Antiviral and Resistance Studies of AG1343, an Orally Bioavailable Inhibitor of Human Immunodeficiency Virus Protease

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AG1343 ([3S-(3'R, 4aR'), 8aR', 2'S', 3'S']-2-[2' hydroxy-3'-phenylthiomethyl-4'-aza-5'-oxo-5'-2'-(2'-'methyl-3'-hydroxy-phenyl)pentyl]-decahydroiso-quinoline-3-N-(4-butylcarboxamide methanesulfonic acid) is a selective, nonpeptidic inhibitor of human immunodeficiency virus (HIV) protease ($K_i = 2 \text{ nM}$) that was discovered by protein structure-based drug design methodologies. AG1343 was effective against the replication of several laboratory and clinical HIV type 1 (HIV-1) or HIV-2 isolates including pyridinone- and zidovudine-resistant strains, with 50% effective concentrations ranging from 9 to 60 nM. In reversibility studies, inhibition of gag (p55) proteolytic processing in HIV-1 particles from cells treated with AG1343 was maintained for up to 36 h after drug removal. The ability of virus to develop resistance to AG1343 was studied by serial passage of HIV-1 NL4.3 in the presence of increasing concentrations of drug. After 28 passages, a variant with a 30-fold reduction in susceptibility to AG1343 was isolated. Molecular analysis of the protease from this variant indicated a double change from a Met to Ile at residue 46 and an Ile to Val or Ala at residue 84 (M46I + I84V, A). Consistent with these findings, reductions in susceptibility were observed for recombinant viruses constructed to contain the single I84V change or the double M46I + I84V substitutions. Resistance, however, was not detected for recombinant viruses containing other key mutations in HIV-1 protease, including a Val to Ile change at residue 32 or a Val to Ala or Phe at residue 82. The potent anti-HIV activity of AG1343 against several isolates suggests that AG1343 should perform well during ongoing human phase II clinical trials.

MATERIALS AND METHODS

Compounds. AG1343 was synthesized at Agouron Pharmaceuticals, Inc. Ro 31-8559 was kindly provided by Roche Research Centre, Welwyn Garden City, England. Zidovudine (AZT) was purchased from Burroughs Wellcome (Research Triangle Park, N.C.).

Cells and virus strains. The following reagents were obtained from the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: CEM-SS, MT-2, and MT-4 human T-cell lines, a chronically infected cell line (HIV-1 IIIB/CEM-SS); and the HIV-1 RF, HIV-1 IIIB, HIV-1 Ba-L, HIV-2 ROD, HIV-1 IIIB A17, HIV-1 G9010-6, and HIV-1 HJ122 strains. HIV-1 Rlo is a primary clinical strain of HIV-1 isolated from a child with AIDS (S). Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of HIV-seronegative donors by centrifugation through lymphocyte separation medium. Following overnight incubation, nonadherent cells (PBMCs) were removed and were stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 3 days. Attached cells (macrophages) were subsequently incubated for 7 days in medium containing 10% human type AB serum and 20 μg of granulocyte-macrophage colony stimulating factor per ml.

HIV-1 protease assay. HIV-1 protease from HIV-1 IIIB was expressed in Escherichia coli and was purified from insoluble inclusion bodies as described previously (13). The proteolytic activity of purified HIV protease was measured by a modified chromogenic assay developed by Richards et al. (35). The synthetic peptide His-Lys-Ala-Arg-Val-Leu-Phe-(para-NO2)-Glu-Ala-Nle-Ser-NH2 (American Peptide Company, Sunnyvale, Calif.) was used as the substrate. The assay was carried out in 0.5 M NaCl-50 mM MES (2-[N-morpholino]ethanesulfonic acid; pH 5.6)-5 mM EDTA [1,1-bis-chloroethylnitrosourea]-2,2'-dichloroethane]-2% dimethyl sulfoxide at 37°C. Cleavage of the scissile bond between leucine and para-nitrophenylalanine was assayed by spectrophotometric monitoring of the decrease in the A405. The initial velocity was determined as the rate of decline in the absorbance during the first 100 s of the enzymatic reaction. Under these conditions, the Michaelis constant ($K_M$) for this substrate is 52 ± 16 μM. For determination of the inhibition of AG1343, a saturating concentration of substrate (200 μM) was used. Between 13 and 20 concentrations of AG1343 were evaluated, and the velocity of the reaction was measured at each concentration as described above. The apparent $K_M$ was determined by computer-assisted non-

