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Adenovirus endopeptidase and papain are inhibited by the same agents

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Abstract

Adenoviruses encode a cysteine protease (AVP) which carries out highly specific cleavages on at least seven viral proteins and two cellular proteins. Virus infectivity is dependent on this function. The three-dimensional positions of the amino acids involved in catalysis display a striking similarity to those of papain, suggesting a similar catalytic mechanism. This similarity has prompted us to compare the effect of papain inhibitors on the two enzymes. AVP and papain activity was tested on a fluorescent peptide substrate as well as on metabolically labeled adenovirus (Ad2) precursor proteins. Hep2 cells infected with Ad2 were exposed to inhibitors and assayed for, (a) infectious virus, (b) in situ Ad2 protease activity, (c) physical particle production and their polypeptide composition.We found that in both substrate systems AVP was sensitive to the papain inhibitors benzamidoacetonitrile, acetamidoacetonitrile and *N*-methoxyphenylalanine glycylnitrile, and that the degree of sensitivity was influenced by the substrate. Unlike papain, AVP was relatively insensitive to E64. In ex vivo tests, Hep2 cells infected with Ad2 were exposed to inhibitors and assayed for, (a) infectious virus, (b) in situ Ad2 protease activity, (c) physical particle production and their polypeptide composition. A 4-fold reduction in virus titer was obtained when the inhibitors were added between 17 and 25 h after infection. Processing of precursor proteins was also inhibited yet the production of physical particles was only reduced 2-fold. These experiments show that papain inhibitors are also capable of inhibiting the adenovirus protease both in vitro and ex vivo, thus forging a possible link between structural similarity and functionality.

Keywords: Adenovirus type 2; Protease; Cysteine; Papain; Inhibitors

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1. Introduction

Proteolytic processing is an essential part of the life cycle of many viruses. Adenoviruses encode a cysteine protease which carries out highly specific cleavages on at least seven viral proteins and two cellular proteins (Weber, 1995). Virus infectivity is absolutely dependent on the proteolytic function (Weber, 1976; Grierson et al., 1994; Weber, 1995; Greber et al., 1996). The enzyme is packaged into virus particles as the integral protein. The ts1 mutant of Ad2 prevents encapsidation of the otherwise active AVP, resulting in unprocessed virions at the nonpermissive temperature which fail to uncoat in a subsequent infection (Cotten and Weber, 1995; Rancourt et al., 1995). Genetic and structural data have identified the active site as His54-Glu71-Cys122 (Rancourt et al., 1994; Ding et al., 1996). Thus, AVP is a thiol protease, a classification consistent with its sensitivity to cysteine protease inhibitors and relative insensitivity to inhibitors of other classes of proteases (Webster et al., 1989; Tihanyi et al., 1993; Weber and Tihanyi, 1994).

The enzyme is stimulated by formation of a disulfide bond between Cys104 of the enzyme and Cys10′ of an eleven amino acid cleavage fragment from the C-terminus of virus protein PVI (Mangel et al., 1993; Webster et al., 1993; Ding et al., 1996). Mangel and his colleagues noted a striking similarity with papain in the three-dimensional positions of the amino acids involved in catalysis, suggesting a similar catalytic mechanism (Ding et al., 1996). The substrate cleavage sites of the two enzymes are quite different: (M,I,L)XGG-X or (M,I,L)XGX-G for AVP and nonspecific for papain with preference for Arg or Lys in the P1′ position. While little work has been done on inhibitors of AVP (Cornish et al., 1995; Sircar et al., 1996), there is a considerable literature on the inhibition of papain (Brisson et al., 1986; Foje and Hanzlik, 1994; Hanzlik and Liu, 1995; Turk et al., 1997). The potential similarity in the catalytic mechanism between the two enzymes has prompted us to compare the effect of papain inhibitors on the two enzymes.

2. Materials and methods

2.1. Cells and virus

Human adenovirus type 2 (Ad2) was grown and titered in monolayer cultures of Hep2 cells using Delbecco's modified minimum essential medium (DMEM) supplemented with 2.5% calf serum. To harvest virus, infected cells were washed once in DMEM without serum and rapidly frozen thawed six times. These cell lysates were clarified by low speed centrifugation and titered. Purified virus was prepared by equilibrium density gradient centrifugation in CsCl. The visible virus band (from three 10 cm petri dishes) was collected from the top, dialysed and the optical density determined by disrupting the virions with 1% sodium dodecyl sulphate (SDS). Virion proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver.

