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Inhibition of adenovirus infection with protease inhibitors

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Abstract

The effect of a series of cysteine and serine protease inhibitors was tested on the growth of human adenovirus type 2 in tissue culture. In accordance with the nature of the adenovirus protease, only the cysteine protease inhibitors were effective in significantly reducing the production of infectious virus. Addition of the inhibitors to the medium 18 h after infection gave IC_{50} of 30, 40 and 80 nM with *N*-ethylmaleimide, leupeptin and E64c, respectively. Several lines of evidence suggest that inhibition of infectious virus formation operated through the inhibition of the viral protease rather than cellular toxicity: (a) the yield of physical particles declined only 4–5-fold, while that of infectious virus declined 3–7 orders of magnitude, (b) these particles contained unprocessed precursor proteins and (c) pulse-chase experiments showed that the inhibitors prevented the efficient processing of viral precursor proteins. We conclude that the cysteine protease inhibitors efficiently depress the formation of infectious adenovirus by inhibiting the viral protease.

Keywords: Adenovirus type 2; Cysteine protease inhibitors; Suppression of adenovirus replication; Inhibition of viral protease

1. Introduction

Adenoviruses have been isolated from many common vertebrates, and some 48 serotypes have been isolated from humans (Ginsberg, 1984). They cause a variety of diseases in animals, and bronchopneumonias, gastroenteritis and keratoconjunctivitis in man (Horowitz, 1985). Isolated case reports have associated the virus with a variety of other afflictions as well (Greenberg, 1991; Zaltzman et al., 1994). The viruses appear to pose little real threat to healthy individuals, but can be life-threatening in immunocompromised patients, the very young and the aging population. Ocular adenoviral infections occur in epidemics worldwide and produce significant patient morbidity, as well as causing substantial economic losses (Gordon et al., 1991). Because of the large number of serotypes and other constraints, vaccines are impractical. A number of agents have been tested for their effect on virus growth or for the control of a virus-caused condition, but so far no systematic approach has been attempted to discover anti-adenoviral agents (Brotz et al., 1978; Cassano, 1991; Cook, 1993; D'Halluin et al., 1980; Eggerding and Raskas, 1978; Hutter, 1990;

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Iseki and Baserga, 1983; Kan-Mitchell and Prusoff, 1979; Murphy et al., 1993; Shortridge and Squires, 1977; Viswalingam, 1993; Ward et al., 1993; Wigand, 1979; Yamazaki and Tagaya, 1980). For this reason we set out to identify viral targets for chemotherapy. Studies on the adenovirus protease suggest that because of its uniqueness and key role in virus growth it might be such a target (Weber, 1990; Weber and Tihanyi, 1994). Proteases have been successfully targeted for chemotherapy in other diseases (Dodwell and Howell, 1993; Ebina and Tsukada, 1991; Hayashi et al., 1991; Ishii et al., 1990; Olaya and Wasserman, 1991; Ovcharenko and Zhirnov, 1994; Rosenthal et al., 1991).

As a first step on the road to exploring the utility of chemotherapy of adenovirus infections by means of anti-proteolytic agents, we tested the efficacy of commercially available protease inhibitors for the suppression of adenovirus replication. The results show that some cysteine protease inhibitors counter adenovirus replication at nanomolar concentrations by inhibiting the viral protease, thereby demonstrating the viability of the approach in vitro.

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 Dulbecco's modified minimun 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 four times. These cell lysates were clarified by low speed centrifugation and titered, or the virus was further purified by equilibrium density gradient centrifugation in CsCl. The visible virus band (from a minimum of three 10-cm petri dishes) was collected from the top, dialyzed and the optical density determined by disrupting the virions with 1% sodium dodecyl sulphate (SDS). Alternatively the viral proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver.