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The human immunodeficiency virus type 1 (HIV-1)-encoded protease is an aspartic protease that is responsible for the cleavage of the gag (p55) and gag-pol (p160) precursor polyproteins to yield both structural proteins of the virus as well as the enzymes necessary for viral replication. The HIV-1 protease is required for the proper processing and maturation of viral particles since certain mutations in the protease gene result in a loss of infectivity and the formation of noninfectious, defective HIV-1 particles (18, 32). Accordingly, HIV-1 protease is considered a promising target for antiviral therapy. Recent data from in vivo and in vitro studies have confirmed the utility of HIV-1 protease inhibitors as potential antiviral therapies. In vitro, many peptidomimetic inhibitors that show potent activity against the virus in cell culture-based assays have been described (4, 8, 19, 24, 41, 44; for a review, see reference 45). Moreover, results from initial human clinical trials with some protease inhibitors have indicated significant reductions in viral load and increases in the number of CD4+ cells after treatment (23, 37, 39). AG1343 ([3S-(3'R, 4aR'), 8aR', 2'S', 3'S']-2-[2' hydroxy-3'-phenylthiomethyl-4'-aza-5'-oxo-5'-2'-(2'-'methyl-3'-hydroxy-phenyl)pentyl]-decahydroiso-quinoline-3-N-(4-butylcarboxamide methanesulfonic acid) is an HIV protease inhibitor that was discovered by using an iterative cycle of crystal structure determination, computer-aided drug design, and chemical synthesis (16). AG1343 has recently begun evaluation in human phase I/II clinical trials. In this report, we describe the in vitro antiviral potency of AG1343 in several different HIV infection models. In addition, we present data describing the in vitro isolation and the characterization of an HIV-1 variant with reduced susceptibility to AG1343.
linear least-squares fitting of the data to the tight binding equation of Morrison (27).

Cell protection assay. The ability of compounds to protect cells against HIV-1 infection was measured by the MTT dye reduction method (2). Briefly, MT-2 cells and CEM-SS cells were infected with HIV-1 IIIB or HIV-1 RF at multiple

itiations of infection of 0.01 and 0.03, respectively. Following a 4-h adsorption period, infected or uninfected cells were resuspended at 5 × 10^6 cells per ml and were incubated with appropriate concentrations of drug. Six days (CEM-SS cells) or 7 days (MT-2 cells) later, MTT (5 mM) was added to the test plates and the amount of formazan produced was quantified spectrophotometrically at 570 nm.

The 50% effective concentration (EC_{50}) was the concentration of drug that increased the percentage of formazan production in virus-infected cells to 50% of that produced by drug-free, uninfected cells. The 50% cytotoxic concentration was the concentration of drug that decreased the percentage of formazan pro-

duced in drug-treated, uninfected cells to 50% of that produced in drug-free, uninfected cells. The therapeutic index was calculated by dividing the 50% cytotoxic concentration by the EC_{50}.

Antiviral assays. PHA-stimulated PBMCs were infected with HIV-1 Rofo at a multiplicity of infection of 0.001. Following a 2.5-h adsorption period, the cells were washed to remove virus inoculum and 2 × 10^6 cells per ml were resus-
pended in medium alone or medium containing the appropriate concentrations of drug. After 14 days of incubation at 37°C, culture supernatants were removed and assayed for reverse transcriptase (RT) as described below. Antiviral efficacy (EC_{50}) was calculated using a logistic regression analysis program and was defined as the concentration of drug that decreased the levels of RT in drug-treated, infected cells to 50% of that produced in drug-free, infected cells. Macrophage cultures were infected with HIV-1 Ba-L at a multiplicity of infection of 0.1. Following a 48-h incubation, the cells were washed and resuspended in medium alone or medium containing the appropriate concentrations of drug. Antiviral efficacy (EC_{50}) was calculated as described above. CEM-SS cells chronically infected with HIV-1 IIIB (HIV-1 IIIB/CEM-SS) were washed to remove virus and were resuspended at 5 × 10^6 cells per ml with appropriate dilutions of drug or medium alone as a control. Three days later, culture supernatants were removed and assayed for RT activity. Antiviral efficacy (EC_{50}) was calculated as described above.