2.2. Inhibitor studies

In pulse-chase experiments infected cells were labeled with 30 μCi/200 μl of [^{35}S]methionine from 23 to 24 h p.i., the isotope removed and inhibitors added directly to the cells for 15 min. followed by medium and excess methionine (chase) to achieve the indicated inhibitor concentrations. Cell lysates were separated by SDS-PAGE and followed by autoradiography.

2.3. Inhibitors

Benzamidoacetonitrile (BAN) is a powerful competitive inhibitor ($K_i = 0.14$ mM) of papain (Sluyterman and Wijdenes, 1973). Acetamidoacetonitrile (AAN) is a reversible inhibitor of papain (Hanzlik et al., 1990. *N*-Methoxyphenylalanine glycylnitrile (MPN) is a specific substrate analog inhibitor of papain which is thought to form a reversible covalent thioimidate bond between the nitrile group and the active site sulfhydryl of papain to rationalize the potency of the inhibition ($K_i < 10^{-3} \times K_m$). Stock solutions of inhibitors were prepared as follows: (a) E64 (Sigma), 5 mM in distilled water; (b) Iodoacetate (Boehringer

Mannheim), 10 mM in distilled water freshly prepared and pH adjusted to 7.5 with Tris–HCl; (c) BAN (mol. wt. 160) initially dissolved in minimal amount of DMSO then 10 mM in DMEM; (d) AAN (mol. wt. 98) also initially dissolved in minimal amount of DMSO then 10 mM in DMEM (Neugebauer et al., 1996); (e) MPN (mol. wt. 234) dissolved in minimal amount of DMSO then 5 mM in DMEM (Neugebauer et al., 1996).

2.4. Enzymes and peptides

Recombinant adenovirus type 2 protease (AVP; EC3.4.22.–) was purified by affinity chromatography from an *E. coli* expression system as described before (Houde and Weber, 1990). Papain (EC 3.4.22.2; 2 × crystallized) was purchased from Sigma.

2.5. Enzyme assays

Quantitative assays were carried out with a fluorescent peptide substrate, rhodamine 110, bis-(L-leucyl-L-arginylglycylglycine amide), tetrahydrochloride (R110), purchased from Molecular Probes, Inc. (4849 Pitchford Ave., Eugene, OR 97402), essentially as described before (Diouri et al., 1995). The reaction mixture contained TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, 40 μM pVIc (enzyme activating peptide GVQSLKRRRCF), 5 μM R110, 1 μg of AVP or papain, in a total volume of 300 μl. Inhibitors were always added subsequent to coupling the activating peptide to the enzyme. Incubation was at 37°C for 18 h and the fluorescence was measured as before (Diouri et al., 1995).

Qualitative assays were done using ts1 infected cell lysates labelled with [^{35}S]methionine at the nonpermissive temperature (39°C) at 24 h p.i. as a source of viral precursor proteins (Keyvani-Amineh et al., 1995). Ts1 is a mutant temperature sensitive for protease activity (Weber, 1976). Any residual protease activity was inactivated by boiling this substrate for 2 min. A 100 μl reaction mixture contained TE buffer (pH 8), 0.5 μg AVP or 0.01 μg papain, the proteins of a ts1 infected cell lysate at the nonpermissive temperature which contained 10 μg of viral precursor protein pVII (as well as other ts1 proteins in relative proportion) and inhibitor at the indicated concentration. The reaction was incubated at 37°C for 18 h and stopped by boiling in lysing solution.

