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Viral Therapy: Prospects for Protease Inhibitors

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Antiviral activities of known protease inhibitors were assayed in virus-infected cell cultures. Some members of the cystatin superfamily, in particular chicken cystatin, were able to block virus replication. In a binding assay, using purified components, chicken and human cystatin were able to bind poliovirus protease with affinities which were reflected in their relative anitviral potencies. Prospects for application of protease inhibitors in clinical viral infections are discussed.

Key words: viral proteases, cystatins, virus inhibitors, protease inhibitors, antivirals

It is now clear that many kinds of viruses are dependent on protein cleavages during replication. The cleavages are often observed during viral capsid assembly but may include additional processing reactions involved in viral nucleic acid metabolism. Those viral systems where some of the proteolytic events are mediated by virus-coded proteases are potential candidates for the application of specific antiproteases as viral inhibitors (for a review see [1]). Since most viral diseases are not life-threatening, it is also anticipated that if antiproteases are to find practical uses as antivirals, they must be designed in some way to be specific for the protease of the infecting virus, and not be potent inhibitors of the many known and unknown proteolytic functions of the host. This is obviously not a simple criterion to meet, without a highly detailed understanding of the structure and mechanism of the viral enzyme, and a complete picture of its interaction with viral protein substrates before and during cleavage reactions. At this time, we have barely made a beginning at the kinds of studies which may provide such detailed information, but the intial results are interesting and encouraging.

MATERIALS AND METHODS

Cells and Viruses

HeLa O cells and WISH cells, poliovirus type 1, Mahoney strain, human rhinovirus type 1A, and vesicular stomatitis virus, New Jersey strain, were obtained

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from the American Type Culture Collection, Rockville, Maryland. Cells were cultured in McCoy 5A medium (GIBCO), containing 10% calf serum, gentamycin, and fungizone.

Labeling of Viral Proteins and Analysis of Products

Cultured cells as monolayers were infected at high multiplicity (20–50 plaque-forming units per cell) and labeled with [^{35}S] methionine or other amino acids at 3–5 hr postinfection. Cells were subsequently lysed with 0.01 M Tris buffer, pH 6.8, containing 1% sodium dodecyl sulfate and 1% mercaptoethanol, and the protein components were resolved by gel electrophoresis and radioautography, as described [2].

Purification of Poliovirus Protease and Inhibitor Binding Assays

Poliovirus protease was recovered from genetically engineered *Escherichia coli* [10], using a combination of chromatographic procedures (Towatari et al, in preparation). Briefly, the bacteria expressing the viral protease were lysed by freeze-thaw in the presence of 1 mg/ml lysozyme, the insoluble portion of the cell was removed by centrifugation, and the viral protease was recovered from the soluble portion of the cell, following chromatography on DEAE-Sephadex A-50, CM Sephadex C-50, gel filtration on Sephacryl 200, adsorbtion chromatography on mercury agarose, and finally an optional step on a Du Pont GF 250 HPLC column. The protease finally recovered was judged homogeneous by gel electrophoresis and by amino-terminal sequence analysis. Activity of the protease was detected by its ability to process high molecular weight viral precursor polypeptides to proteins which comigrated with bonafide viral proteins from infected cells [2].

Binding of protease to inhibitors was measured in a solid-phase assay. The inhibitors were prepared in various dilutions and blotted onto nitrocellulose filter paper BA83(Schleicher and Schuell) in dilute phosphate buffer. A solution containing the pure protease (10 μg/ml) was then reacted with the filter for 60 min at 37°C, and then the filter was washed extensively with phosphate buffer containing 1% bovine serum albumin to remove non-specifically bound protease molecules. The filter was then reacted with rabbit antibodies to the protease, and the antibody bound was decorated with [^{125}I] protein A and visualized by radioautography.

Chicken cystatin was isolated from chicken egg white [3]. Human cystatin C was purified from human serum [4] and human Stefin B from spleen by using the procedure given in [5]. Pancreatic trypsin inhibitor was obtained from Mobay Pharmaceutical and rat alpha-1-macroglobulin was a gift of Dr. K. Lonberg-Holm.

RESULTS

Recently we reported the inhibition of poliovirus protein cleavages and virus replication in HeLa cells in culture, if the cysteine proteinase inhibitor from chicken egg white (chicken cystatin) was present in the tissue culture medium during infection [6]. In the present study, we have extended the protocol to include two additional viruses, human rhinovirus 1A and vesicular stomatitis virus. The rhinovirus was predicted to show a sensitivity to the inhibitor, since it is closely related to poliovirus and is also believed to encode its own cysteine protease required for replication. By comparison, the vesicular stomatitis virus does not carry out extensive proteolytic

processing and should serve as a negative control for the action of the protease inhibitor. This was indeed the case, as shown in the infectivity data summarized in Table I.

