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ScienceDirect
Virology 356 (2006) 217–224
VIROLOGY
www.elsevier.com/locate/yviro

Determinants of activity of the HIV-1 maturation inhibitor PA-457

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Received 8 June 2006; returned to author for revision 6 July 2006; accepted 14 July 2006
Available online 22 August 2006

Abstract

3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid, also termed PA-457 or DSB, is a novel HIV-1 inhibitor that blocks virus maturation by disrupting cleavage of the capsid precursor, CA-SP1. To better define the molecular target for PA-457, we prepared a panel of mutant viruses with point deletions spanning the CA-SP1 cleavage domain and characterized each of these viruses for PA-457 sensitivity. Our results indicate that amino acid residues in the N-terminal half of SP1 serve as determinants of PA-457 activity, while residues in the C-terminal half of SP1 were not involved in compound activity. These findings support and extend previous observations that PA-457 is a specific inhibitor of CA-SP1 cleavage and identify the CA-SP1 domain as the primary viral determinant for this novel inhibitor of HIV-1 replication.

Keywords: PA-457; HIV; Maturation inhibitor; CA-SP1 cleavage; Viral determinants

Introduction

HIV-1 assembly is driven largely by the Gag precursor protein $Pr55^{Gag}$. Following synthesis, $Pr55^{Gag}$ is transported to the plasma membrane where virus assembly occurs (Freed, 1998; Morita and Sundquist, 2004; Swanstrom and Wills, 1997). Through a complex combination of Gag–lipid, Gag–Gag, and Gag–RNA interactions, a multimeric budding structure forms at the inner leaflet of the plasma membrane. The budding virus particle is ultimately released from the cell surface in a process that is promoted by an interaction between the late domain in the p6 region of Gag (Gottlinger et al., 1991; Huang et al., 1995) and host proteins, most notably TSG101 and other cellular factors (Demirov et al., 2002a; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001).

Concomitant with particle release, the viral protease (PR) cleaves both $Pr55^{Gag}$ and $Pr160^{GagPol}$ to generate the mature Gag proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6, two small Gag spacer peptides (SP1 and SP2), and the mature *pol*-encoded enzymes PR, reverse transcriptase (RT), and integrase (IN). Gag and GagPol cleavage triggers a structural rearrangement termed maturation, during which the immature particle transitions to a mature infectious viral particle characterized by an electron-dense, conical core. The efficiencies with which PR cleaves its target sequences vary widely, resulting in a highly ordered Gag and GagPol processing cascade (Erickson-Viitanen et al., 1989; Kräusslich et al., 1988; Mervis et al., 1988).

The sequential nature of Gag processing can be disrupted by altering the amino acid sequence at cleavage sites within Gag (Krausslich et al., 1995; Wiegers et al., 1998) and even partial inhibition of Gag processing profoundly impairs virus maturation and infectivity (Kaplan et al., 1993). Mutating key residues in the p6 late domain (Demirov et al., 2002b; Gottlinger et al., 1991; Huang et al., 1995) or inhibiting the interaction between p6 and TSG101 (Demirov et al., 2002a; Garrus et al., 2001) also delays Gag processing, and increases levels of the Gag cleavage

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doi:10.1016/j.virol.2006.07.023

intermediates $p25^{CA\text{-}SP1}$ and $p41^{MA\text{-}CA}$ in virions. It has also been reported that deletions in the dimer initiation site (DIS) of the viral genomic RNA lead to an accumulation of the capsid precursor p25 and a defect in virus maturation (Liang et al., 1998; Liang et al., 1999).

Recently, we and others reported on 3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid (PA-457), a potent HIV drug candidate that employs a novel mechanism of action to block Gag processing and inhibit virus replication (Li et al., 2003; Zhou et al., 2004b). PA-457 potently inhibits replication of both prototypic and clinical HIV-1 isolates and retains this antiviral activity against viruses resistant to the three classes of approved drugs targeting the viral enzymes RT and PR. Interestingly, the compound is HIV-1 specific with no activity observed against the related retroviruses HIV-2 or SIV. PA-457 inhibits virus replication by specifically disrupting the conversion of the Gag capsid precursor protein ($p25^{CA\text{-}SP1}$) to mature capsid protein ($p24^{CA}$) resulting in the release of immature, non-infectious virus particles. Using *in vitro* resistance selection experiments, the determinants of PA-457 activity have been mapped to the region flanking the CA-SP1 cleavage site (Li et al., 2003; Zhou et al., 2004b).