3. Results and discussion

3.1. Effect of inhibitors on enzyme activity

To compare the relative potency of inhibition of the adenovirus protease (AVP) with papain, in addition to the general thiol inhibitors, iodoacetamide (IAA) and E64, two simple nitrile analogs of papain substrates, benzamido acetonitrile (BAN) and acetamido acetonitrile (AAN) and one peptide nitrile, *N*-methoxy phenylalanylo-glycylnitrile (MPN), were chosen. The fluorescent peptide substrate LRGG-rhodamine-GGRL (R110) employed in the enzyme assay, is specific for AVP but is also efficiently digested by papain (Table 1). For maximal activity, AVP requires the eleven amino acid pVIc peptide as cofactor. The treatment noted as pVIc raised the basal activity of the enzyme from 976 to 31 009 fluorescence units. The activated enzyme was used in the inhibition studies and this value was taken as 100% enzyme activity. We found that AVP was sensitive to each of the three nitrile inhibitors to an extent exceed-

Table 1
Effect of papain inhibitors on adenovirus protease activity in vitro

Treatment	Relative enzyme activity[a]			
	AVP	(%)	Papain	(%)
–	976		8005	100
pVIc	31 009	100		
AAN	3821	12	1472	18
BAN	1585	5	1007	12
MPN	1405	4	874	11
IAA	994	3	606	7
E64	22 857	71	640	8

[a] The reactions contained 1 μg enzyme, 7 μM R110 fluorescent peptide substrate, 66 μM inhibitor, 40 μM pVIc. Reactions were incubated overnight and the numbers indicate fluorescence intensity. Results are from at least three determinations. Standard deviations were 9–13%.

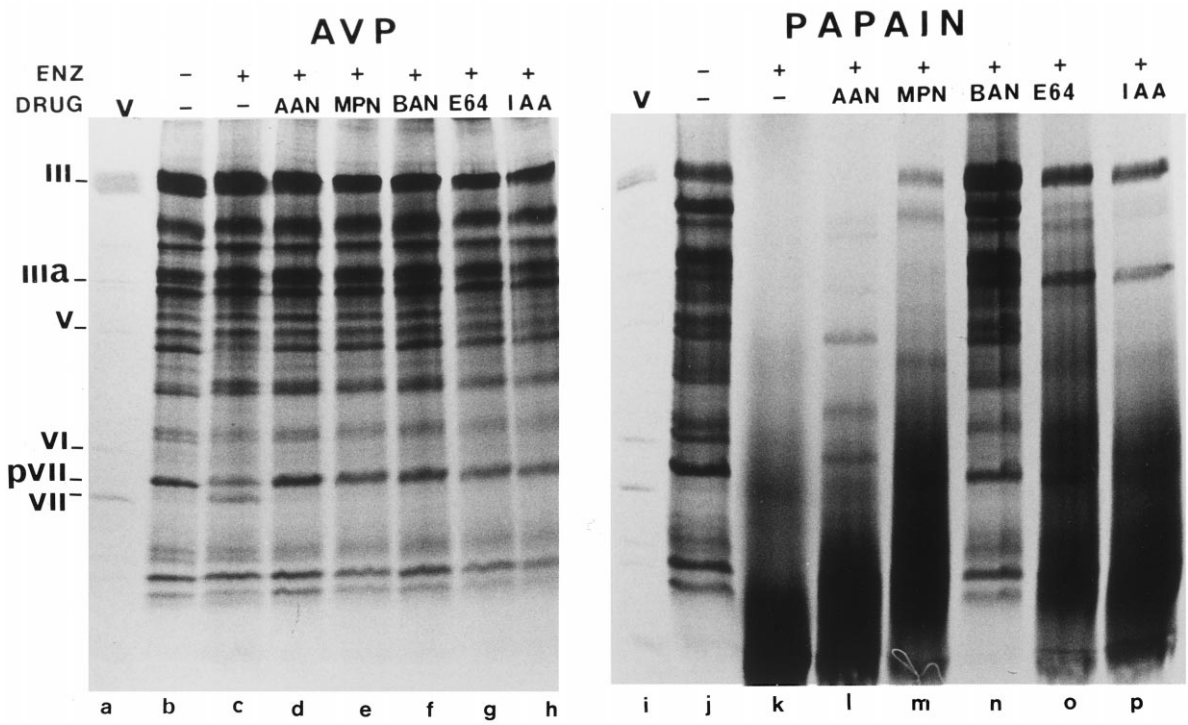

Fig. 1. The effect of inhibitors on the digestion of adenovirus proteins by AVP and papain in vitro. The reaction mixtures contained ^{35}S-labeled ts1 proteins (10 μg of protein PVII), 0.5 μg of AVP or 0.01 μg of papain and 50 μM of inhibitor, as indicated on the figure. The reactions were incubated at 37°C for 18 h, then stopped by boiling in lysing solution and subjected to SDS-PAGE and autoradiographed. V, ^{35}S-met labeled Ad2 marker virus.

ing that of papain. The relative insensitivity of AVP to E64, an agent which has been shown to be an excellent irreversible inhibitor of papain, has been noted before (Tihanyi et al., 1993).