RT assay. A microtiter plate-based RT assay was used. Briefly, tritiated thymi
dine triphosphate ([3H]TTP; Amersham Corp., Arlington Heights, Ill.) was calculated as described above. Following a 48-h incubation, the cells were washed and resuspended in medium alone or medium containing the appropriate concentrations of drug. After 14 days of incubation at 37°C, culture supernatants were removed and assayed for RT activity. Antiviral efficacy (EC_{50}) was calculated as described above.

Western blot analysis. Polyproteins present in SDS-solubilized virions were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. Following transfer onto Hybond-ECL nitrocellulose (Amersham Corp.), the gels were washed and were also stored at


cell lines. The level of p24 in cell-free supernatants were assayed with a commercial kit (Du Pont NEN, Boston, Mass.). The level of p24 was determined in drug-treated, uninfected cells to 50% of that produced in drug-free, uninfected cells. The therapeutic index was calculated by dividing the 50% cytotoxic concentration by the EC_{50}.

RESULTS

Activity against HIV-1 protease. AG1343 (Fig. 1) showed potent inhibition against HIV-1 protease with a Ki of 1.71 ± 0.62 nM. Under the same conditions, pepstatin A, a general inhibitor of aspartic proteases, and Ro 31-8959, an inhibitor of aspartic proteases, including pepsin, renin, and gastrin at concentrations up to 1

TABLE 1. In vitro antiviral activity of AG1343 in acute and chronic HIV infection models

Virus strain Cell type EC_{50} (nM)

HIV-1 RF CEM-SS 31
HIV-1 IIIB MT-2 43
HIV-1 Ba-L Macrophage 23
HIV-1 Rofo PBMC 10
HIV-1 IIIB A17 MT-2 30
HIV-1 IIIB MT-2 10
HIV-1 G910-6 MT-2 60
HIV-1 IIIB (chronic infection) CEM-SS 39
HIV-2 ROD CEM-SS 9

a In vitro efficacy studies of AG1343, Ro 31-8959, and AZT were performed using different virus strains including the monocyteotropic strain HIV-1 Ba-L; the human lymphocytic-accumulating strain HIV-1 Rofo; the primate-rodent strain HIV-1 IIIB A17 (28); AG1343-susceptible and AG1343-resistant strains HIV-1 IIIB and HIV-1 G910-6, respectively (21); and chronically infected HIV-1 IIIB/CEM-SS cells; HIV-1 RF, HIV-1 IIIB, and HIV-2 ROD are laboratory strains.

a Results represent means from two or more experiments. ND, not deter-

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µM and showed very weak affinity to cathepsin E (74,000 nM) and cathepsin D (435 nM).

**Antiviral efficacy against acute and chronic HIV infections.** The antiviral efficacy of AG1343 was examined against a variety of laboratory and clinical strains of HIV-1 and HIV-2 in several different infection models, and the results are summarized in Table 1. By using MTT dye reduction as an endpoint, AG1343 was able to protect both CEM-SS and MT-2 cells against acute HIV-1 RF- and HIV-1 IIIB-induced cell killing, with EC_{50}s ranging from 31 to 43 nM. The 50% cytotoxic concentration of AG1343 ranged from 23 to 28 µM, yielding a favorable therapeutic index of 526 to 916. No significant reduction in antiviral activity was observed when this assay was performed in the presence of up to 50% fetal bovine serum or 50% human serum (data not shown). The mean EC_{50} in a variety of acute infection models was 59 nM (range, 7 to 130 nM; data not shown).