We next compared the relative antiviral activities of several different protease inhibitors, using the assay system described in Table I. The results are summarized in Table II.

Since the antiviral activity results for the protease inhibitors are complex, and are partly dependent on ability of the inhibitor to penetrate to the host cell interior in an active form, it seemed desirable to compare directly the binding of the inhibitors to a viral protease by using purified components.

The procedure for the binding assay is described in detail in Materials and Methods. The data are summarized as follows: Chicken cystatin has 50–100 times

TABLE I. Antiviral Activities of Chicken Cystatin*

Virus	Yield (pfu/ml)		% Inhibition
	Untreated	With cystatin	
Poliovirus type 1	$1.6 \pm 0.4 \times 10^8$	$2.8 \pm 0.5 \times 10^7$	82
Rhinovirus type 1A	$7.9 \times 1.2 \times 10^6$	$1.8 \pm 0.3 \times 10^6$	77
VSV	$6.6 \pm 1.1 \times 10^8$	$6.1 \pm 0.9 \times 10^8$	< 10

*Hela cells were infected with one of the viruses at a multiplicity of 0.1. After virus attachment, the medium (serum-free) was adjusted to contain 1 mg/ml of avian cystatin. Incubation was continued for up to 8 hr at 37°C; then the infected cells were frozen and thawed 2× and plaque titrations were carried out [1] to determine infectivity titers. pfu, plaque-forming units. VSV, vesicular stomatitis virus.

TABLE II. Relative Antiviral Activities of Selected Proteinase Inhibitors VS Poliovirus*

Inhibitor	Relative antiviral potency (ED_{50})
Chicken cystatin	5
Human cystatin C	1
Stefin B	0.1
Pancreatic trypsin inhibitor (bovine)	Not detected
Alpha-1-macroglobulin (murine)	Not detected

*In order to determine relative antiviral activities of each of the protease inhibitors, Hela cells were infected with 0.01 plaque-forming units per cell of poliovirus type 1. After completion of a 60-min period at 35°C to permit virus attachment and early events to proceed, the cell cultures were placed in medium containing a known concentration of a proteinase inhibitor, beginning with 100 μM, and ranging downward in threefold dilutions. The infected cells were incubated for 8 hr at 35°C and were then washed 3× with phosphate-buffered saline, and finally dissolved in 0.1% SDS. The virus yield was determined in each case by plaque titration on Hela cells. ED_{50} levels were extrapolated for each proteinase inhibitor, relative to untreated cultures, from the reduction in virus yield determined for each inhibitor at the several inhibitor concentrations tested.

greater affinity for poliovirus protease than does human cystatin C. The human Stefin B showed no detectable binding to the enzyme by our assay procedure. Greatest binding was found with alpha-macroglobulin, as might be predicted, since in this case the binding is covalent and essentially irreversible.

DISCUSSION

This study has shown that some protein inhibitors of cysteine proteases are able to block virus replication when added to infected cells in culture and that in general, the more effective they were as antivirals, the greater their ability to bind to a viral protease. An exception to this is alpha-1-macroglobulin, which has the greatest binding to the purified protease, but no measurable antiviral activity. We interpret this as an inability of the large (ca 700 kD) inhibitor to penetrate intact cells, while the smaller (ca 10 kD) cystatins are able to do so [6].

Since the cystatins [7,8] are so-called endogenous inhibitors, it is curious why viruses such as poliomyelitis are able to replicate. One possible explanation is that the inhibitors may actually be present in very low concentrations and may be largely complexed with endogenous cellular proteases. A more interesting possibility is that viral proteases may have been selected to be resistant to the inhibitory effect of proteins such as Stefin B, which are the predominant intracellular form of these inhibitors. The more effective inhibitors, such as chicken or human cystatin C, are believed to be largely or exclusively extracellular. Similarly, the macroglobulins, which are very effective protease inhibitors, are present in high concentrations in serum and other extracellular fluids, but are unable to penetrate to the interior of infected cells, where they must be present if they are to effectively prevent viral protein cleavages.

With the availability of highly purified cysteine protease inhibitors [7,8] and recent advances in preparation of viral proteases from recombinant DNA systems [9] (Towatari et al, in preparation), the interaction between viral proteases and inhibitors can be studied in greater detail. It may then be possible to identify small regions of cystatins which retain inhibitory activity, or to synthesize new versions of cystatins with improved binding affinities for viral proteases and enhanced ability to enter cells. If these criteria can be met, then cystatins may eventually be clinically useful antiviral agents.

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