Acta Biochimica Polonica

Vol. 49 No. 4/2002

1005–1012

QUARTERLY

Inhibition of proteolytic processing of adenoviral proteins by ε-aminocaproic acid and ambenum in adenovirus-infected cells★

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Received: 19 November, 2002; revised:19 September, 2002; accepted: 04 December, 2002

Key words: adenovirus, ε-aminocaproic acid, ambenum, adenoviral proteins

Maturation of adenovirus particles is markedly affected by proteolytic processing. The possibility for blocking the conversion of precursor structural core protein (preVII) into mature structure protein VII by officinal drugs ε-aminocaproic acid and ambenum has been demonstrated in Hep-2 cells infected with adenovirus. Proteolytic processing may be regarded as one of the targets for inhibiting adenovirus reproduction.

Adenoviruses are ubiquitous agents infecting humans of all ages, but predominantly infants and children. They cause acute respiratory diseases, pharyngoconjunctival fever, bronchopneumonias, keratoconjunctivitis, gastroenteritis and hemorrhagic cystitis in man (Horwitz, 1985). Adenoviral infections are quite common. Adenoviruses are able to persist in the body for a long time in the latent state causing immunosupressive effects (Horwitz, 2001). A frequency of disseminated of adenoviral diseases in immunocompromised and immunocompetent children, and in adults, has been reported (Munoz *et al.*, 1998).

Recently some success has been attained in elaborating novel drugs designed for effective chemotherapy of adenoviral infections. High antiviral activity of such compounds has been demonstrated in both *in vitro* and *in vivo* studies (De Clercq *et al.*, 1987; Nosach *et al.*, 1989; 2000; Gordon *et al.*, 1991; 1996a, 1996b; De Clercq, 1997; Mentel *et al.*, 1997; 2000;

★Presented at the 8th International Symposium on Molecular Aspects of Chemotherapy, September, 2001, Gdańsk, Poland

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Abbreviations: ε-ACA, ε-aminocaproic acid; MTD, maximal tolerated dose; preVII, precursor of protein VII; p.i., post infection.

Dyachenko *et al.*, 1999; Alexeeva *et al.*, 2000; Zarubaev *et al.*, 2000; Romanowski *et al.*, 2001; Hillenkamp *et al.*, 2002).

On the basis of (*S*)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)cytosine, the new drug Cidofovir (Gilead Sciences) possessing a broad spectrum of antiviral activity has been developed. The topical administration of Cidofovir (Forvade) was shown to be useful in the treatment of adenovirus-associated keratoconjunctivitis. However, the employment of this drug does not preclude the generation of resistant strains of the virus (Gordon *et al.*, 1996a).

Unfortunately, drugs for treating other forms of adenoviral infections are not yet available. Therefore, the search for new compounds capable of affecting different stages of adenoviral reproduction is very urgent. Taking into account the important role of proteolytic processing for viral reproduction, one of the possible targets for chemotherapy of viral infections is the blocking of proteolytic cleavage of viral proteins. Several structural proteins of adenoviruses (IIIa, VI, VII, VIII, 55K) are synthesised as precursors and are incorporated as such into the virions. Then, inside the virions they are subjected to proteolytic cleavage by the viral protease at the final stage of morphogenesis. Such proteolytic cleavage converts the precursors into structural viral polypeptides resulting in full maturation of the infectious viral particles (Anderson *et al.*, 1973; Weber, 1976; Anderson, 1990; Weber, 1995).

Earlier *in vitro* studies showed that the adenovirus protease may be the target for inhibition of adenoviral reproduction (Bhatti & Weber, 1979; Weber, 1976; 1995). The ability of some commercial protease inhibitors to depress adenovirus replication in cell culture was already investigated (Nosach *et al.*, 1994; Sircar *et al.*, 1996; 1998). In this article we have analysed whether several officinal drugs used in medicine as fibrinolysis inhibitors, such as ε-aminocaproic acid (ε-ACA) and *para*-aminobenzoic acid (ambenum), are able to block the proteolytic processing of adenoviral proteins.

