) for 1–4 h, washed twice with PBS and challenged with undiluted infectious allantoic fluid (MOI = 10–50 PFU/cell). After adsorption for 1 h at 4 or 37°C in the presence or absence of PC, the cells were washed twice with PBS and were incubated with culture medium. At hourly intervals after infection, medium was re-

placed with PC-containing medium. At 8 h after infection the cells and supernatants were pooled after one freeze–thaw cycle and HA and infectious titres were determined. Virus titers were determined by plaque assay or CPE assay by end-point titration (Reed and Muench, 1938). The significance of differences in infectious virus titres was estimated using the Student's *t*-test.

2.7. Viral protein synthesis

The method was as described by Hay and Zambon (1984). The cell monolayers in duplicate wells of 24-well plates were pre-treated for 1 h at room temperature with PC, washed twice with PBS and challenged with undiluted infectious allantoic fluid (MOI = 10–50 PFU/cell). After adsorption for 1 h at room temperature in the presence of PC, the cells were washed twice with PBS and drug-containing medium added. After incubation for 5 h at 37°C the monolayers were labelled for 1 h with 10 μCi of ^{35}S-methionine/well, lysed and proteins were analysed by electrophoresis on a 12% polyacrylamide gel.

2.8. Immuneprecipitation

Cell lysates were immuneprecipated with anti-HA MAb (HC2) and immune complexes analysed by SDS gel electrophoresis as described by Sugrue and Hay (1991).

2.9. Selection of resistant variants

A/Rostock was serially passaged in the presence of inhibitory concentrations of PC and resistant plaques were isolated in the presence of drug (Hay et al., 1985).

3. Results

3.1. Cytotoxicity

Confluent CEF monolayers treated for 72 h with PC at concentrations of 12.5–50 μg/ml did not show any visible changes in cell morphology or cell density, whereas 100 μg/ml of PC caused microscopically detectable alterations. To evaluate the effect of PC on proliferating cells, MDCK and CEF cells were grown in drug-containing medium (12.5–200 μg/ml) and after 72 h cell viability was determined by the MTT assay. PC at concentrations of 50 μg/ml or less did not reduce significantly the $OD_{540,\ 690}$ values for drug treated cells compared to the control (Fig. 1). The IC_{50} for MDCK cells was 64 μg/ml and for CEF cells 72 μg/ml.

3.2. Virucidal activity

To investigate the direct inactivating effect of PC, A/Rostock was treated for 1 h at room temperature with concentrations of PC ranging from 12.5 to 500 μg/ml. Concentrations of PC 50 μg/ml or less did not cause any reduction in HA titre or virus infectivity determined by plaque assay. A total of 100 μg/ml PC reduced HA titre 4-fold and plaque infectious titre by 1 log and concentrations of PC >200 μg/ml abolished completely the biological activity of the virus.

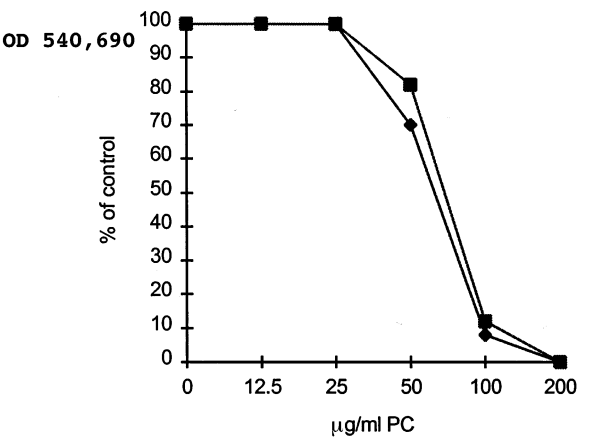

Fig. 1. To evaluate the effect of PC on CEF (■) and MDCK (◆) cell growth cells were seeded with increasing doses of PC (12.5–200 μg/ml), incubated for 72 h and cell viability determined by the MTT assay (Pauwels et al., 1988). The optical densities ($OD_{540,\ 690}$) of drug treated cells are expressed as a percentage of control cells. The values are the average of two independent experiments.

