

## In Vitro Antiviral Activity of AG7088, a Potent Inhibitor of Human Rhinovirus 3C Protease

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**AG7088 is a potent, irreversible inhibitor of human rhinovirus (HRV) 3C protease {inactivation rate constant ( $k_{\text{obs}}/[I]$ ) =  $1,470,000 \pm 440,000 \text{ M}^{-1} \text{ s}^{-1}$  for HRV 14} that was discovered by protein structure-based drug design methodologies. In H1-HeLa and MRC-5 cell protection assays, AG7088 inhibited the replication of all HRV serotypes (48 of 48) tested with a mean 50% effective concentration ( $\text{EC}_{50}$ ) of  $0.023 \mu\text{M}$  (range,  $0.003$  to  $0.081 \mu\text{M}$ ) and a mean  $\text{EC}_{90}$  of  $0.082 \mu\text{M}$  (range,  $0.018$  to  $0.261 \mu\text{M}$ ) as well as that of related picornaviruses including coxsackieviruses A21 and B3, enterovirus 70, and echovirus 11. No significant reductions in the antiviral activity of AG7088 were observed when assays were performed in the presence of  $\alpha_1$ -acid glycoprotein or mucin, proteins present in nasal secretions. The 50% cytotoxic concentration of AG7088 was  $>1,000 \mu\text{M}$ , yielding a therapeutic index of  $>12,346$  to  $>333,333$ . In a single-cycle, time-of-addition assay, AG7088 demonstrated antiviral activity when added up to 6 h after infection. In contrast, a compound targeting viral attachment and/or uncoating was effective only when added at the initiation of virus infection. Direct inhibition of 3C proteolytic activity in infected cells treated with AG7088 was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of radiolabeled proteins, which showed a dose-dependent accumulation of viral precursor polypeptides and reduction of processed protein products. The broad spectrum of antiviral activity of AG7088, combined with its efficacy even when added late in the virus life cycle, highlights the advantages of 3C protease as a target and suggests that AG7088 will be a promising clinical candidate.**

The *Picornaviridae* family consists of more than 200 different viruses, which are associated with a wide variety of medically important diseases including the common cold, aseptic meningitis, conjunctivitis, encephalitis, and respiratory disease (reviewed in references 7, 39, and 44). Human rhinoviruses (HRV), which include over 100 different virus serotypes, are the most important etiological agents of the common cold. Although HRV-induced upper respiratory illness is often mild and self-limiting, HRV infection may ultimately result in sinusitis, otitis media, and lower respiratory tract illnesses including exacerbations of asthma, cystic fibrosis, and bronchitis in individuals with underlying respiratory disorders (3). Although no effective antiviral therapies for either the prevention or treatment of diseases caused by HRV infection are currently available, considerable progress in the discovery and development of new antirhinoviral drugs directed towards a novel target, the HRV 3C protease, has recently been made (11–15, 19, 22, 26, 36, 41, 47, 51–53).

The HRV 3C protease is responsible for the cleavage of viral precursor polypeptides into structural and enzymatic proteins which are essential for viral replication. DNA sequence comparisons among HRV serotypes, and even among several related picornaviruses, have identified a significant degree of homology within the 3C coding region including the strict conservation of the active-site residues, thus providing an additional rationale for targeting drug discovery efforts (8, 16, 20, 21, 29, 31, 38, 45, 48, 49, 55). AG7088 is a potent, irreversible inhibitor of HRV 3C protease that was discovered by protein structure-based drug design methodologies (12, 35, 36) and is

currently undergoing evaluation in phase I clinical studies with humans. In this study, we describe the in vitro activity of AG7088 against a variety of different HRV serotypes as well as related picornaviruses in different cell-based systems, as well as its cytotoxicity in these systems.

### MATERIALS AND METHODS

**Compounds.** AG7088 and pleconaril (17) were synthesized at Agouron Pharmaceuticals, Inc. Pirodavir (1) was kindly provided by Janssen Research Foundation (Beerse, Belgium), and WIN 51711 (40) was kindly provided by Sterling Winthrop Research Institute (Collegeville, Pa.). Ganciclovir (Syntex Corp., Palo Alto, Calif.) was obtained from a local pharmacy, and acyclovir was purchased from Sigma (St. Louis, Mo.).

**Cells and virus strains.** All numbered HRV serotypes, echovirus type 11 (EV 11), enterovirus type 70 (ETV 70), coxsackievirus types A21 (CAV 21) and B3 strain Nancy (CVB 3), human cytomegalovirus (HCMV) strain AD169, and herpes simplex virus type 1 (HSV-1) strain McIntyre were purchased from the American Type Culture Collection (ATCC; Manassas, Va.). HRV Hanks and a nasal lavage from a patient challenged with HRV Hanks were kindly provided by Ronald Turner from the Medical University of South Carolina, Charleston, S.C. HRV and coxsackievirus stocks were propagated, and antiviral assays were performed, in H1-HeLa cells (ATCC) incubated at 34 and 37°C, respectively. ETV 70, EV 11, and HCMV stocks were propagated, and antiviral assays were performed, in MRC-5 (ATCC) cells at 37°C. HSV-1 stocks were propagated, and antiviral assays were performed, in Vero (ATCC) cells incubated at 37°C. Vero cells were grown in minimal essential medium (Life Technologies, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah). H1-HeLa cells and MRC-5 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum.