2.2. In vitro assay of PVII processing

Recombinant adenovirus protease was purified from an *E. coli* expression system as described before (Tihanyi et al., 1993). The substrate consisted of purified, disrupted ts1-39 C virions labeled with ^{35}S-Met. Reaction volume was 40 μl in TE buffer (pH 8.0), and inhibitors were added from stock solutions. The reactions were incubated at 37°C overnight, then lysing solution was added, the sample was boiled for 2 min and subjected to SDS-PAGE. Cleavage of PVII to VII was determined by autoradiography.

2.3. Inhibitors

The following solvents were used to make the stock solutions of the inhibitors: E64, E64c and E64d (Sigma), 5 mM in distilled water; *N*-ethylmaleimide (NEM, Boehringer Mannheim), 10 mM in phosphate buffer, pH 7.8; Iodoacetate (IAA, Boehringer Mannheim), 10 mM in distilled water, freshly prepared and pH set at 7.5 with Tris; egg white cystatin (Sigma), 1 mg/ml in Tris buffer, pH 8; soybean trypsin inhibitor (SBTI, Sigma), 1 mg/ml in phosphate buffer; MDL 28170 (Marion Merrell Dow, Inc.), initially dissolved in a minimal amount of DMSO, then 1 mM stock made in DMEM; aprotinin (Sigma), dissolved directly in DMEM; chymostatin (Boehringer Mannheim), dissolved in 10 μl DMSO, then in DMEM; pepstatin (Sigma), dissolved in 10 μl DMSO, then in DMEM; antipain (Sigma), dissolved in DMEM; phenylmethylsulfonylfluoride (PMSF, Sigma), initially dissolved in 40% ethanol, then in DMEM.

3. Results and discussion

In preliminary experiments several cysteine protease inhibitors were tested in time course experiments to determine the point in virus infection which is most susceptible to inhibition. We found that infectious virus yield was maximally depressed when the inhibitors were added at about 18–20 h after infection. The target of inhibition was most likely the viral protease, as the

protease is synthesized late in infection. A number of protease inhibitors were therefore tested for their ability to inhibit virus growth by adding the drug to the medium at different concentrations at 18 h after infection and titering virus yield at 48 h. The results are shown in Fig. 1 (——). As expected, cysteine protease inhibitors were clearly more effective than serine protease inhibitors. The shape of the inactivation curves appeares to be biphasic. As none of these inhibitors are specific to adenovirus protease, the drastic inhibition of the production of infectious virus could be due to cellular toxicity caused by the inhibitors. This was checked two ways: (a) cells were grown in the presence of the agents for 48 h at the highest concentrations used in Fig. 1, no cytopathic effects being observed, and (b) the physical particle yields from the above cell lysates were determined. Virus was purified by CsCl equilibrium density gradient centrifugation. The virus band was collected and the optical density determined. Fig. 1 (– – –) shows the results obtained with six inhibitors. None of the inhibitors depressed total virus yield more than 5-fold. This contrasts sharply with the inhibition of infectious virus yield seen above, which extended to several orders of magnitude. For example, 1 μM NEM reduced virus yield by only 30%, but reduced infectious virus yield by five orders of magnitude. These results show that the cysteine protease inhibitors did not significantly affect the production of virus particles, but that they did have a profound inhibitory effect on the infectivity of these particles. The simplest explanation of these results is that the inhibitors reduced the efficiency of the proteolytic cleavages that are required to achieve full infectivity (Weber, 1990). It has been shown before with the protease defective ts1 mutant that virus assembly is not affected by the absence of proteolytic processing and that such unprocessed virions are not infectious (Weber, 1976; Rancourt et al., 1995).