MATERIALS AND METHODS

Cells and virus. Human adenovirus type 2 (Ad2) was grown in monolayer cultures of Hep-2 cells, using the medium comprising 45% of medium 199, 45% of Eagle's medium, and 10% of heat inactivated bovine serum. To harvest the virus, infected cells were frozen and thawed three times. The titre of the virus was expressed in inclusion-forming units per ml (IFU/ml) (Weber, 1972; Alexeeva *et al.*, 2000).

Inhibitors. ε-Aminocaproic acid (ε-ACA, $H_2N(CH_2)_5COOH$) and ambenum (*para*–aminobenzoic acid, *p*-$H_2NCH_2C_6H_4COOH$) were synthesised at the Institute of Organic Chemistry of the NAS of Ukraine. Substances of microbial origin, were obtained from the Institute of Antibiotics and Enzymes (Russia). The substances were dissolved in Eagle's medium and sterilised by filtration through 0.2 μm Millipore filters. Only fresh solutions were used.

Antiviral assays. Cells were grown in tubes with or without strips of cover glasses for 48 h. Subsequently, the cells were infected with Ad2 and incubated for 1 h at room temperature. The unabsorbed virus was removed by washing with Hanks Balanced Salt Solution (HBSS) and the substances under study, dissolved in the maintenance medium (Eagle medium without serum), were added at the appropriate concentrations. The infected cells which were not treated with the assayed substances were used as positive controls of virus infection. The cells were incubated for 48 h at 37°C. Subsequently, the infected cells grown on the strips of cover glasses were fixed with 96% ethanol, washed with HBSS, and stained with 0.01% acridine orange solution. The number of infected cells with DNA-containing inclusion bodies was counted using a fluorescence microscope (Alexeeva *et al.*, 2000). Sta-

tistical significance was assessed using the Students' *t*-test. Infected cells grown in tubes without cover glasses were frozen and thawed three times and the titer of the virus was estimated.

The maximal tolerated dose (MTD) of inhibitors was determined microscopically.

Evaluation of the influence of ε-ACA and ambenum on the processing of adenoviral proteins. The cells were grown at 37°C at the bottom of scintillation-count flasks. After 2 days the cells were infected with adenovirus (10–20 IFU/cell), and after adsorption of the virus for 1 h, the cells were rinsed with HBSS, and Eagle's medium containing 30 and 15 mg/ml ε-ACA or 5 mg/ml ambenum was added. Twenty two hours after infection ^{14}C-protein hydrolysate (10 μCi/ml) was added to the medium and the cells were further incubated for 18 h. The infected cells maintained in the medium without inhibitors were used as the control. After 18 h of incubation with ^{14}C-protein hydrolysate (40 h p.i.), the cells were washed twice with the cold buffer (0.01 M Tris/HCl, 0.1 M NaCl, pH 7.4), suspended in the same buffer, and pelleted by centrifugation at 1500 r.p.m. for 10 min at 4°C. Then 50–100 μl of the dissociation buffer containing 5% SDS, 10% 2-mercaptoethanol and 6 M urea was added to the cell pellet and the samples were heated at 100°C for 3 min, and analyzed by SDS/PAGE and autoradiography.

Analysis of virions synthesised in the presence of ε-ACA. Cells were grown in 1 l flasks at 37°C and after 2 days were infected with adenovirus (10–20 IFU/cell). Upon 1-h adsorption of the virus, the cells were washed with HBSS and incubated in Eagle's medium without serum (virus control) or in the presence of 15 and 30 mg/ml ε-ACA. The next day the medium was discarded and the same fresh medium was added. After 48 h the cells were detached in the minimal volume of the medium, pelleted by centrifugation, and resuspended in 0.05 M Tris/HCl (pH 8.0) containing 0.5 M NaCl and 0.5% Triton X-100. This suspension was incubated overnight at 4°C and centrifuged at low speed to remove cellular debris. The virus was purified by centrifugation in CsCl density gradient (1.2–1.4 g/cm^3) for 4 h at 39 000 r.p.m. (4°C) in Beckman centrifuge. The viral band was collected through the needle of a syringe and trichloroacetic acid was added to 10% final concentration. After 2 h incubation at 4°C the samples were centrifuged for 15 min at 1500 r.p.m. The pellet was washed 3 times with acetone and dissolved in the dissociation buffer. Samples were analysed by SDS/PAGE with 94, 67, 43, 30, 20, and 14 kDa proteins as markers.