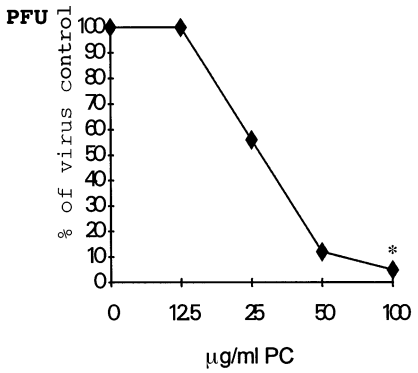

Fig. 2. The experiments were done in duplicate as described in Section 2. The mean number of plaques in the virus control (non-drug treated, virus infected cells) was 135 ± 11.3 ($n = 8$). The results are presented as a percentage of virus control and are the mean values from four independent experiments.* Minute plaques were observed.

3.3. Effect of PC on virus replication

In all experiments non drug treated, mock infected cells were used as cell control and non drug treated, virus infected cells as virus control. Rimantadine hydrochloride (0.1–1 μg/ml) was used as a positive control for inhibition of virus replication.

The in vitro anti-influenza virus effect of PC was studied in a number of assays of the replication of A/Rostock in CEF. The expression of HA on the surface of infected cells, determined by an ELISA with anti-HA MAb, was inhibited by PC. In experiments employing multiple or single cycles of virus replication, the respective EC_{50}s were 2.1 and 10.5 μg/ml. PC inhibited the CPE of A/Rostock in CEF with an EC_{50} of 3.6 μg/ml. In an infectious virus yield reduction assay, the concentration of PC which partially inhibited HA production and reduced the plaque titre by 1 $\log_{10}$PFU/0.5 ml was 3.1 μg/ml; the reduction of infectivity increased up to 3 $\log_{10}$PFU/0.5 ml at concentration of approximately 50 μg/ml. In a plaque reduction assay in CEF A/Rostock plaques were reduced in size and number by concentrations of PC of 25–100 μg/ml (Fig. 2). The EC_{50} in this assay was higher, 32 μg/ml, possibly because PC, like some other plant compounds, does not diffuse well in the agarose overlay (Balde et al., 1990) or because it binds non-specifically to BSA.

3.4. Time of addition studies

The inhibitory effect of PC was determined following addition at different times relative to virus infection. In a single cycle of virus replication, inhibition of virus infectivity was most pronounced when PC (20 μg/ml) was added at between 0 and +3 h after virus infection (Table 1). Pre-treatment of cells with the preparation for 1–4 h, its presence during adsorption or after 3 h of infection did not reduce virus production. The slight inhibition observed when the adsorption was performed at 37°C, allowing internalisation of viral particles, was not significant. The HA and infectious titres of unabsorbed virus in the inoculum were estimated in the same experiments and were shown not to differ significantly from control (results not shown).

3.5. Inhibition of viral protein synthesis

At 20 μg/ml, PC inhibited the synthesis of ^{35}S-methionine labelled virus proteins without affecting cellular protein synthesis (Fig. 3). Inhibition of HA synthesis, analysed by immuneprecipitation, was more clearly discernible and the minimum inhibitory concentration was estimated to be approximately 10 μg/ml (results not shown).

3.6. Selection of PC resistant mutants

Methods which readily select rimantadine-resistant mutants were used. After 11 passages of A/Rostock in CEF in the presence of increasing inhibitory concentrations of PC (10–50 μg/ml) the virus (R-11), harvested from the 11th passage, was less susceptible to inhibition by PC. Direct selection of resistant plaques from the sensitive virus stock by three successive plaque titrations in the presence of 50–100 μg/ml of PC yielded a more resistant variant (Rostock R). Similar results were obtained for R-11 and Rostock R with regard to their growth characteristics (production of HA, expression of HA, cytopathogenicity and

plaque formation) and susceptibility to the inhibitory effects of PC. The lower susceptibility of Rostock R to inhibition by PC compared to

Table 1
Inhibitory effect of PC on a single cycle of A/Rostock replication in CEF

Addition of PC (20 μg/ml)	Reduction in virus titre[a]	
	HA titre (δ $\log_2$ HA)	Infectious titre (δ $\log_{10}$TCID$_{50}$/0.1 ml)
Pretreatment[b] (1 h)	n.d.	n.d.
Pretreatment[b] (4 h)	n.d.	n.d.
Adsorption[c] (1 h, 4°C)	n.d.	n.d.
Adsorption[c] (1 h, 37°C)	2	0.75
Post infection[d]		
0	4	2.5
+1	4	2.25
+2	5	2.87
+3	2	2.62
+4	2	n.d.
+5	2	n.d.
+6	2	n.d.
+7	n.d.	n.d.