The antiviral efficacy of AG1343 was also demonstrated against other virus strains in different host cell types (Table 1). By using the levels of RT as an endpoint, AG1343 demonstrated potent activity against the replication of both the monocytotropic HIV-1 Ba-L strain and a low-passage clinical isolate (HIV-1 RoJo) when the drug was assayed in either primary macrophages or PBMCs isolated from whole human blood, respectively. AG1343 was also effective in inhibiting the replication of HIV-2 ROD in CEM-SS cells and in reducing the levels of RT released from CEM-SS cells chronically infected with HIV-1 IIB. As expected, AZT which targets an early, preintegration stage in the viral life cycle, was ineffective in inhibiting RT release from chronically infected cells at concentrations up to 1,000 nM (Table 1).

To examine the potential utility of AG1343 in the treatment of patients who have had prior exposure to RT inhibitors, the efficacy of AG1343 was tested against the AZT-resistant strain HIV-1 G910-6 (21). AG1343 was effective at inhibiting the replication of HIV-1 G910-6, with an EC_{50} of 60 nM (Table 1). The AZT resistance phenotype of HIV-1 G910-6 was confirmed by the demonstration of a >4,966 fold increase in the EC_{50} in response to AZT compared with that for its matched predrug isolate, HIV-1 HI12-1 (21). AG1343 was also effective against the replication of HIV-1 A17, a strain of HIV-1 highly resistant to nonnucleoside RT inhibitors including pyridinone, nevirapine, and TIBO [tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one] compounds (28).

**Inhibition of gag (p55) processing following drug removal.** Inhibition of HIV-1 proteolytic processing results in the formation of defective particles containing precursor gag (p55) and little or no processed p24. Resumption of processing has been shown to occur following removal of drug from defective particles (4, 25, 41). To examine the reversibility of the inhibition of p55 processing by AG1343, virions from AG1343-treated, chronically infected HIV-1 IIB/CEM-SS cells were purified away from drug. Resumption of proteolytic processing in HIV-1 particles over time was examined by Western blot analysis with a monoclonal antibody which recognizes both p24 and p55. As Fig. 2 illustrates, virions purified from cells treated with medium alone consisted almost exclusively of processed p24 protein, indicative of mature virions. In contrast, at time zero, virions from cells treated with 7.5 µM AG1343 contained almost exclusively p55 but lacked processed p24, indicative of immature virions. Subsequent incubation for 36 h in the absence of drug resulted in a partial reduction in p55 levels, the appearance of processing intermediates, but a complete absence of processed p24.

**Isolation of an HIV variant with reduced susceptibility to AG1343.** In vitro serial passage studies of HIV-1 in the presence of increasing concentrations of various protease inhibitors have resulted in the isolation of variant HIV-1 strains with reduced susceptibilities to the inhibitor used for selection. The phenotype of resistance to these inhibitors has been attributed to a few specific changes in the protease gene region (10–12, 15, 17, 24, 29–31, 33, 40, 42). As a means of studying the resistance of HIV-1 to AG1343, wild-type virus was serially passaged in the presence of increasing concentrations of drug. Following 22 passages, a variant which had a sevenfold reduced susceptibility to AG1343 was isolated (Table 2). Subsequent genotypic analysis demonstrated an Arg to Gin change at residue 8 (R8Q) in one of six clones, an Asp to Asn change at residue 35 (D35N) in four of six clones, an Ala to Val change at residue 71 (A71V) in three of six clones, and an Ile to Val change at residue 84 (I84V) in one of six clones (Fig. 3). After an additional six passages (passage 28), a variant with a 30-fold reduced susceptibility to AG1343 was isolated (Table 2). Molecular analysis of the protease gene from this variant identified a double change from a Met to Ile at residue 46 and an Ile to Val or Ala at residue 84 (M46I + I84V,A) in six of six clones, a Leu to Pro change at residue 63 (L63P) in three of six clones, and an A71V change in two of six clones. The D35N change, which was observed in an earlier passage (passage 22), was not detected in any clones examined from the passage 28 variant.