**Enzyme assays.** The proteolytic activity of HRV 14 3C protease was measured by a continuous fluorescence resonance energy transfer assay as described previously (11–15). In brief, cleavage of the substrate peptide was monitored by the appearance of fluorescent emission at 490 nm (following excitation at 336 nm) in a Perkin-Elmer LS50-B spectrophotometer. Data were analyzed with the nonlinear regression analysis program ENZFITTER, which calculates a first-order rate constant for the inactivation of HRV 14 3C protease. Protease selectivity assays were performed with commercially available proteases (at approximately 10 nM concentrations) essentially as described by the supplier. Human liver cathepsin B, porcine erythrocyte calpain I, and human neutrophil elastase were purchased from Calbiochem (San Diego, Calif.), bovine chymotrypsin and hu-

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man thrombin were purchased from Boehringer Mannheim (Indianapolis, Ind.), and bovine trypsin was purchased from Sigma.

**Cell protection assay.** The ability of compounds to protect cells against infection was measured by a dye reduction method (54). Briefly, H1-HeLa and MRC-5 cells were resuspended at  $2 \times 10^5$  and  $5 \times 10^4$  cells per ml, respectively, in medium containing appropriate concentrations of compound or medium only. In some experiments, assays were performed in the presence or absence of either human  $\alpha_1$ -acid glycoprotein (AAG) or type 1-S bovine submaxillary gland bovine mucin (Sigma), both at a final concentration of 1 mg/ml in medium containing 10% fetal bovine serum. Cells were infected with HRV, CAV 21, and CVB 3 at a multiplicity of infection (MOI) of 0.004 to 0.5, 0.03, and 0.08, respectively, or mock-infected with medium only. An MOI of 2.0 to 6.0 was used in assays utilizing HRV 25. MRC-5 cells were infected with EV 11 or ETV 70 at an MOI of 0.003 or 0.004, respectively, or mock-infected with medium only. Vero cells were resuspended at  $1.5 \times 10^5$  cells per ml and infected with HSV-1 at an MOI of 0.05 or mock-infected with medium only. One to five days later, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma) with phenazine methosulfate (Sigma) was added to the test plates and the amount of formazan produced was quantified spectrophotometrically at a test reference of 450 nm and a reference wavelength of 650 nm. Data were expressed as the percentage of formazan produced in infected, compound-treated cells compared to formazan produced in wells of uninfected, compound-free cells. The 50% effective concentration ( $EC_{50}$ ) was calculated as the concentration of compound that increased the percentage of formazan produced in infected, compound-treated cells to 50% of that produced by uninfected, compound-free cells. The 50% cytotoxicity concentration ( $CC_{50}$ ) was calculated as the concentration of compound that decreased the percentage of formazan produced in uninfected, compound-treated cells to 50% of that produced in uninfected, compound-free cells. The therapeutic index was calculated by dividing the cytotoxicity ( $CC_{50}$ ) by the antiviral activity ( $EC_{50}$ ).

**Time-of-addition assay.** Subconfluent monolayers of H1-HeLa cells in six-well plates were infected with HRV 14 at an MOI of 15. After 1 h of adsorption, cell monolayers were washed three times with phosphate-buffered saline (PBS) and replenished with medium. AG7088 (0.5  $\mu$ M) or WIN 51711 (3.0  $\mu$ M) was added at concentrations 20-fold above the  $EC_{50}$  (as determined by the cell protection assay) at the time of infection and at various times thereafter. Eight hours after infection, samples were processed by three freeze-thaw cycles followed by sonication for 15 s and clarification by centrifugation (5 min at  $15,000 \times g$  at 4°C). Clarified cell and supernatant lysates were stored at -70°C for subsequent analysis for infectious virus.

**Virus yield assay.** Infectious virus titers were determined by a virus plaque assay. Briefly, 0.2 ml of serial 10-fold dilutions of virus were allowed to adsorb onto monolayers of H1-HeLa cells. After 1 h of adsorption, the cell monolayers were washed twice with PBS and overlaid with medium containing 0.5% Sea-Plaque agarose (FMC Bioproducts, Rockland, Maine). After 3 days of incubation at 34°C, the cell monolayers were fixed with EAF (65% ethanol, 22% acetic acid, and 4% formaldehyde) and stained with 1% crystal violet, and virus plaques were enumerated. Data were expressed as PFU per milliliter.

**Analysis of proteolytic processing.** The ability of AG7088 to inhibit HRV 14 3C-mediated proteolytic processing was assessed by polyacrylamide gel electrophoresis (PAGE) of radiolabeled sodium dodecyl sulfate (SDS)-solubilized lysates of HRV 14-infected cells. Initially, H1-HeLa cells were infected with HRV 14 at an MOI of 10. Eight and one-half hours after infection, the cells were washed with PBS and the medium was replaced with methionine- and cysteine-deficient medium (Life Technologies). At 9 h after infection, appropriate concentrations of compounds were added. After a 30-min exposure to compounds, 50  $\mu$ Ci of [ $^{35}$ S]Met-[ $^{35}$ S]Cys (Expre- $^{35}$ S  $^{35}$ S protein label; New England Nuclear, Boston, Mass.) was added. One hour later, the monolayers were washed twice with cold PBS and lysed in 250  $\mu$ l of radioimmunoprecipitation assay buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), sonicated, and stored at -70°C for subsequent analysis. Proteins present in the solubilized cell lysates were resolved by 12% PAGE. Following electrophoresis, gels were stained with Coomassie brilliant blue, destained, and treated with Amplify (Amersham, Arlington Heights, Ill.). Gels were air dried overnight in cellulose sheets and exposed to film at -80°C.

