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Simple In Vitro Translation Assay To Analyze Inhibitors of Rhinovirus Proteases

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We have developed a simple in vitro translation method to analyze compounds that inhibit the rhinovirus 3C protease in peptide substrate assays but demonstrate no antiviral activity. This complementary assay, which provides both qualitative and quantitative results, detects the inhibition of the 3CD protease in the native polyprotein form.

The virus family *Picornaviridae* comprises approximately 180 serotypes of human pathogens. These include the rhinoviruses (RV), which are the primary causes of the common cold (8), and the enteroviruses (whose prototype is poliovirus [PV]), which cause such diverse diseases as meningitis, encephalitis, conjunctivitis, and respiratory disease. These viruses have positive-sense, single-stranded RNA genomes. Upon infection of susceptible cells, the viral RNA is translated into a single large polypeptide, which is subsequently cleaved in a series of bi- and monomolecular events by two virus-encoded proteases, 2A and 3C (or its precursor form, 3CD). The activities and sequence requirements of these proteases have been extensively studied (reviewed in references 9 and 14). Both the 2A and 3C proteases cleave their own N termini; 3C (or 3CD) protease is responsible for seven additional cleavages. Because this cleavage cascade is vital for viral replication, these proteases are attractive targets for the development of antiviral drugs. Furthermore, they are highly specific for cleavage of viral polyprotein substrates (13), lessening the likelihood that protease inhibitors will show nonspecific toxic effects.

RV and PV 3C proteases have been expressed in bacteria and purified to homogeneity by standard biochemical techniques (2, 3, 7, 17). Enzyme activity is monitored with small peptides containing the required glutamine-glycine scissile bond as substrates. Using a variety of screening assays, we and other researchers have discovered several classes of 3C inhibitors (5, 11, 12, 16). Although many of these inhibitors show significant activity against the isolated enzyme, high levels of antiviral activity have not yet been observed. There are several possible explanations for this inconsistency. First, it is not known whether 3C or 3CD is the predominant active form of the enzyme during virus infection. At least some PV cleavages, notably those within the capsid precursor polypeptide, are carried out only by 3CD (19). Second, it is possible that the small peptide substrates used in 3C inhibitor screens are inadequate substitutes for the natural substrates. Third, enzyme inhibitors must be able to cross the cell membrane in order to demonstrate antiviral activity in cell culture. Finally, because high-volume protease screens are typically designed to detect a decrease in a fluorescent signal, they are subject to errors caused by nonspecific quenching. To help distinguish among these factors, we have developed a cell-free translation method that measures inhibition of the 3CD protease in the context of the natural substrate. We have found this translation assay to be a valuable complement to the primary enzyme screens and whole-cell antiviral assays currently in use.

The C-terminal portion of the RV14 genome was derived from a cDNA clone generously provided by R. Rueckert, University of Wisconsin—Madison. The cDNA was digested with *Nco*I and *Sal*I, releasing a 5,494-bp fragment containing the region from viral nucleotide 4309 (encoding approximately one-third of the 2C gene) through the 3′ noncoding region. This DNA was inserted in frame into the polylinker region of the pCITE-1 vector (Novagen, Madison, Wis.), a vector designed to enhance the translation of foreign DNA by fusing it to the internal ribosomal binding site of encephalomyocarditis virus. This approach precludes the use of supplemental HeLa cell extract, which is generally required for efficient in vitro translation of RV and PV RNA (1, 4). The pCITE construct was linearized by digestion with *Mlu*I and transcribed in vitro with T7 polymerase (18). Translation assays were conducted with rabbit reticulocyte lysates by standard procedures (Novagen) with 2 μg of RNA transcript and 20 μCi of [^{3}H]leucine (Amersham, Arlington Heights, Ill.). A blank reaction lacking the transcript was used to detect background radioactivity due to translation of endogenous mRNA in the lysate. Acetone-precipitated proteins were analyzed on sodium dodecyl sulfate–12% acrylamide Laemmli gels, fixed for 30 min in 25% methanol–10% acetic acid–1% glycerol, enhanced with Enlightning solution (NEN Du Pont, Boston, Mass.), and visualized by autoradiography. The inhibition of 3CD cleavage was approximated by scanning densitometry with an LKB Ultroscan XL Enhanced Laser Densitometer (Pharmacia-LKB, Piscataway, N.J.).