**Susceptibility of recombinant HIV-1 virus strains containing mutations in the protease gene.** To confirm the relevance of these changes in conferring the drug resistance phenotype, recombinant mutant HIV-1 NL4.3 strains containing single or double mutations in the protease gene were constructed and examined for their susceptibilities to AG1343. As the results in Table 3 indicate, and consistent with results from the serial

<table>
<thead>
<tr>
<th>Virus</th>
<th>Conc (µM)</th>
<th>EC_{50} (µM)</th>
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<tr>
<td>HIV-1 NL4.3</td>
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<td></td>
</tr>
<tr>
<td>P1</td>
<td>0.001</td>
<td>0.080</td>
</tr>
<tr>
<td>P22</td>
<td>0.300</td>
<td>0.560 (7×)</td>
</tr>
<tr>
<td>P28</td>
<td>1.600</td>
<td>2.400 (30×)</td>
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* Wild-type HIV-1 NL4.3 was serially passaged in the presence of increasing concentrations of AG1343, as indicated. Values in parentheses (resistance levels) represent the ratio of the EC_{50} for virus isolated following serial passage (P) for the indicated times to the EC_{50} for wild-type HIV-1 NL4.3, as determined by the susceptibility assay in MT-4 cells described in Materials and Methods.
passage study, a reduction in susceptibility was detected for viruses containing the single mutation I84V or the double mutation R8Q, K + M46L and M46I + I84V. No change in susceptibility was observed for viruses containing the single mutations M46L, L63P, or A71V. This latter observation is consistent with other reports (12, 24, 31) which also indicate the appearance of these changes in virus variants without a reduction in susceptibility. Other reductions in susceptibility were observed for variants that had not arisen during serial passage. These include variants containing a change from Leu to Met at residue 90 (L90M) as well as variants containing the double mutations R8K + V82I and V32I + V82A (Table 3). Furthermore, significant reductions in the susceptibilities of virus strains containing single mutations previously shown to confer resistance to other protease inhibitors, e.g., V32I, a Gly to Val change at residue 48 (G48V), or a Val to Ala or Phe change at residue 82 (V82A,F) (15, 17, 29–31, 33, 40), were not detected for AG1343. Recombinant viruses containing some double or triple changes in the protease gene were not viable and hence could not be tested (data not shown).

**DISCUSSION**

HIV-1 protease inhibitors represent a promising new class of anti-HIV agents that have recently entered clinical trials. Preliminary data from those studies indicate that as a class, protease inhibitors can be distinguished from the currently approved RT inhibitors, e.g., AZT, zalcitabine, didanosine, and stavudine by their lack of significant toxic side effects as well as the magnitude of early antiviral effect (23, 37, 39).

AG1343 is a novel and potent HIV-1 protease inhibitor that was discovered on the basis of the experimentally derived three-dimensional structure of HIV-1 protease (16). In the present study we evaluated the in vitro antiviral efficacy of AG1343 in several different HIV infection models using both primary and continuous cell lines. AG1343 proved to be effective at inhibiting the replication of laboratory, clinical, and RT inhibitor-resistant isolates for which EC50 ranged from 9 to 60 nM. The antiviral potency of AG1343 against both AZT- and nonnucleoside inhibitor-resistant isolates provides a rationale for the treatment of patients who have had prior exposure to this class of inhibitors. As with other protease inhibitors and unlike RT inhibitors which are only effective early in the viral life cycle at a stage prior to integration, AG1343 was effective at inhibiting the release of RT from cells chronically infected with HIV.