The above results were obtained with an artificial peptide substrate. Because the type of substrate may influence relative cleavage efficiency, we repeated the inhibition experiments with authentic protein substrates in the form of metabolically labeled adenovirus structural proteins. Enzyme concentrations and incubation time were adjusted with each enzyme to obtain readable results. Fig. 1 shows the results of a typical experiment. The inhibition of AVP is best seen in the case of the pVII core protein which is normally cleaved to VII (Fig. 1, lane c). Papain cleaves all viral proteins to small peptides (Fig. 1, lane k). Again all of the inhibitors efficiently stopped AVP activity. This assay, however, does not lend itself to quantitative comparisons. In the case of papain, the relative efficiency of the inhibitors is easily assessed qualitatively: AAN being the least efficient, followed by MPN, IAA and E64 in increasing potency of inhibition. BAN appeared to be the most potent inhibitor in this assay (Fig. 1, lane n).

3.2. Effect of inhibitors on virus infection

The effect of BAN, MPN and AAN on the yield of infectious virus was tested by adding the inhibitors to the medium during virus infection. Inhibitors were added (at 50 μM final concentration) at different times after infection to determine the optimal period of sensitivity. Infectious virus was titered 48 h after infection. A 4-fold reduction in virus titer was obtained when the inhibitors were added between 17 and 25 h after infection. This effect quickly disappeared when the inhibitors were added before or after these times.

Lower concentrations of inhibitors (1, 5, 10 μM) showed no detectable effect. Higher concentrations were cytotoxic.

The reduction in virus production in the presence of the inhibitors could be due to either non-specific effects or due to the inhibition of the viral protease. To distinguish between these alternatives we looked first at protease activity in infected cells and secondly, at the production of virus particles. In the first instance infected cells were pulse labeled between 23 and 24 h with [^{35}S]methionine and then chased in the presence or absence of inhibitor. Virus protein processing was examined by electrophoresis followed by autoradiography.

Examples of results obtained with BAN and MPN show that the processing of the precursors to capsid proteins VI and VII was nearly completely inhibited (Fig. 2, lanes e, j) when the concentration of inhibitors was raised to 50 μM. The inhibition of processing was dose-dependent and BAN was the most effective, showing a 25% reduction in processing of PVII to VII at 10 μM, increasing to 100% at 50 μM (Fig. 2, lanes d, e). These results are consistent with the interpretation that the viral protease is inhibited. They, however does not rule out the possibility that the observed reduction in cleavage was due to non-specific toxicity. This possibility was addressed by the second approach which consisted in assessing the production of virus particles; the rationale being that unhealthy cells produce less virus. Inhibitors were added to infected cell cultures at 18 h after infection and the viruses were purified at 48 h after infection. The viral bands at density 1.34 g/ml were collected from the CsCl gradients and the optical density was determined. BAN at 0, 1, 50 μM gave 2.0, 1.8, 1.0 OD_{260} units of virus, respectively and MPN at 0, 1, 10 and 50 μM gave 0.7, 0.7, 0.6, 0.5 OD_{260} units of virus, respectively. These figures show a maximum 2-fold reduction in virus production, whereas the reduction in infectious virus titer was respectively 1-, 4-, 64-fold for BAN and 1-, 1-, 3-, 11-fold for MPN. Consequently we suggest that the inhibitors cause minimal toxicity, but efficiently inhibit infectious virus production by inhibiting proteolytic processing. This conclusion predicts that a certain proportion of the viruses produced in the presence of these inhibitors are noninfectious and contain uprocessed precursor proteins. Fig. 3 shows stained electropherograms from one of the experiments, clearly demonstrating the presence of pVI and pVII precursor proteins at the inhibitory concentration of BAN and MPN (50 μM; lanes d, e and j). Drug induced toxicity was also verified on uninfected cell cultures by means of plating efficiency. The size and number of colonies remained unaffected at drug (AAN, BAN, MPN) concentrations up to 100 μM. Taken together these experiments strongly support our contention that these papain inhibitors are also capable of efficiently inhibiting the adenovirus protease.