The inhibition of purified 3C protease in vitro was determined by a fluorescence assay previously used for human immunodeficiency virus type 1 (HIV-1) protease (15) with the following modifications: the peptide substrate (biotin-Arg-Ala-Glu-Leu-Gln-Gly-Pro-Tyr-Asp-Glu-Lys-fluorescein isothiocyanate) was used at a concentration of 4.1 μM, the enzyme concentration was 0.12 μM, and the reaction was conducted for 15 h at 22°C in Tris buffer (0.02 M Tris, 0.15 M NaCl, 0.01 M dithiothreitol, 0.5 mg of bovine serum albumin per ml, pH 8). Inhibitor specificity was confirmed by secondary assays with HIV-1 protease as described previously (15); inhibitors that also demonstrated activity against HIV-1 protease were disregarded.

Antiviral activity was tested in HeLa cell monolayers grown in 96-well plates in minimum essential medium containing

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TABLE 1. Inhibitory activity as determined by three assays

Compound[a]	% Inhibition of:		Antiviral activity	
	Peptide cleavage[b]	Translation activity[c]	IC_{50} (μg/ml)	TI[d]
DMSO control	0	0	>500	ND[e]
Peptide aldehyde	100	63	178	55
1[f]	0	0	0.04	250
2	0	0	ND	ND
3	91	62	>500	<1
4	100	58	22	5
5	97	Toxic	45	2
6	82	0	>500	<1
7	100	64	>500	<1
8	100	Toxic	>500	<1
9	89	16	23	4
10	16	0	>500	<1

[a] Compound numbers correspond to those in Fig. 2A.
[b] Percent inhibition of purified 3C protease as measured by decreased cleavage of a fluorescently labeled peptide substrate (see text).
[c] Percent inhibition of normal protein processing in an in vitro translation assay as determined by densitometric scans of autoradiograms. Percent inhibition is defined as follows: [amount of uncleaved polyprotein/(amount of cleaved 3CD + amount of uncleaved polyprotein)] × 100. "Toxic" indicates that translation was completely inhibited.
[d] TI, therapeutic index (defined as TC_{50}/IC_{50}). By convention, true antiviral activity requires a TI of ≥10.
[e] ND, not determined.
[f] Enviroxime, an antiviral compound that targets a replicative protein other than 3C protease (10), was used as a control.