RESULTS AND DISCUSSION

Our cytomorphological studies have shown that ε-ACA at a concentration of 30 mg/ml and ambenum at a concentration of 5 mg/ml were not toxic for Hep-2 cells, and caused neither destruction of the cell monolayer nor alteration of cell morphology.

In antiviral assays in cell culture, the minimal inhibitory concentration (MIC) or the 50% inhibitory dose (ID_{50}) of the substances under study is generally estimated according to routinely used methods. Estimation of MIC or ID_{50} is based on the assessment of the reproduction of viruses in drug-treated and non-treated cells employing assays suitable for different viruses, such as plaque assay, analysis of DNA synthesis, transformation, antigen expression, and fluorescent focus (De Clercq *et al.*, 1987; Hu & Hsiung, 1989; Mentel *et al.*, 1997). In this study, and in several previous ones, we have assessed reproduction of adenovirus by counting cells containing virus-induced intranuclear inclusion bodies (Fig. 1) (Nosach *et al.*, 2000; Alexeeva *et al.*, 2000), a technique approved for determination of virus infectivity (Weber, 1972; Landau *et al.*, 1982; Durham & Johnson, 1984).

The data on inhibition of virus reproduction by the substances under study in cells infected

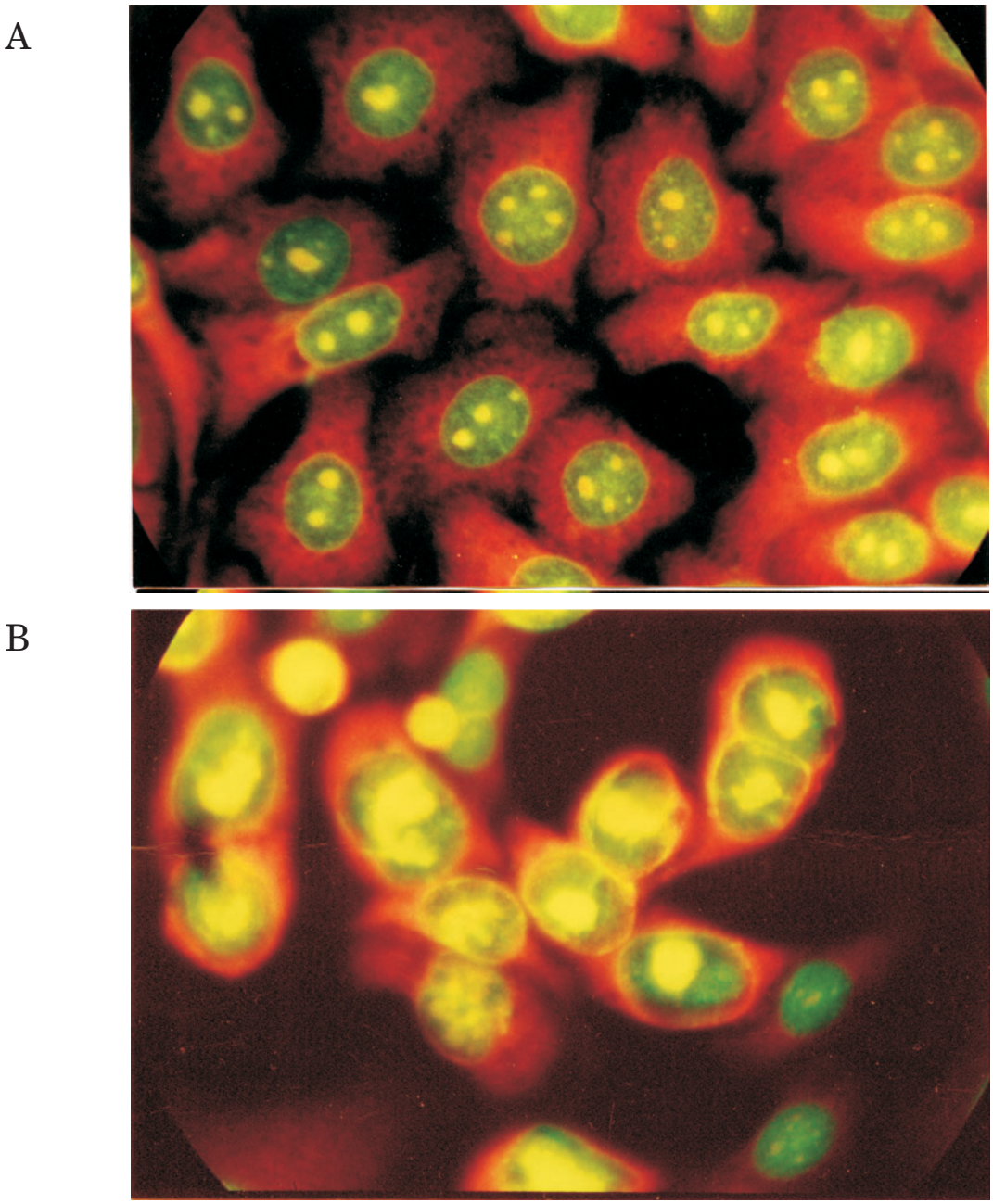

Figure 1. Intranuclear inclusion bodies in adenovirus-infected cells.

Luminescent microscopy of cells stained with 0.01% acridine orange solution. A, non-infected Hep-2 cells; B, cells infected with human adenovirus type 2 (48 h p.i.).

at different multiplicity of infection are given in Table 1. It is shown that both ε-ACA and ambenum reduced the number of infected cells, in most cases by no more than 50%. The most pronounced inhibition occurred at relatively lower multiplicity levels.