The gag (p55) and gag-pol (p160) regions of the HIV-1 genome are initially translated into large precursor polyproteins which are subsequently cleaved by HIV-1 protease to yield structural and regulatory proteins found in the virion (9). Interference with HIV-1 proteolytic processing following treatment with peptidomimetic compounds or by the introduction of specific mutations in the protease gene results in the formation of defective, noninfectious HIV-1 particles which contain unprocessed p55 (18, 32). Purification of defective HIV-1 particles from drug-treated cells and subsequent incubation in drug-free medium have been shown to result in the resumption of proteolytic processing (4, 25, 41). Since this phenomenon may mimic the situation in the clinical setting if suitable levels in plasma are not sustained, it was of interest to determine the properties of AG1343 in this regard. Results from in vitro kinetic studies showed that inhibition of proteolytic processing

![Table 3](image_url)
by AG1343 was only partially restored by 36 h after drug removal. This suggests that virus may be slow to recover from AG1343 treatment, a potentially positive feature should drug levels in the blood temporarily fall below the therapeutic range. These results are similar to that described for other inhibitors, e.g., U-75875, (3, 20), but are in contrast to those reported for certain other compounds, e.g., U-81749, L-735, 524, and BMS 186,318 (4, 25, 41), which are rapidly and completely reversed following removal of drug. The mechanism of this irreversibility is not known. There is no evidence, on the basis of the chemical structure of AG1343 (Fig. 1), to indicate an irreversible or covalent interaction with the active site of HIV-1 protease. Maintenance of inhibition of p55 gag processing could perhaps result from an enhanced accumulation of AG1343 in purified virions.

The development of virus strains which contain mutations in specific gene regions following treatment with either RT or protease inhibitors in vivo (7, 14, 21–23, 36) severely limits the utility of chronic treatment of HIV-infected individuals. Development of resistance to protease inhibitors has been studied extensively in vitro by isolating and characterizing resistant HIV-1 variants and by examining the susceptibilities of viral strains constructed to contain specific changes in the protease gene. We have characterized a variant isolated in vitro following 28 passages. The variant has a reduced susceptibility to AG1343 and contains an IS4V mutation. This IS4V mutation has been shown to confer resistance to other protease inhibitors including RP 312 (10) and ABT-538 (24). These results are consistent with those of molecular modeling studies, which indicate a substantial contact between residue 84 and 84' in the S1 and S1' binding sites of the enzyme with the P1 thiophenyl and the P1' decahydroisoquinoline of AG1343, respectively. Substitution of an isoleucine with the smaller amino acid, valine, may result in a loss of contact between the enzyme and AG1343, and hence reduced binding. Consistent with the results reported by others (12, 24, 31), additional changes were observed, e.g., M46I, A71V, and L63P; however, these were not associated with reductions in susceptibility to AG1343.

Preliminary in vitro data suggest that protease inhibitors may fall into specific classes depending on their resistance patterns (26). This is in contrast to preliminary in vivo data which suggest that treatment of HIV-1-infected patients with at least one inhibitor, L-735, 524, results in the emergence of HIV-1 strains containing multiple mutations which appear cross-resistant to many different protease inhibitors (7). In our study we found little or no reduction in susceptibility to AG1343 of recombinant HIV-1 strains which contain single mutations in the protease gene which have previously been shown to confer resistance to other protease inhibitors. More specifically, these include a R8Q or R8K substitution described for A-77003 (15), and either a V32I, a M46L, or a V82A/F change for A-77003 (12, 17), an G48V change described for Ro 31-8959 (15), and either a V32I, a M46L, or a V82A/F change described for L-735, 524, ABT-538, SC-52151, and BMS 186,318 (24, 31, 33, 40). Combinations of some of these mutations in recombinant viruses, e.g., V32I + V82A, did, however, result in a fourfold reduction in susceptibility to AG1343. The significance of the reductions in susceptibility described for viruses containing single and double mutations may be more apparent following the determination of the mutations and analysis of resistance associated with viral isolates arising during clinical studies.

In summary, we have described a novel HIV-1 protease inhibitor that has potent and selective activity against the enzyme as well as in a variety of different HIV-1 and HIV-2 infection models. This activity, coupled with its high degree of oral bioavailability (38), lack of toxicity in preclinical studies (43), and favorable pharmacokinetic profiles in human phase I clinical studies (34), support further evaluation of this compound in human phase II trials. These studies are ongoing.

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