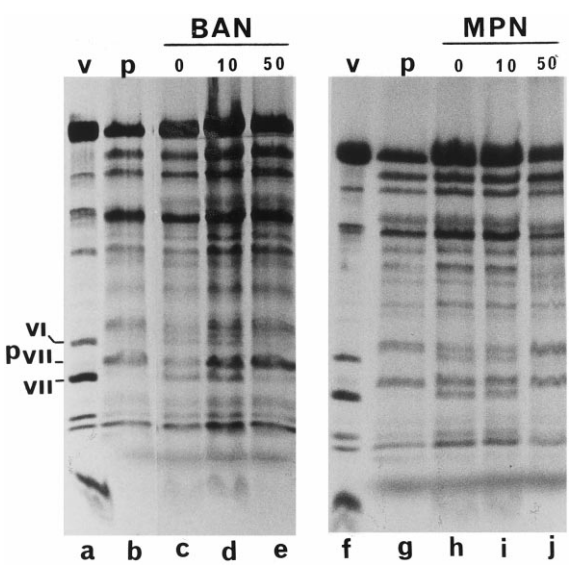

Fig. 2. Effect of BAN and MPN on the proteolytic cleavages in adenovirus infected cells. Infected cells were pulse-labeled with [^{35}S]methionine from 23 to 24 h post-infection (lanes b–e and g–j) and then chased in the presence of 0, 10 or 50 μM BAN (lanes c–e) or MPN (lanes h–j) until 48 h. Cell lysates were prepared and the proteins separated by SDS-PAGE and autoradiographed. V, [^{35}S]methionine labeled Ad2 marker virus

The structural similarity in the active sites of papain and AVP have been suggested to predict a similar catalytic mechanism (Ding et al., 1996). Here we have shown that these similari-

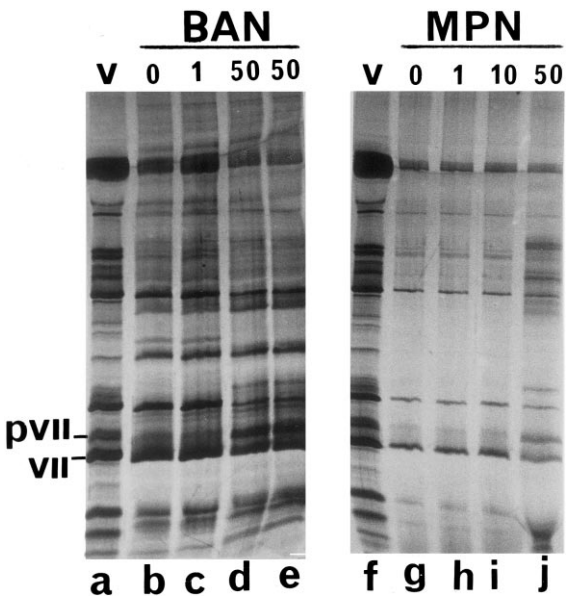

Fig. 3. Effect of BAN and MPN on virus production and maturation. BAN and MPN were added directly on infected cells for 15 min. at 18 h post-infection, then fresh culture medium was added to achieve the final concentrations indicated on the figure (0–50 μM). 48 h after infection at 37°C the cells were harvested and the virus was purified by CsCl density gradient centrifugation and subjected to SDS-PAGE and stained with silver. The optical density of the virion band at 1.34 g/ml CsCl density was established as follows: lanes b–e, 2.0, 1.8, 1.0, 1.0, respectively, and lanes g–j, 0.7, 0.7, 0.6, 0.5, respectively. V, Ad2 virus marker.

ties between the two enzymes appear also to extend to modes of inhibition: three substrate analogues which are competitive inhibitors of papain also inhibited AVP. This knowledge should aid in the design of more powerful and specific inhibitors of AVP as therapeutic agents to control adenovirus infections.

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