We have also analysed the synthesis of viral proteins in infected cells treated with inhibitors. Prolonged (18 h) labelling of proteins synthesised in adenovirus infected cells with ^{14}C-protein hydrolysate showed inhibition of processing of protein VII precursor (preVII) into the structural protein VII by both ε-ACA (30 mg/ml) and ambenum (5 mg/ml). The data obtained are presented in Fig. 2, lanes 2 and 3. Upon short-term labelling (1 h after 40 h p.i.) only preVII protein appeared both in the presence and absence of ε-ACA and

Table 1. The influence of proteolysis inhibitors on the percentage of infected cells

Virus	Compound, concentration (mg/ml)	% of infected cells in absence of compounds, $\chi \pm m$	% of infected cells in presence of compounds, $\chi \pm m$	Inhibition of infected cells number (%)
Ad 1	ε-ACA, 30	50.0 ± 2.23	23.0 ± 1.88*	54
		68.0 ± 2.08	40.2 ± 2.19*	42
Ad 2	ε-ACA, 30	75.2 ± 1.93	45.0 ± 2.22*	40
		78.3 ± 1.85	53.3 ± 2.23*	33
		85.0 ± 1.59	77.0 ± 1.88**	10
	Ambenum, 5	53.3 ± 2.23	24.3 ± 1.90*	55
		55.2 ± 2.22	31.0 ± 2.06*	44
		85.0 ± 1.59	70.0 ± 2.04*	18

*Difference between percentage of infected cells incubated with and without inhibitor is significant $P < 0.001$; **difference between percentage of infected cells incubated with and without inhibitor is significant $P < 0.01$.

ambenum (Fig. 2, lanes 4–6). It is worth noting that blocking of preVII processing has been demonstrated at relatively high infectious dose of the virus when the number of infected cells in the presence of ambenum and ε-ACA decreased insignificantly. At the same time the effects of the ambenum and ε-ACA on the yield of the virus were more evident, 37% and 11%, respectively, of the yield in cultures without the inhibitors. The substance of microbial origin also has been shown to inhibit the proteolytic processing of preVII (Fig. 3).

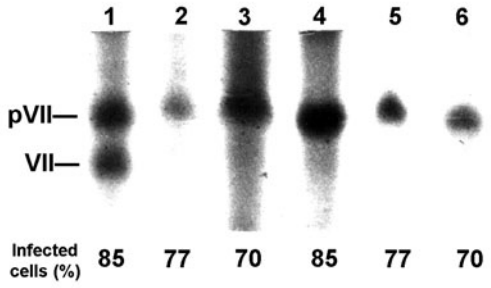

Figure 2. SDS/PAGE of proteins synthesised in Ad2-infected Hep-2 cells incubated in the presence of ε-ACA (30 mg/ml) and ambenum (5 mg/ml).