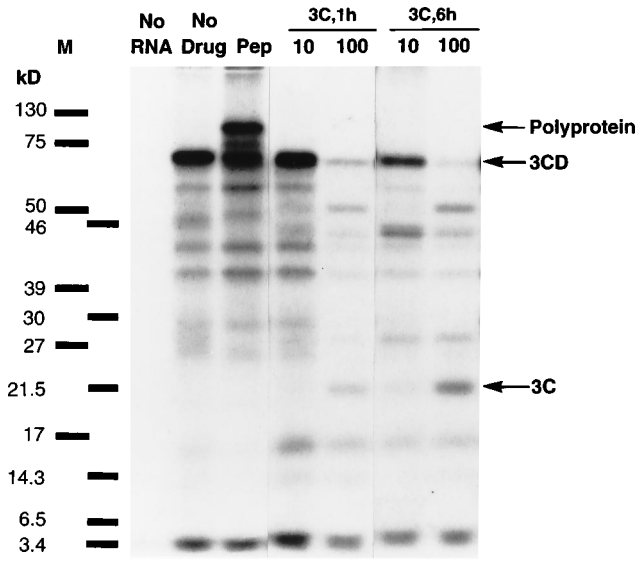

FIG. 1. Appearance of uncleaved polyprotein (106 kDa) due to inhibition of N-terminal cleavage of 3CD protease by 100 μg of peptide aldehyde inhibitor (Pep) per ml. To confirm the identities of the 3C and 3CD bands, exogenous 3C (10 or 100 μg/ml) was added to the translation mixture at the start of the reaction, and the mixture was incubated for 1 or 6 h. The intensity of the 3CD band decreased between 1 and 6 h; this decrease was accompanied by an increase in free 3C protease. The identity of these two bands was further confirmed by Western blot analysis (not shown). A new 52-kDa band (likely the 3D polymerase) also appeared; however, the lack of 3D antibody precluded its confirmation by Western blotting. M, molecular mass markers.

Earle's balanced salt solution, 1% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Fifty PFU of RV14 was inoculated into each well and allowed to attach at room temperature for 1 to 2 h. A duplicate set of uninfected wells was used to control for drug cytotoxicity. Medium containing serial dilutions of the compound was added, and the cultures were incubated for 2 to 3 days at 34°C (until extensive cytopathic effects were evident in the drug-free cultures). To quantitate antiviral activity, 50 μl of freshly-prepared XTT-PMS medium [1 mg of 2,3-bis(methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 (XTT) per ml and 25 nM phenazine methosulfate (PMS) in serum-free minimum essential medium] was added to each well, and the plates were incubated at 37°C for 2 to 3 h. Color development, indicating the presence of metabolically active cells, was detected spectrophotometrically (A_{450}). The concentration of drug required to prevent 50% of the cytopathic effect (50% inhibitory concentration [IC_{50}]) was calculated from the linear portion of each dose-response curve. Compound toxicity (50% toxic concentration [TC_{50}]) was recorded as the concentration of drug that resulted in a 50% cytopathic effect in uninfected cell controls.

In general, we used the in vitro translation assay to analyze inhibitors which had shown activity against purified 3C in the fluorescence assay but had no antiviral activity (Table 1). For a control inhibitor, we used a tetrapeptide aldehyde designed to mimic the S1 to S4 positions of the minimum sequence required for cleavage of the substrate (6). This peptide (butoxycarbonyl-Val-Leu-Phe-Gln-CHO) had shown both good activity against the purified enzyme and weak activity against RV14 in cell culture (12). All inhibitors were dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 20 to 0.4 mg/ml and diluted 40-fold in the reaction mixture (final concentration of DMSO, 2.5%). Although DMSO had no detectable effect on translation efficiency, 2.5% DMSO was added to all drug-free control samples.

In initial experiments, we determined that the peptide aldehyde control could significantly inhibit the release of 3CD (72 kDa) from the full-length polyprotein (expected size, 106 kDa [Fig. 1]). The N-terminal cleavage of 3CD was sufficiently rapid that no full-length polyprotein was ever detected in the absence of inhibitor. Although several cleavage products were evident, a band corresponding to free 3C protease (20 kDa) was absent; this suggested that the 3CD could not efficiently cleave itself further. To ensure that we had properly identified the 3CD band, we added 10 or 100 μg of purified 3C protease per ml (3) to the standard translation mixture and incubated the mixture for either 1 h (standard conditions) or 6 h at 30°C. In the presence of 100 μg of exogenous 3C per ml, a 20-kDa new translation product appeared by 1 h of incubation and became more prominent after 6 h of incubation (Fig. 1); correspondingly, the 3CD bands were diminished. Additional 3C processing was evident as well. We confirmed the identities of the 3C and 3CD bands by Western blot (immunoblot) analysis (ECL system; Amersham), using a polyclonal anti-3C antibody produced in rabbits (data not shown). A new 52-kDa band, probably representing the appearance of free 3D, also increased in intensity between 1 and 6 h of incubation; however, we lacked the antibody necessary to confirm its identity by Western blot analysis. The peptide aldehyde inhibited N-terminal cleavage of 3CD at concentrations as low as 10 μg/ml (data not shown); we typically used it at 100 μg/ml. For a control of inhibitor specificity, we verified that the peptide aldehyde could not inhibit processing of the polyprotein from encephalomyocarditis virus (this RNA was provided as control material by Novagen) (data not shown).