**HCMV antiviral assay.** The antiviral activity of AG7088 against HCMV AD169 replication in MRC-5 cells was determined by an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody MAb directed against the HCMV major immediate-early gene product (MAb 810; Chemicon, Temecula, Calif.). Briefly, following a 2-h virus adsorption, the inoculum was removed and medium containing the appropriate concentrations of compound was added. Five days after infection, the MRC-5 monolayers were incubated with MAb 810, followed by goat anti-mouse antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, Calif.), and viral antigen was then detected spectrophotometrically at 650 nm with the tetramethylbenzidine liquid substrate system (Sigma). The  $EC_{50}$  was calculated as the concentration of compound that reduced the optical density to 50% of that of the virus control. The  $CC_{50}$  was measured by the XTT reduction method as described above.

**Statistical analyses.** Determination of statistical significance was made by using Student's unpaired *t* test with the Statview (SAS Institute, Inc., Cary, N.C.) software program.

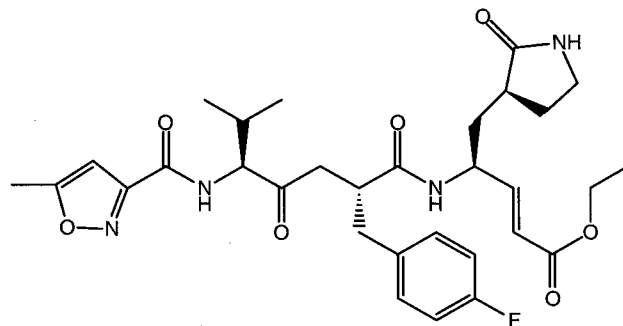


FIG. 1. Chemical structure of AG7088.

## RESULTS

**Activity against HRV 3C protease.** AG7088 is a ketomethylene-containing peptidomimetic compound which incorporates an unsaturated ethyl ester Michael acceptor (Fig. 1). AG7088 has demonstrated potent and irreversible inhibition of HRV 3C protease with an inactivation rate constant ( $k_{obs}/[I]$ ) of  $1,470,000 \pm 440,000 \text{ M}^{-1} \text{ s}^{-1}$  for HRV 14. Enzyme inhibition was shown to be specific for the viral 3C protease since 10  $\mu$ M AG7088 produced no significant inhibition against a variety of serine or cysteine proteases, e.g., human elastase, human thrombin, bovine trypsin, bovine chymotrypsin, human and bovine cathepsin B, and porcine calpain. In addition, lack of reactivity with nonenzymatic thiols was demonstrated by AG7088's stability when incubated in the presence of dithiothreitol (5 mM) (12).

**Activity against HRV serotypes and cytotoxicity of AG7088.** The efficacy of AG7088 against a panel of 48 different HRV serotypes was evaluated in a cell protection assay utilizing H1-HeLa cells (Fig. 2; Table 1). These included representative virus strains derived from minor and major receptor groups (50) as well as from two antiviral groups (A and B) previously defined based on differing susceptibilities to capsid-binding molecules (2). Pirodavir and pleconaril, compounds which inhibit virus capsid attachment and/or uncoating (1, 17), were included for comparison. Results indicate that AG7088 was active against all HRV serotypes (48 of 48) tested with a mean  $EC_{50}$  of 0.023  $\mu$ M (range, 0.003 to 0.081  $\mu$ M) and a mean  $EC_{90}$  of 0.082  $\mu$ M (range, 0.018 to 0.261  $\mu$ M). These values were comparable to or significantly better than those obtained with both pirodavir and pleconaril; pirodavir inhibited the replication of 42 of 47 (89%) HRV serotypes tested with a mean  $EC_{50}$  of 0.329  $\mu$ M (range, 0.003 to 4.770  $\mu$ M), and pleconaril inhibited the replication of 42 of 45 (93%) HRV serotypes tested with a mean  $EC_{50}$  of 0.822  $\mu$ M (range, 0.003 to 8.122  $\mu$ M). Furthermore, although AG7088 was able to inhibit approximately 80% of the HRV serotypes tested with  $EC_{50}$  and  $EC_{90}$  of less than or equal to 0.038 and 0.110  $\mu$ M, respectively,  $EC_{50}$  of less than or equal to 0.579 and 0.862  $\mu$ M and  $EC_{90}$  of less than or equal to 5.84 and 3.96  $\mu$ M were necessary for pleconaril and pirodavir, respectively, to inhibit this same percentage of HRV serotypes (Fig. 2). Consistent with results obtained with pirodavir, AG7088 demonstrated comparable levels of activity against HRV serotypes derived from either major or minor receptor groups as well as from antiviral groups A or B (Table 1). In contrast, significant reductions in activity against HRV serotypes classified in antiviral group A were observed for pleconaril.