Cells were infected with A/Rostock and at 8 h after infection supernatants were pooled after one freeze–thaw cycle and the HA and infectivity titres were determined. Values represent the differences between control and PC-treated virus titres and are the mean values of four independent experiments. The significance of the diference in infectious titres is estimated by the Student's t-test.

[a] Virus control titres, $6\ \log_2$HA, $4 \pm 0.3\ \log_{10}$TCID$_{50}$/0.1 ml ($n = 8$).

[b] CEF cells were incubated with PC-containing medium for 1 or 4 h, washed twice with PBS and challenged with undiluted infectious allantoic fluid of A/Rostock (MOI = 10–50 PFU/cell). After adsorption for 1 h at 37°C the cells were washed twice with PBS and were incubated with culture medium without PC.

[c] PC was added at the time of adsorption at 4 or 37°C; after 1 h the cells were washed twice with PBS and culture medium without PC was added.

[d] After virus adsorption cells were incubated in drug-free medium and at every hour post infection medium was replaced with PC (20 μg/ml) containing medium in one set of wells.

n.d. (no difference) indicates that hemagglutination and infectious titres of control and PC-treated cell supernatants were indistinguishable.

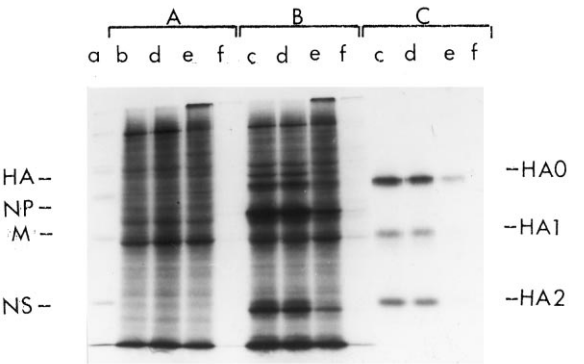

Fig. 3. The experiment was done as described in Section 2. ^{35}S-labelled proteins were from uninfected CEF cells (A), CEF infected with A/Rostock at an MOI = 10–50 PFU/cell, total protein (B) or immune precipitates with MAb to HA (HC2) (C). Lane a, molecular weight markers; lane b, uninfected cell control; lane c, infected cell control; lanes d, e and f, cells treated with 4, 20 and 100 μg/ml PC, respectively. The major virus protein bands are identified on the left: HA, haemagglutinin; NP, nucleoprotein; M, matrix protein; NS, non structural protein. The HA bands are identified on the right. HAO, uncleaved HA; HA1 and HA2 subunits of HA.

parent virus was consistently observed in various assays (Table 2).

Table 2
Comparative sensitivity of A/Rostock and A/Rostock R to inhibition by PC

Assay	Minimal inhibitory concentration (μg/ml)	
	A/Rostock	A/Rostock R
Contact assay	100	200
ELISA[a]	2.1	20
Infectious virus yield reduction[b]	3.1	25
Plaque formation reduction[a]	32	>100
CPE reduction[a]	3.6	25
Inhibition of protein synthesis[c]	10	50

The assays were performed as described in Section 2.

[a] EC_{50}, 50% effective concentration.

[b] EC_{90}, 90% effective concentration.

[c] Minimal concentration causing visible inhibition.