Inhibitors were routinely tested at 500 μg/ml (Fig. 2A). Compounds showed no inhibition (compounds 1 and 2 [Fig. 2A, lanes 1 and 2]), weak inhibition (compounds 6, 9, and 10), or strong inhibition (compounds 3, 4, and 7) of 3CD release; drug toxicity was manifested as the absence of any translation

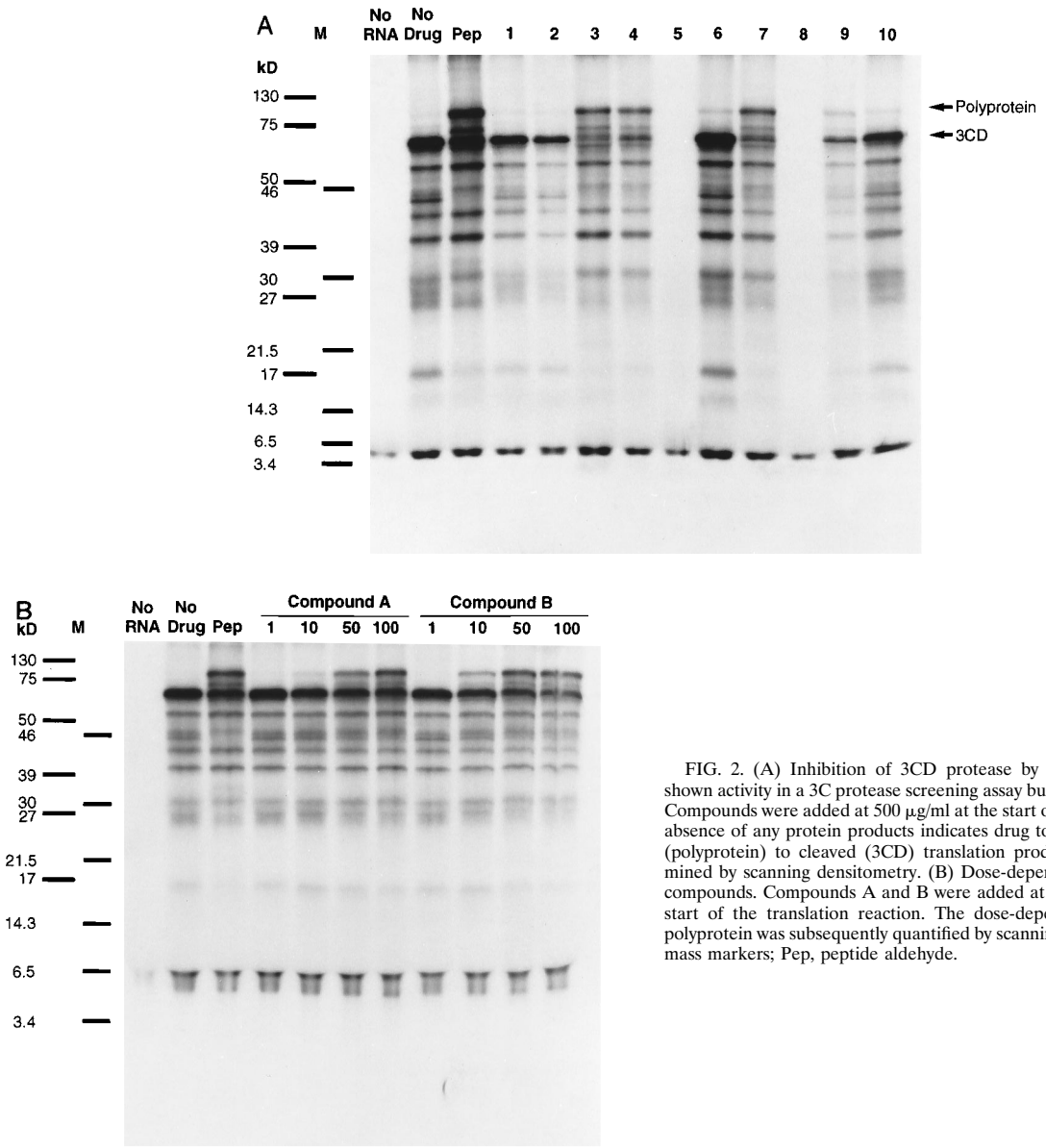

FIG. 2. (A) Inhibition of 3CD protease by various compounds that had shown activity in a 3C protease screening assay but lacked activity in cell culture. Compounds were added at 500 μg/ml at the start of the translation reaction. The absence of any protein products indicates drug toxicity. The ratio of uncleaved (polyprotein) to cleaved (3CD) translation products was subsequently determined by scanning densitometry. (B) Dose-dependent inhibition by two active compounds. Compounds A and B were added at 1, 10, 50, or 100 μg/ml at the start of the translation reaction. The dose-dependent increase in uncleaved polyprotein was subsequently quantified by scanning densitometry. M, molecular mass markers; Pep, peptide aldehyde.

products (compounds 5 and 8). The inhibition of protein processing was quantitated by scanning densitometry. Comparative results for inhibitors 1 to 10 in the three complementary assays are shown in Table 1. Compound 1 (enviroxime) served as a control inhibitor that shows excellent antiviral activity but is known to inhibit a viral target other than 3C (10). Compound 2 was negative in both the peptide substrate fluorescence screen and the translation assay. Compounds 3 to 10 inhibited the cleavage of peptide substrates in the enzyme assay but showed no antiviral activity because of an unfavorable toxicity profile. In these cases, the in vitro translation assay was able to distinguish true 3C inhibitors (compounds 3, 4, 7, and 9) from apparent false positives (compounds 6 and 10). Moreover, compound 9 was shown to be less active than the other three inhibitors.

Compounds which appeared to be strong 3CD inhibitors were examined at 1, 10, 50, and 100 μg/ml to determine whether they had dose-response relationships (Fig. 2B and Table 2). It was interesting that no active inhibitors were able to halt protein processing completely; both a normal-length 3CD cleavage product and an aberrant one were produced. The appearance of normal-length 3CD probably indicates that