AG7088 also demonstrated comparable activity against a low-passage clinical isolate, with an  $EC_{50}$  and  $EC_{90}$  of 0.006

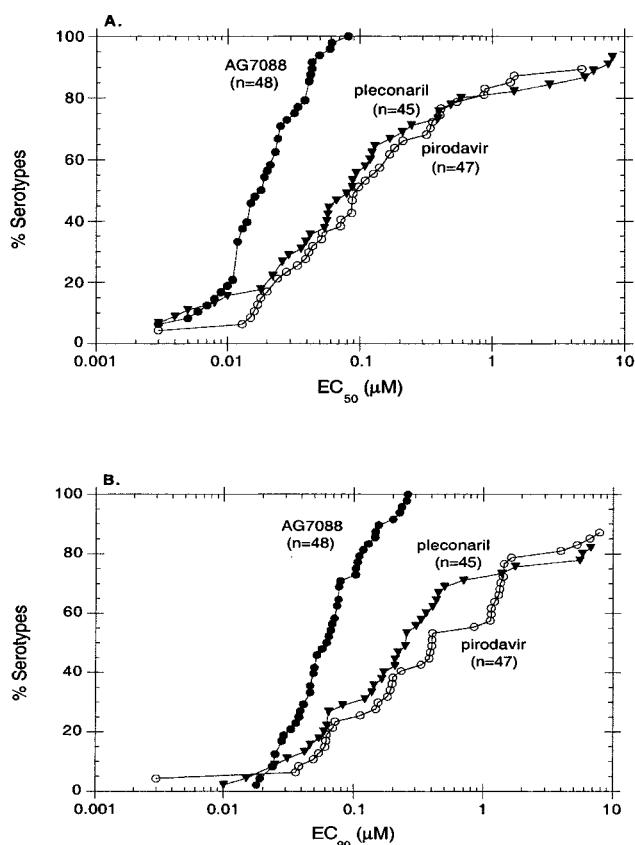


FIG. 2. In vitro activity of AG7088 against HRV serotypes. EC<sub>50</sub> (A) and EC<sub>90</sub> (B) of AG7088, pleconaril, and pirodavir for 48, 45, and 47 HRV serotypes, respectively, were determined by measuring XTT dye reduction following 2 to 5 days of infection of H1-HeLa cells as described in Materials and Methods.

and 0.030 μM, respectively, as well as against HRV 14 replication in a different host cell type, i.e., MRC-5 cells, with an EC<sub>50</sub> and EC<sub>90</sub> of 0.004 and 0.009 μM, respectively. The specificity of AG7088 for HRV was also demonstrated by the absence of activity (EC<sub>50</sub> > 100 μM) against other heterologous viruses, e.g., HCMV strain AD169 and HSV-1 strain McIntyre (data not shown).

Cytotoxicity in H1-HeLa cells was measured in parallel with the determination of antiviral activity. Results indicated that the EC<sub>50</sub> of AG7088 required for antiviral activity (range, 0.003 to 0.081 μM) was significantly less than the CC<sub>50</sub> of >1,000 μM, yielding a therapeutic index of >12,346 to >333,333. CC<sub>50</sub> of 150 and 77 μM were observed for pirodavir and pleconaril, respectively, yielding therapeutic indices of <15 to 49,967 and <8 to 25,667, respectively.

**Antiviral activity of AG7088 after HRV infection.** A time-of-addition assay was performed to determine the efficacy of AG7088 when added at various times after virus infection. For this purpose, H1-HeLa cells were infected with HRV 14 at a high MOI to achieve a single cycle of virus replication and levels of infectious virus were determined 8 h later. Results indicated that the addition of AG7088 to infected cells could be delayed up to 6 h after infection without a significant loss of in antiviral activity (Fig. 3). In contrast, a compound (WIN 51711) which binds to virus capsids and acts by inhibiting virus uncoating (40) was inhibitory only when provided at the initiation of the virus life cycle; a loss of virus suppression was observed when addition of the compound was delayed until 2 h after infection.

**Inhibition of HRV 3C proteolytic processing.** To confirm that the in vitro antiviral activity of AG7088 was derived from a direct inhibition of HRV 3C-mediated proteolytic processing, HRV14-infected H1-HeLa cells were treated with AG7088 (2.0 to 0.5 μM) and radiolabeled polyproteins were resolved by SDS-PAGE. Pleconaril was included as a negative control. SDS-PAGE analysis (Fig. 4) indicated a dose-dependent accumulation of large HRV 14 precursor polyproteins with a concomitant reduction of low-molecular-weight cleavage products in cells treated with AG7088 but not pleconaril. Polyprotein cleavage products predicted to accumulate following inhibition of 3C-mediated proteolytic processing, e.g., P1 (97 kDa) and P2-P3 (146 kDa) were observed. Likewise, a predicted reduction in the polyprotein cleavage products, e.g., VP1 (33 kDa), VP0 (37 kDa), 2C (38 kDa), and 3CD (72 kDa), was also observed.