The cells were labelled for 18 h with ^{14}C-protein hydrolysate added to the medium 22 h p.i. (lanes 1–3), and for 1 h at 48 h after infection (lanes 4–6). Lanes 1 and 4, infected cells incubated without inhibitors; lanes 2 and 5, infected cells incubated with ε-ACA (30 mg/ml); lanes 3 and 6, infected cells incubated with ambenum (5 mg/ml).

As is seen from data presented in Figs. 2 and 3 ε-ACA, ambenum and the substance of microbial origin block the conversion of preVII into core protein VII. In control virus-infected cultures incubated without inhibitors, there is an extensive accumulation of protein VII. The proteolytic cleavage of preVII results in the

Figure 3. SDS/PAGE of proteins synthesised in Ad2-infected Hep-2 cells incubated with the inhibitor of microbial origin (5 mg/ml).

The cells were labelled for 18 h with ^{14}C-protein hydrolysate added to the medium 22 h p.i. Lane 1, infected cells incubated with the inhibitor of microbial origin (5 mg/ml); lane 2, infected cells incubated without the inhibitor.

loss of one Met-containing tryptic peptide (Anderson *et al.*, 1973).

At the final stage of virion assembly, the viral protease cleaves many precursor proteins (Weber, 1976; 1995; Bhatti & Weber, 1979). A series of such cleavages is a prerequisite for completion of virion maturation, resulting in conversion of "young virions" into mature virus particles.

It is possible that ε-ACA and ambenum also inhibit the proteolytic processing of other proteins. preVII is simply the most abundant of them and also the most easily isolated.

We have also established that the virions synthesised in presence of ε-ACA and purified in CsCl gradient are characterised by a decreased content of protein VII (Fig. 4).

Adenoviral protease is a 23 kDa polypeptide coded by the L3 region within the late transcription unit 60-61.7. This enzyme is believed to represent a cysteine protease (Webster *et al.*, 1989; Anderson, 1990; Thanyi *et al.*, 1993; Sircar *et al.*, 1996). The virion contains just a few copies of this enzyme (Anderson, 1990). It has been shown recently that adenoviral protease is required not only for virion maturation, but also for the penetration of the virus into the cells (Cotton & Weber, 1995; Graber *et al.*, 1996; Rusindana-Umunyana *et al.*, 2000). It has been established (Graber *et al.*, 1996) that inhibitors of cysteine protease located inside the capsid block degradation of the capsid-stabilizing protein VI and prevent virus uncoating at the nuclear membrane.

We have shown here that the decrease in the number of infected cells incubated with ε-ACA or ambenum was rather insignificant. These data may be explained by taking into account that virus-induced intranuclear inclusion-bodies are due not only to the presence of mature infective virions. Such inclusion-bodies may also represent sites of accumulation of structural components. Therefore, protease inhibitors may decrease profoundly the infectivity of viral particles without affecting the number of structural particles being produced (Sircar *et al.*, 1996; 1998).

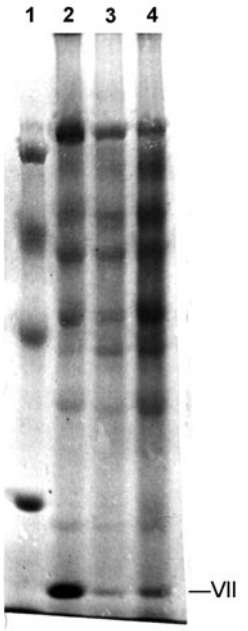

Figure 4. The effect of ε-ACA on virus maturation.

The purified virus was subjected to SDS/PAGE. The gels were stained with Coomassie Blue. Lane 1, molecular mass markers: 94, 67, 43 and 30 kDa; lane 2, adenovirus purified from cells incubated in inhibitor-free medium; lane 3 and 4, adenovirus purified from cells incubated in the presence of ε-ACA, 30 mg/ml and 15 mg/ml, respectively.

We have demonstrated the ability of fibrinolysis inhibitors, such as ε-ACA and ambenum, to block the proteolytic cleavage of adenoviral polypeptides in infected cells; these polypeptides can be considered the target of the inhibitors antiviral activity. These drugs have previously been shown to effectively inhibit also the reproduction of the influenza virus (Lozitsky *et al.*, 1994).

The data obtained suggest the possibility of employing the officinal medicinal drugs, such as ε-ACA and ambenum, as anti-adenoviral agents.

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