3.7. Susceptibility of different influenza viruses to PC

The sensitivities of representative influenza viruses to the inhibitory action of PC was assessed by ELISA of the reduction in HA expression on the surface of PC-treated infected cells or by the reduction of the virus-induced CPE after multiple cycles of virus replication. The concentration dependence of the inhibitory effect of PC was strain-dependent ($EC_{50} = 0.4–10.5$ μg/ml; SI = 7.3–160) (Table 3). EC_{50} values estimated in ELISA and CPE reduction assays were comparable. Most sensitive to inhibition by PC was the replication of A/eq/Miami/63 in MDCK cells ($EC_{50} = 0.4$ μg/ml; SI = 160) and least sensitive was that of A/Beijing/352/89. The replication of the rimantadine-resistant mutants A/Rostock (41R) in CEF and A/Krasnodar (R) in MDCK cells were as susceptible to the virus-inhibitory effect of PC as that of the parent viruses.

Table 3
Susceptibility of influenza viruses to PC

Virus strain	IC_{50}^{b} (μg/ml)	EC_{50}^{c} (μg/ml)	SI[d]
A/Victoria/36/88 (H1N1)	64	2.2	29.1
A/Gabrovo/539/79 (H1N1)[a]	(MDCK)	5.0	12.8
A/Singapore/1/57 (H2N2)[a]		2.3	27.8
A/Krasnodar/101/59 (H2N2)[a]		4.5	14.2
A/Krasnodar (R)[a]		4.8	13.3
A/Beijimg/352/89 (H3N2)		10.5	6.1
A/Sofia/92/72 (H3N2)[a]		8.5	7.3
A/eq/Fontainbleu/78 (H3N8)		3.8	16.8
A/eq/Miami/63 (H3N8)		0.4	16.0
A/ck/Rostock/34 (H7N1)	72	2.1	34.3
A/ck/Rostock/34[a]	(CEF)	3.6	20
A/Rostock (41R)		2.8	25.7
A/ck/Weybridge/27 (H7N7)		5.2	13.8
A/ck/Weybridge/27[a]		6.1	11.8

The susceptibility to PC-inhibitory action was evaluated as described in Section 2 by ELISA with MAb to HA of the corresponding virus strains and CPE-reduction assay. Monolayers were infected with 10–100 $TCID_{50}$/0.1 ml.
[a] CPE-reduction assay.
[b] IC_{50}, 50% cell toxicity concentration determined by MTT assay (Fig. 1).
[c] EC_{50}, concentration reducing by 50% the optical density values (OD_{450}; ELISA) or the virus-induced CPE in PC-treated virus-infected cells in relation to virus control. The values of EC_{50} are the mean of three to six experiments.
[d] SI (selectivity index) is the ratio of IC_{50} to EC_{50}.

4. Discussion

The present studies concerning the in vitro selectivity and specificity of the anti-influenza virus action of a partially standardised plant polyphenolic complex, isolated from the Bulgarian medicinal plant *G. sanguineum* L., were important in view of the pronounced protective effect of the substance against lethal experimental influenza infection in mice (Manolova et al., 1987). PC (3 mg/kg) administered intranasally 6 h before infection with A/Aichi/2/68 (H3N2) reduced mortality (index of protection = 67%) and prolonged the survival time (3 days) of infected animals. When PC was administered by aerosol in a dose of 20 mg/ml, applied 24 h before and 2, 24, 48 and 72 h after virus infection, the index of protection was 64% (Serkedjieva and Manolova, 1992). Administration of PC in combination with rimantadine hydrochloride produced a synergistic protective effect (index of protection = 77.8%) (Gegova et al., 1993).

The results presented here showed that at concentrations below the IC_{50}, PC inhibited the replication of A/Rostock/34, assayed by a reduction in HA-expression in virus-infected cells (ELISA), virus-specific cytopathogenicity to CEF and a decrease in the production of infectious virus (Table 2, Fig. 2). PC inhibited virus replication when added between 0 and 3 h after infection (Table 1); pre-treatment of cells, addition during adsorption or at late stages of viral infection did not affect