**Effect of protein on the antiviral activity of AG7088.** Since evaluation of AG7088 in human clinical studies involves delivery by the intranasal route, it was of interest to evaluate the potential effects of proteins present in nasal secretions, AAG and mucin, on the in vitro antiviral activity of AG7088 in a H1-HeLa cell protection assay. Although the physiological concentration of AAG or mucin in nasal fluid is not known with certainty, AAG at a concentration of 1 mg/ml, which represents a physiologically relevant concentration in plasma (30), and mucin at a concentration of 1 mg/ml, which represents the maximum soluble concentration, were resuspended in medium containing 10% fetal bovine serum for these experiments. Under these conditions, no statistically significant differences between the antiviral activity of AG7088 in the presence of either AAG or mucin ( $P > 0.05$ ; Table 2) and the antiviral activity of AG7088 in the absence of AAG or mucin were observed. The mean EC<sub>50</sub> and EC<sub>90</sub> for AG7088 against HRV 14 in the absence of AAG were 0.021 and 0.040 μM, respectively, while in the presence of AAG, the mean EC<sub>50</sub> and EC<sub>90</sub> were 0.043 and 0.108 μM, respectively. Similarly, the EC<sub>50</sub> and EC<sub>90</sub> in the absence of mucin were 0.059 and 0.160 μM, respectively, while in the presence of mucin the EC<sub>50</sub> and EC<sub>90</sub> were 0.030 μM and 0.074 μM, respectively. No cytotoxicity was observed up to concentrations of 1 and 10 μM AG7088 in the presence of either AAG or mucin, respectively.

**Activity of AG7088 against related picornaviruses.** The efficacy of AG7088 against four related picornaviruses was also examined. Pirodavir and pleconaril were included for comparison. In H1-HeLa or MRC-5 cell protection assays, AG7088 was active against all four picornaviruses tested, with EC<sub>50</sub> ranging from 0.007 to 0.183 μM and EC<sub>90</sub> ranging from 0.033 to 0.340 μM (Table 3). These values were comparable to or significantly better than those obtained with both pirodavir and pleconaril; pirodavir inhibited the replication of two of the four (50%) picornaviruses tested with EC<sub>50</sub> of 4.833 and 0.443 μM, and pleconaril inhibited the replication of three of the four (75%) picornaviruses tested with EC<sub>50</sub> ranging from 0.037 to 1.012 μM. SDS-PAGE analysis of CAV 21-infected cells treated with AG7088 indicated a dose-dependent accumulation of large viral precursor polyproteins with a concomitant reduction of low-molecular-weight cleavage products, confirming that the in vitro antiviral efficacy of AG7088 is due to a direct inhibition of picornavirus 3C protease (data not shown).

## DISCUSSION

As members of one of the largest families of medically important human pathogens, HRVs are the single major cause of the common cold. HRV-induced upper respiratory illness has also been associated with serious medical complications in