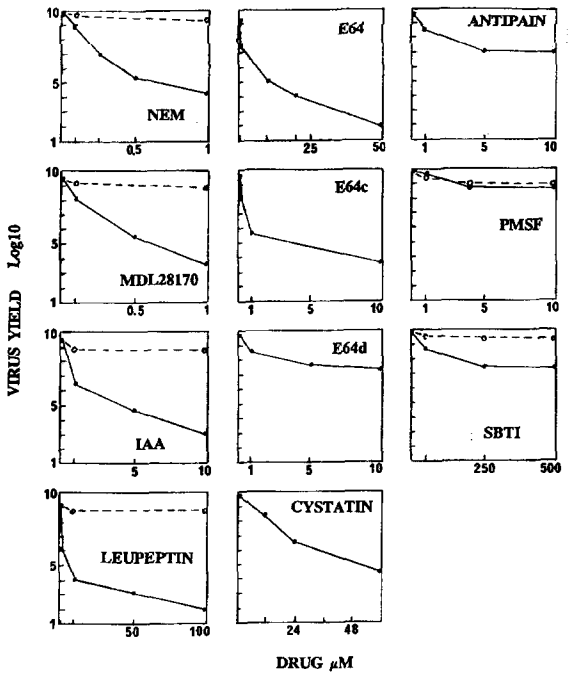

Fig. 1. The effect of protease inhibitors on virus yield. Monolayers of Hep2 cells were infected with Ad2 (m.o.i. of 10) and drug was added to the medium at 18 h after infection. At 48 h the cells were lysed and the clarified supernatant either titered for PFU (——) or centrifuged to equilibrium in a CsCl gradient, the virus band collected and its optical density (280 nm) measured (– – –). The virus yield is expressed on a log scale either as PFU/ml, or percentage relative optical density. Control experiments, using the solvents in which the drugs were dissolved, had no measurable effect on virus infection and were therefore not shown.

These results demonstrate that the observed antiviral effect of these agents is unlikely to be due to cellular toxicity, nor even to inhibition of any other viral function, because the synthesis of physical particles was nearly normal. The greatly decreased infectious virus yield could have been due entirely to the inhibition of the viral protease, thus giving rise to noninfectious immature virions. If this were the case, then the virions synthesized in the presence of cysteine protease specific inhibitors should display the corresponding immature virion polypeptide pattern. The results bore out this prediction, as evidenced by the incomplete cleavage of viral precursor proteins pVI, pVII and 11K when virus was grown in the presence of the cysteine protease inhibitors MDL 28170, NEM and leupeptin (Fig. 2, a–i). Similar results were obtained with IAA (not shown). By contrast, the serine protease inhibitors PMSF (not shown) and SBTI had little effect on virus production and maturation, even at substantially higher