virus replication. These results suggest that early synthetic events in viral multiplication are most sensitive to the inhibitory effect of the substance. At concentrations greater than 50 μg/ml, PC reduced the infectivity of the infecting virus before or at the time of adsorption, possibly due to non-specific binding to viral glycoproteins or to cell membrane components (Okuda et al., 1992). Electron microscopic studies are in progress to examine this possibility. To gain an insight into the effects of PC on intracellular stages in virus replication the effect on virus-specific macromolecular synthesis was studied. Virus-specific protein synthesis was selectively inhibited, shown by the reduction of total protein and HA-synthesis (Fig. 3). The effect was dose-dependent and most pronounced when the preparation was added after virus infection; the minimum inhibitory concentration was 10 μg/ml (Serkedjieva, 1995). In RH cells infected with influenza virus A/WSN/33 (H1N1) PC (62 μg/ml) was shown to inhibit virus-specific RNA synthesis by approximately 50% (Serkedjieva and Tonew, 1996). At concentrations >200 μg/ml PC exhibited a strong extracellular virucidal effect. Thus at non-virucidal concentrations PC may also bind non-specifically to the virus membrane (Okuda et al., 1992). In this way inhibition of macromolecular synthesis may be secondary to the direct interaction of the substance with the virus glycoproteins. Alternatively, the inhibition of virus infectivity might be attributed to multiple mechanisms of action, e.g. specific inhibition of an early stage in viral intracellular multiplication and non-specific interference with virus-cell interactions. In this regard, the selective influenza virus inhibitors amantadine and rimantadine hydrochloride interfere with more than one stage in the replication of influenza viruses (Hay and Zambon, 1984).

Further evidence for the selectivity of the antiviral activity was provided by the selection of mutants with reduced sensitivity to PC, either by multiple passages in the presence of the drug or following purification of resistant plaques. Although the lower sensitivity to PC-inhibition of the resistant variants of A/Rostock was consistently observed (Table 2), the difference relative to the parent virus was insufficient to provide a suitable basis for genetic studies. Furthermore, the differential sensitivities of different strains of human, equine and avian influenza viruses to PC in cell culture is consistent with anti-viral selectivity (Table 3).

The virus inhibitory effect of PC is related to the presence of large quantities of polyphenol compounds. Analysis of PC showed that it contained biologically active compounds including tannins (34%), flavonoids (0.17%), catechins and proanthocyanidines (2 mg/kg) (Serkedjieva et al., 1997). In order to evaluate the active ingredients PC was fractionated by extraction with solvents of increasing polarity and separation of the extracts by column and thin layer chromatography. By this bioassay-guided fractionation more than 60 extracts, fractions and individual compounds were obtained and screened for anti-influenza virus activity. Only the *n*-butanol extract and four fractions from the ethyl acetate extract showed antiviral effect comparable to that of the initial methanol extract; but none exhibited increased antiviral activity. The identification of individual compounds showed that flavonoids-aglycones and glycosides (quercetin, quercetin 3-*O*-galactoside, morin, myricetin, kaempferol, rhamnasin, retusin and apigenin), phenolic acids (caffeic, ellagic and quinic), gallotannins, catechins and maltol were present in the PC extract (Ivancheva et al., 1992, 1996). Studies on the further evaluation of the active constituents are in progress.

Many plant extracts and compounds of plant origin have been shown to possess anti-influenza virus activity (Vanden Berghe et al., 1986), only a few of which exhibited a protective effect in experimental influenza infection in vivo, e.g. polyphenols, caffeic acid (Pollikoff et al., 1966) and gossipol from *Gossipium hirsutum* L. (Vickanova et al., 1970), flavonoid fractions from *Epilobium hirsutum* L. (Ivancheva et al., 1992), a flavonoid (F36) from *Scutelaria baicalensis* L. (Nagai et al., 1992) and a flavonoid from Euphorbiaceae (Sidwell et al., 1994). The mechanism of the antiviral action of F36 has been studied and been shown to interfere with the membrane fusion and budding of viral particles by inhibition of influenza A virus sialidase (Nagai et al., 1995).

The modest selective anti-influenza activity of PC in the in vitro studies contrasts with its significant protective effect in experimental influenza A virus infection in mice. The therapeutic effect of PC in vivo still remains to be explained. PC has been shown to exhibit a stimulating effect on cell type immune responses and to induce a low production of serum interferon in mice after intraperitoneal application (Manolova et al., 1989). Thus, the protective effect of PC in the murine infection model may be attributed to a combination of more than one biological activity-selective antiviral action, non-selective immunomodulating activity and some non-specific biological and pharmacological interactions known for natural polyphenols, such as protein binding, radical scavenging and antioxidant activities.

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