TABLE 1. In vitro activity of AG7088 against HRV serotypes<sup>a</sup>

HRV serotype	EC <sub>50</sub> (μM) of:			EC <sub>90</sub> (μM) of:		
	AG7088	Pirodavir	Pleconaril	AG7088	Pirodavir	Pleconaril
1a <sup>c,e</sup>	0.041 ± 0.025	0.353 ± 0.311	0.388 ± 0.299	0.256	0.375, 2.497	0.653, 2.894
2 <sup>c,e</sup>	0.012 ± 0.005	0.013 ± 0.009	<0.003	0.030, 0.027	0.060 ± 0.050	0.054
3 <sup>b,d</sup>	0.017, 0.019	0.015, 0.052	0.056, 0.055	0.027, 0.048	0.021, 0.087	0.178, 0.097
8 <sup>b,d</sup>	0.023 ± 0.019	>10 ± 0	5.016 ± 0.944	0.049 ± 0.037	>10 ± 0	>10 ± 0
9 <sup>c,d</sup>	0.017, 0.006	0.004, 0.026	<0.003, 0.007	0.083, 0.031	0.027, 0.439	0.100, 0.562
10 <sup>c,d</sup>	0.024 ± 0.007	0.041 ± 0.016	0.123	0.147 ± 0.091	0.112 ± 0.037	0.302
11 <sup>c,d</sup>	0.081 ± 0.133	0.141 ± 0.204	1.465 ± 1.586	0.021, 0.289	1.644 ± 2.036	6.710 ± 5.699
12 <sup>c,d</sup>	0.041	0.018	0.109	0.110	0.062	0.256
13 <sup>b,d</sup>	0.012	0.183	0.579	0.079	0.405	5.600
14 <sup>b,d</sup>	0.013 ± 0.012	0.044 ± 0.045	0.058 ± 0.043	0.050 ± 0.048	0.154 ± 0.192	0.208 ± 0.169
15 <sup>c,d</sup>	0.020 ± 0.022	0.517, 0.166	0.495, 0.478	0.103 ± 0.107	0.906, 1.995	1.200, 1.585
16 <sup>c,d</sup>	0.015 ± 0.003	0.051 ± 0.016	ND	0.077 ± 0.021	0.191 ± 0.073	ND
17 <sup>b,d</sup>	0.014, 0.013	0.148, 0.654	>10, >10	0.027, 0.029	0.275, 2.626	>10, >10
19 <sup>c,d</sup>	0.013, 0.007	0.211 ± 0.295	0.161, 0.076	0.029, 0.042	1.226 ± 1.338	0.487, 0.383
21 <sup>c,d</sup>	0.011 ± 0.005	0.008, 0.023	0.012, 0.045	0.076 ± 0.024	0.021, 0.054	0.028, 0.063
22 <sup>c,d</sup>	0.034 ± 0.016	0.042, 0.005	0.010, 0.010	0.066 ± 0.025	0.084, 0.014	0.034, 0.027
23 <sup>c,d</sup>	0.038 ± 0.043	0.043, 0.150	0.018 ± 0.023	0.225 ± 0.313	0.062, 0.739	0.042 ± 0.030
24 <sup>c,d</sup>	0.049 ± 0.035	0.072, 0.261	0.048, 0.370	0.074	0.390	0.144
25 <sup>c,d</sup>	0.061 ± 0.047	>10 ± 0	0.054 ± 0.014	0.201 ± 0.147	>10 ± 0	0.250 ± 0.237
28 <sup>c,d</sup>	0.043 ± 0.026	0.046, 0.131	0.014, 0.057	0.118 ± 0.067	0.119, 0.246	0.031, 0.096
29 <sup>c,e</sup>	0.025 ± 0.018	0.018, 0.016	0.050, 0.027	0.062 ± 0.039	0.044, 0.028	0.095, 0.068
30 <sup>c,e</sup>	0.036, 0.013	0.025, 0.119	0.089, 0.084	0.071, 0.033	0.079, 0.316	0.338, 0.562
31 <sup>c,e</sup>	0.021 ± 0.014	4.621, 4.919	0.019, 0.025	0.039 ± 0.024	7.943, 7.833	0.053, 0.071
32 <sup>b,d</sup>	0.015 ± 0.007	0.418, 0.400	0.118, 0.140	0.052 ± 0.022	0.840, 1.896	0.536, 0.876
36 <sup>c,d</sup>	0.013 ± 0.007	0.087 ± 0.067	0.018 ± 0.013	0.064 ± 0.080	1.323 ± 0.572	0.062 ± 0.023
38 <sup>c,d</sup>	0.069, 0.048	0.030, 0.222	0.019, 0.025	0.208, 0.082	0.112, 0.700	0.261, 0.080
39 <sup>c,d</sup>	0.051, 0.012	0.053, <0.003	ND	0.095, 0.052	0.022, 0.100	ND
40 <sup>c,d</sup>	0.043 ± 0.043	0.020	0.026	0.129 ± 0.110	0.069	0.059
45 <sup>b,d</sup>	0.023 ± 0.007	>10, >10	>10, >10	0.076 ± 0.021	>10, >10	>10, >10
49 <sup>c,e</sup>	0.012, 0.018	ND	ND	0.062, 0.408	ND	ND
52 <sup>b,d</sup>	0.016 ± 0.003	0.530, 0.547	6.244, >10	0.046 ± 0.037	0.856, 0.848	>10, >10
53 <sup>c,d</sup>	0.006 ± 0.004	0.093, 0.124	0.061, 0.114	0.025 ± 0.018	2.371, 0.316	0.197, 0.243
54 <sup>b,d</sup>	0.012	0.880	2.721	0.261	>10	>10
56 <sup>c,d</sup>	<0.003	<0.003	0.093	0.033	<0.003	0.407
59 <sup>c,d</sup>	<0.003	0.071	0.394	0.018	1.136	5.843
63 <sup>c,d</sup>	0.024 ± 0.019	0.039 ± 0.013	0.168 ± 0.016	0.071 ± 0.068	0.201 ± 0.033	0.362 ± 0.100
67 <sup>c,d</sup>	0.019 ± 0.016	0.052 ± 0.012	0.066 ± 0.026	0.046 ± 0.027	0.148 ± 0.101	0.165 ± 0.064
68 <sup>c,d</sup>	0.042 ± 0.043	1.470 ± 0.079	0.004 ± 0.002	0.107 ± 0.082	5.290 ± 4.079	0.022, 0.008
73 <sup>c,d</sup>	0.019 ± 0.011	0.087 ± 0.051	0.057 ± 0.013	0.046 ± 0.027	0.268, 0.495	0.209 ± 0.023
75 <sup>c,d</sup>	0.011, 0.003	1.624, 0.100	0.109, 0.049	0.024, 0.023	7.017, 0.909	0.270, 0.241
78 <sup>c,d</sup>	0.005	1.379	<0.003	0.019	6.629	0.010
81 <sup>c,d</sup>	0.012, 0.004	0.617, 0.019	0.357, 0.133	0.025, 0.013	2.248, 0.060	0.718, 0.282
84 <sup>b,d</sup>	0.020, <0.003	0.050, 0.231	7.504 ± 4.323	0.071, 0.027	1.413, 0.903	>10 ± 0
86 <sup>b,d</sup>	0.012, 0.005	0.045, 0.127	0.048, 0.036	0.029, 0.052	0.183, 0.479	0.178, 0.066
87 <sup>b,f</sup>	0.060, 0.021	>10, >10	>10, >10	0.121, 0.087	>10, >10	>10, >10
89 <sup>c,d</sup>	0.015, 0.009	0.041, 0.007	<0.003	0.026, 0.024	0.112, 0.031	0.025
95 <sup>b,d</sup>	0.028 ± 0.017	>10, >10	4.977, 6.651	0.068 ± 0.053	>10, >10	>10, >10
Hanks <sup>c,d</sup>	<0.003, <0.003	<0.003	0.003, 0.013	0.026, 0.021	<0.003	0.023, 0.027

<sup>a</sup> Antiviral activity was determined by measuring XTT dye reduction following 2 to 5 days of infection of H1-HeLa cells as described in Materials and Methods. Results represent the means ± standard deviations (3 to 33 experiments) or individual values (1 or 2 experiments). ND, not determined.