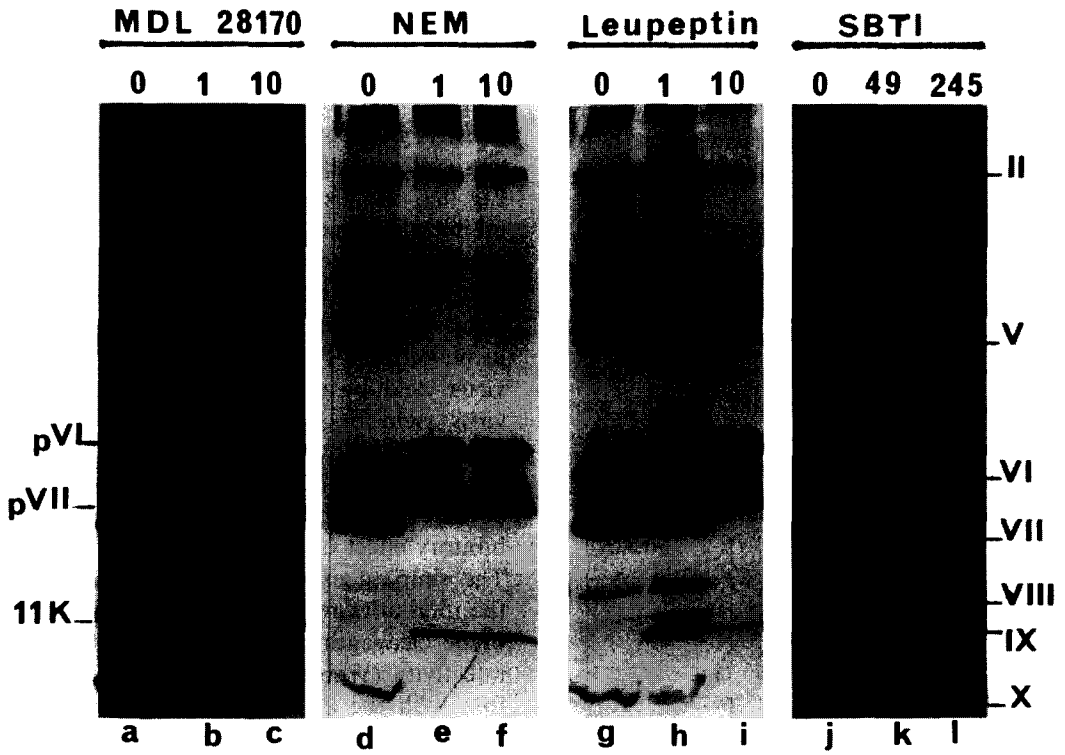

Fig. 2. The effect of protease inhibitors on virus maturation. The purified viruses from the experiment described in Fig. 1 were subjected to SDS-PAGE and the proteins stained with silver nitrate. The drug concentrations (μM) present during virus infection are shown above each lane. The roman numerals to the right of lane 1 identify selected virion proteins. pVI, pVII and 11K are precursors of VI, VII and X, respectively.

concentrations (Fig. 2, j–l). This lack of effect of serine protease inhibitors may have been due to lack of inhibition of the viral cysteine protease, poor penetration of the infected cell or instability of the inhibitor, or a combination of these factors.

To compare the relative efficiency of the inhibitors we calculated the IC_{50}s from the data in Fig. 1 (Table 1). The most effective inhibitors were *N*-ethylmaleimide ($IC_{50} = 30$ nM), leupeptin ($IC_{50} = 40$ nM) and E64c ($IC_{50} = 80$ nM). It is interesting that the relatively minor changes in the E64 analogues had a significant impact on their capacity to inhibit adenovirus replication. Cystatin ($IC_{50} = 3000$ nM), though a cysteine protease inhibitor, was as ineffective as the serine protease inhibitors PMSF ($IC_{50} = 3000$ nM) and SBTI ($IC_{50} = 11\,500$ nM). The proteinaceous nature of cystatin and SBTI may have prevented their penetration of infected cells, thus accounting for the lack of effect.

One of these inhibitors, leupeptin is an aldehyde of the tripeptide acetyl-Leu-Leu-Arg, derived from a microbial source. We assume that the peptide must penetrate the infected cell to gain access to the viral protease, if indeed the observed inhibition is due to reduced protease activity and not to some other mechanism, such as protease trafficking.

In the course of virion assembly the viral protease cleaves six precursor proteins (Weber, 1995). PVII is the most abundant of these and also the most easily quantifiable. We therefore examined the effect of some of the inhibitors on the cleavage, by the viral protease, of viral substrate protein PVII, by an in vitro assay. The cysteine protease inhibitors reduced the cleavage of PVII at the corresponding IC_{50} (Table 1). None of the serine protease inhibitors nor the aspartate-

Table 1
Effect of protease inhibitors on adenovirus growth and on processing of PVII

Inhibitor and molecular weight	Type	IC_{50} [a] (nM)	Conc. (μM) [b]	Inhibition (%)
NEM (125)	Cysteine	30	1	100
MDL 28170 (382)	"	120	1	40
IAA (186)	"	200	5	58
E64 (357)	"	140	10	21
E64c (314)	"	80	5	51
E64d (342)	"	250	1	39
Cystatin (12000)	"	3000	1	10
Leupeptin (426)	Cys/ser	40	100	19
Antipain (605)	Cys/ser	240	ND	ND
PMSF (174)	Serine	3000	10	0
SBTI (20100)	"	11500	490	0
Aprotinin (6500)	"	ND	50	0
Chymostatin (605)	"	ND	10	0
Pepstatin (686)	Aspartate	ND	50	0

[a] The IC_{50} were calculated from the data in Fig. 1.
[b] Drug concentration at which maximal inhibition of PVII processing was obtained, without causing cellular toxicity.