TABLE 2. Dose-dependent inhibition of in vitro translation

Compound[a]	% Inhibition of translation activity in presence of drug at[b]:				
	1 μg/ml	10 μg/ml	50 μg/ml	100 μg/ml	500 μg/ml
A	0	0	25	37	62
B	0	16	38	48	66

[a] Compound letters correspond to those in Fig. 2B.

[b] Percent inhibition of normal protein processing in an in vitro translation assay as determined by densitometric scans of autoradiograms. Percent inhibition is defined as follows: [amount of uncleaved polyprotein/(amount of cleaved 3CD + amount of uncleaved polyprotein)] × 100.

the interaction of the inhibitor with the enzyme is inefficient. On the other hand, the presence of an aberrant processing product may reflect an unusual characteristic of the 3C enzyme: aberrant products can also result when 3C has been mutated to an inactive form (20).

In conclusion, we have developed a simple in vitro translation method for exploring inhibitors of RV and enterovirus 3C protease. This assay could be easily adapted to detect the N-terminal cleavage of 2A protease as well. This assay is intended to complement, not replace, the standard peptide cleavage and antiviral assays. We have used this assay to help differentiate true 3C inhibitors from compounds that nonspecifically quench the fluorescence signal detected in our primary screening assay. Furthermore, the in vitro translation assay can help in structure-activity relationship analyses by providing 3C inhibitory data despite weak antiviral activity which is readily masked by cellular toxicity. Because the transcript RNA encodes the 3CD protease in its native polyprotein form, we can study protease inhibitors in a context which is more relevant to virus infection than standard enzyme assays are. In addition, since our assay is cell free, the compound is not required to traverse the cell membrane; nevertheless, toxic effects of the drug can still be monitored. Finally, the translation assay can provide both qualitative and quantitative results.

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