<sup>b</sup> Antiviral group A (2).

<sup>c</sup> Antiviral group B (2).

<sup>d</sup> Major receptor group (50).

<sup>e</sup> Minor receptor group (50).

<sup>f</sup> Neither major nor minor receptor group (50).

individuals with underlying respiratory disorders (3). Although potent in vitro antirhinoviral activity has been described for numerous compounds to date (reviewed in references 5, 9, and 37), in only a few instances have reductions in clinical symptoms and/or virus infection been achieved in clinical trials (3, 9). The vast majority of these compounds act by binding to virus capsids and inhibiting either virus attachment or subsequent uncoating. However, recent reports have described novel inhibitors of 3C protease. The latter include peptide

aldehydes (19, 22, 47, 53), isatins (52), and homophthalimides (51). Recently a class of irreversible inhibitors incorporating Michael acceptors, which exhibits potent cell-based antiviral activity with little to no cellular cytotoxicity, has been described (11–15, 26). In this study, we describe the antiviral activity and cytotoxicity of AG7088, a potent, peptidomimetic inhibitor of HRV 3C protease that has recently begun to be evaluated in human clinical studies.

In cell-based assays, AG7088 demonstrated comparable an-

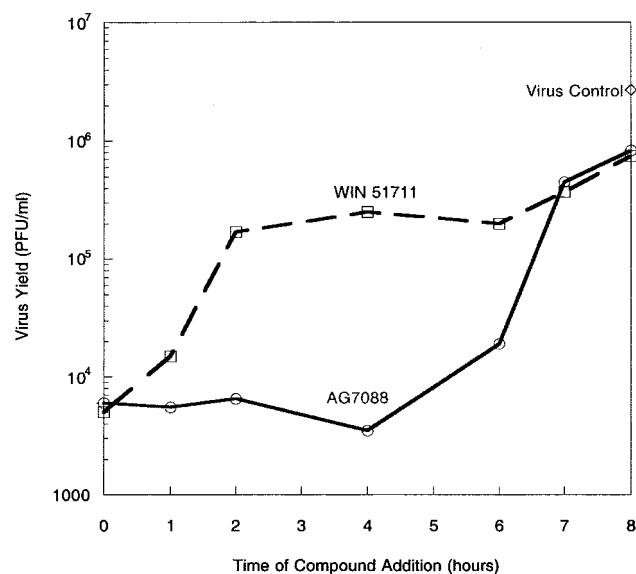


FIG. 3. Antiviral activity of AG7088 when added at various times after virus infection. H1-HeLa cells were infected with HRV 14 at an MOI of 15. AG7088 (0.5  $\mu$ M) and WIN 51711 (3.0  $\mu$ M) were added at various times after infection. Virus control represents infected cells incubated with medium only. Eight hours after infection, cell lysates and supernatants were collected and the level of infectious virus was determined as described in Materials and Methods.

tiviral potencies (27-fold range in  $EC_{50}$ ) against all 48 HRV serotypes tested. These results were in contrast to those observed with the capsid-binding compounds tested. The latter compounds demonstrated a significantly wider range in potency (1,590- to 2,707-fold range in  $EC_{50}$ ) and activity against most but not all HRV serotypes tested. The finding of comparable ranges in the activity of AG7088 against numerous HRV serotypes is consistent with DNA sequence analyses performed on 3C protease-coding gene regions, which demonstrate a significant level of homology in substrate/inhibitor binding regions (8, 16, 20, 21, 29, 31, 36, 38, 45, 48, 49, 55). This same level of homology, reflected in the 3C protease-coding regions derived from other picornaviruses, is also consistent with levels of activity demonstrated against four related picornaviruses.

Nasal secretions are biochemically complex and contain many serum proteins and mucous glycoproteins (33, 43, 46) including acid glycoproteins (34, 42) (unpublished observations). Since human clinical studies of AG7088 involve delivery by the intranasal route, it was of interest to determine the potential effects of mucin and AAG, proteins that are present in nasal washings. AAG is a major serum glycoprotein that has been shown to reduce the *in vitro* antiviral activity of several human immunodeficiency virus protease inhibitors (4, 28, 32). AAG has also been detected in various concentrations in nasal lavages (unpublished data). No significant reductions, however, in the antiviral efficacy of AG7088 in the presence of either AAG or mucin were observed.