specific pepstatin had any effect on the processing of PVII. Consequently the observed IC_{50} on virus growth was probably exerted through the inhibition of some other viral or cellular function. Several types of experiments were conducted to further examine the mechanism of viral inhibition. The inhibition of PVII processing by the inhibitors was examined directly by pulse-chase experiments. Cells were infected as above and then pulse-labeled with ^{35}S-Met for one hour followed by an 18 h chase in the presence of different concentrations of inhibitor. The protein patterns were examined by SDS-PAGE and autoradiography. A typical result depicting only the highest drug concentration is shown in Fig. 3. The processing of the PVII protein, this being the most clearly observable, is completely inhibited during the chase in the presence of 0.5 μM NEM. For each inhibitor tested, the degree of inhibition of PVII processing was quantitated (Table 1). These results confirm those described above and show that the cysteine protease inhibitors effectively inhibit virion protein processing, while the serine protease inhibitors have no observable effect. The most effective inhibitor was again NEM (compare with IC_{50} of 30 nM). We conclude therefore, that the anti-adenoviral effect of the cysteine protease inhibitors operates to a large extent through the inhibition of the viral protease.

The present results demonstrate that cysteine protease inhibitors are effective agents for the control of adenovirus infection in tissue culture. At present there are no specific chemotherapeutics directed against adenoviruses. Adenoviral keratoconjunctivitis has been treated with limited success by means of polyvinylpyrrolidone–iodine (Hutter, 1990). Ribavirin treatment has been attempted in cases of adenovirus-associated cystitis following transplantation (Cassano, 1991; Murphy et al., 1993). The need for specific anti-adenoviral agents is indicated in such cases. The unique sequence of the adenovirus protease and its high degree of conservation among different viral serotypes offers an attractive target for the development of specific protease inhibitors (Rancourt et al., 1994; Weber, 1995; Weber and Tihanyi, 1994). In the pursuit of this objective, we are currently exploring the power of combinatorial peptide libraries expressed in filamentous phages to identify lead compound inhibitors of the adenoviral protease.

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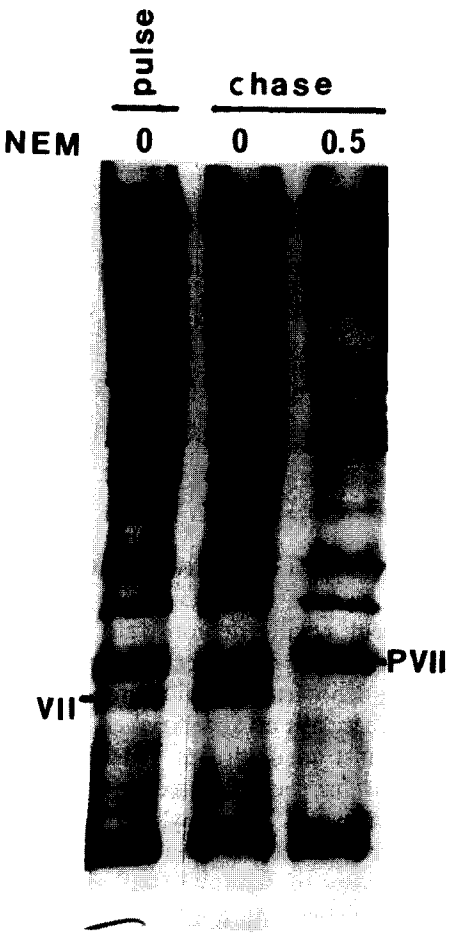

Fig. 3. Effect of NEM on virion polypeptide processing. Ad2 infected Hep2 cells were labeled with ^{35}S-Met for 1 h (lane 1) at 18 h after infection and then chased in the absence (lane 2) or presence (lane 3) of NEM for 18 h. Cell lysates were separated by SDS-PAGE and autoradiographed. Note the complete inhibition of PVII processing in the presence of 0.5 μM NEM.

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