HRVs, as members of the picornavirus family, encode a single large polyprotein precursor (reviewed in references 7, 10, 23, 27, 39, and 44) that depends on the virally encoded 3C protease for all posttranslational cleavages with the exception of an initial autocatalytic cleavage by the 2A protease to release P1, the precursor to the viral capsid proteins, an alternative cleavage by 2A to generate 3C' and 3D' products, and a late autocatalytic cleavage of VP0, which occurs during final viral assembly. The necessity of the 3C protease throughout the virus life cycle was confirmed in experiments that demon-

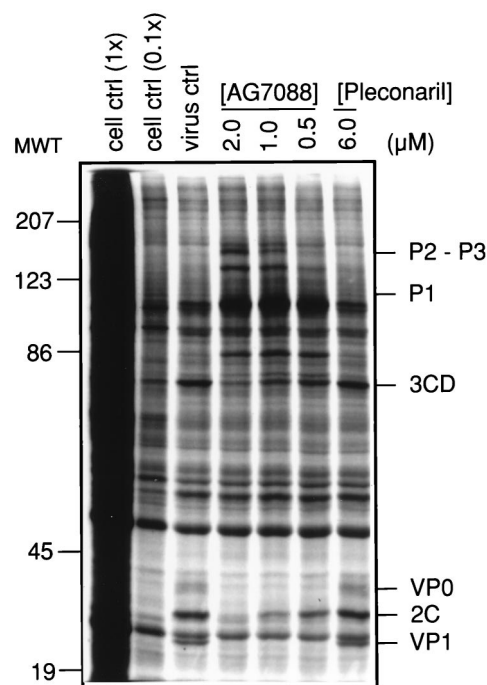


FIG. 4. Inhibition of HRV 14 3C-mediated proteolytic processing by AG7088. SDS-solubilized lysates were prepared from uninfected cells (cell ctrl; 0.1 $\times$  indicates 1/10 the amount of uninfected cell lysate analyzed in lane designated 1 $\times$ ) or infected cells treated with AG7088, pleconaril, or medium only (virus ctrl), and equal amounts of protein were analyzed by PAGE as described in Materials and Methods. P2-P3, P1, 3CD, VP0, 2C, and VP1 designate HRV-specific polypeptides. MWT, molecular weight (in thousands).

strated that the ability of AG7088 to suppress virus replication when added throughout a single cycle of virus replication in a time-of-addition assay. That the antiviral activity of AG7088 was due directly to its inhibition of 3C protease was demonstrated by SDS-PAGE analysis of infected cell lysates, which indicated a dose-dependent accumulation of large HRV 14 precursor polyproteins with a concomitant reduction of low-molecular-weight cleavage products. Profiles of polyproteins predicted to accumulate or be reduced were consistent with the cleavage profile observed when certain amino acid substitutions are introduced into the 3C protease catalytic site (6, 18, 24, 25).

These studies describe the antiviral activity and cytotoxicity of a novel inhibitor of HRV 3C protease. As an irreversible

TABLE 2. *In vitro* antiviral efficacy of AG7088 in the presence of AAG or mucin<sup>a</sup>

Treatment <sup>b</sup>	$EC_{50}$ ( $\mu$ M) <sup>c</sup>	$EC_{90}$ ( $\mu$ M) <sup>c</sup>	$CC_{50}$ ( $\mu$ M)	Therapeutic index <sup>d</sup>
No AAG	0.009, 0.033	0.031, 0.048	>1	>48
AAG	0.043 $\pm$ 0.020	0.108 $\pm$ 0.038	>1	>23
No mucin	0.059 $\pm$ 0.019	0.086, 0.234	>10	>169
Mucin	0.030 $\pm$ 0.019	0.074 $\pm$ 0.033	>10	>333

<sup>a</sup> Antiviral efficacy was determined by measuring XTT dye reduction following 2 or 3 days of infection of H1-HeLa cells with HRV 14 as described in Materials and Methods.

<sup>b</sup> Experiments were conducted in the presence or absence of AAG or mucin (1 mg/ml), both resuspended in medium containing 10% fetal bovine serum.

<sup>c</sup> Results represent the means  $\pm$  standard deviations (three or four experiments) or individual values (one or two experiments).

<sup>d</sup> Therapeutic index =  $CC_{50}/EC_{50}$ .

TABLE 3. In vitro activity of AG7088 against picornavirus infection<sup>a</sup>

Virus strain	AG7088		Pirodavidir		Pleconaril	
	EC <sub>50</sub> (μM)	EC <sub>90</sub> (μM)	EC <sub>50</sub> (μM)	EC <sub>90</sub> (μM)	EC <sub>50</sub> (μM)	EC <sub>90</sub> (μM)
CAV 21	0.147 ± 0.024	0.314 ± 0.073	4.833	>10	0.037 ± 0.048	0.148 ± 0.150
CVB 3	0.183 ± 0.161	0.340 ± 0.321	>10	>10	>10	>10
EV 11	0.014 ± 0.003	0.033 ± 0.015	>10	>10	0.165	0.275
ETV 70	0.007 ± 0.006	0.066 ± 0.119	0.443	0.899	1.297, 0.726	2.726, 2.492

<sup>a</sup> Antiviral efficacy was determined by measuring XTT dye reduction following 1 to 5 days of infection as described in Materials and Methods. Results represent the means ± standard deviations (three to six experiments) or individual values (one or two experiments).

inhibitor, AG7088 may be capable of forming covalent interactions with other proteins and inducing possible toxicities in vivo. The potential for these types of interactions have, however, been minimized by selection of a Michael acceptor with only mild chemical reactivity (12) and certainly can be explored in appropriate clinical trial studies. In summary, the potent activity against all HRV serotypes tested combined with the ability to inhibit virus replication throughout a single cycle of virus replication indicates that AG7088 is a promising new clinical candidate.

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