In the current report, we further define the molecular determinants of PA-457 activity. This work involved preparing a comprehensive panel of single residue point deletion mutants spanning the entire CA-SP1 boundary domain (GHKARVL-AEAMSQVTNPATIM), and characterizing each of the non-defective mutants with respect to infectivity, replication, Gag processing and PA-457 sensitivity. The results reported here are consistent with results from previously reported resistance selection experiments that identified the first (A^{364}) and third (A^{366}) residues of SP1 as critical to PA-457 activity (Li et al., 2003; unpublished results). Significantly, the results of the current study extend the earlier work and identify additional SP1 residues involved in mediating the PA-457 antiviral effect. Not unexpectedly, our data indicated that the Gag residues associated with compound activity reside primarily in the N-terminal domain of SP1. The results reported here demonstrate that multiple SP1 residues serve as viral determinants of PA-457 activity and further support the hypothesis that the CA-SP1 cleavage domain serves as the primary viral determinant for this potent inhibitor of HIV-1 replication.

Results

Effects of CA-SP1 point deletions on virus production and Gag processing

To further define the molecular determinants of PA-457 activity, we generated a series of point deletions spanning the CA-SP1 boundary region. This deletion-based mutagenesis approach has been successfully used to characterize various functional domains of retroviral Gag including MA, CA, NC, and the late-assembly domain (Dorfman et al., 1994; Nelle and Wills, 1996; Ott et al., 2005; Parent et al., 1995; Puffer et al., 1997; Reil et al., 1998; Srinivasakumar et al., 1995; Xiang et al., 1996; Yuan et al., 1999; Yuan et al., 2000). Mutant viruses with single residue point deletions spanning the Gag CA-SP1 cleavage domain (residues $G^{357}H^{358}K^{359}A^{360}R^{361}V^{362}L^{363}$-$A^{364}E^{365}$-$A^{366}M^{367}S^{368}Q^{369}V^{370}T^{371}N^{372}P^{373}A^{374}T^{375}I^{376}M^{377}$) were generated in the context of the HIV-1 molecular clone NL4-3 (Fig. 1). As the Gag CA-SP1 region has been closely associated with viral assembly and budding (Accola et al., 1998; Liang et al., 2002), our initial efforts in characterizing these viruses involved determining the effect of the point deletions on viral particle production. HeLa cells were transfected in parallel with DNA from the parental and variant NL4-3 proviral constructs. Virus production was measured at 48 h post-transfection by p24 antigen capture. The results of these experiments demonstrated that the majority of viruses containing point deletions within the C-terminal domain of CA (ΔG^{357}, ΔH^{358}, ΔK^{359}, ΔA^{360}, ΔR^{361} and ΔL^{363}) or at the N-terminus of SP1 (ΔA^{364}) were defective for viral particle production (Fig. 2). For each of these viruses, a two-log or greater reduction in virus production relative to the parental NL4-3 was observed. Viral particle production by capsid mutant ΔV^{362} was less affected, being about 10% that of the parental NL4-3; however, this virus exhibited a defect in CA-SP1 processing as evidenced by the significant accumulation of virion-associated p25 by Western blot (Fig. 3). It should be noted that cell-associated virus expression of each of these point deletion variants was comparable to the parental NL4-3 (unpublished results) and the reduced level of particle production is apparently not due to a reduction in Gag expression by the transfected cells. These data demonstrate the importance of individual residues within the C-terminal domain of CA and the single residue at the N-terminus of SP1 to Gag processing and viral particle assembly and release.

In contrast to these results, parallel transfections with SP1 point deletion-containing proviral DNA constructs (ΔE^{365},

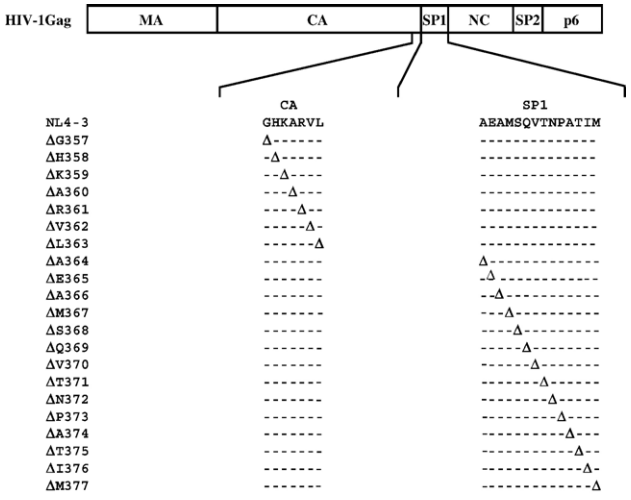

Fig. 1. A schematic representation of the Gag protein and the CA-SP1 boundary sequences of the HIV-1 NL4-3 and point deletion mutants used in this study. The sequences flanking the CA-SP1 cleavage site for HIV-1 NL4-3 are shown at the top of the figure. Numbering the residues of CA-SP1 cleavage domain is based on the standard nomenclature system for the Gag polyprotein of HXB2 virus (Kuiken et al., 2000). A dashed line (-) represents residues that are identical to the parent NL4-3, a delta (Δ) represents CA-SP1 cleavage domain residues that are deleted in the point deletion mutants.

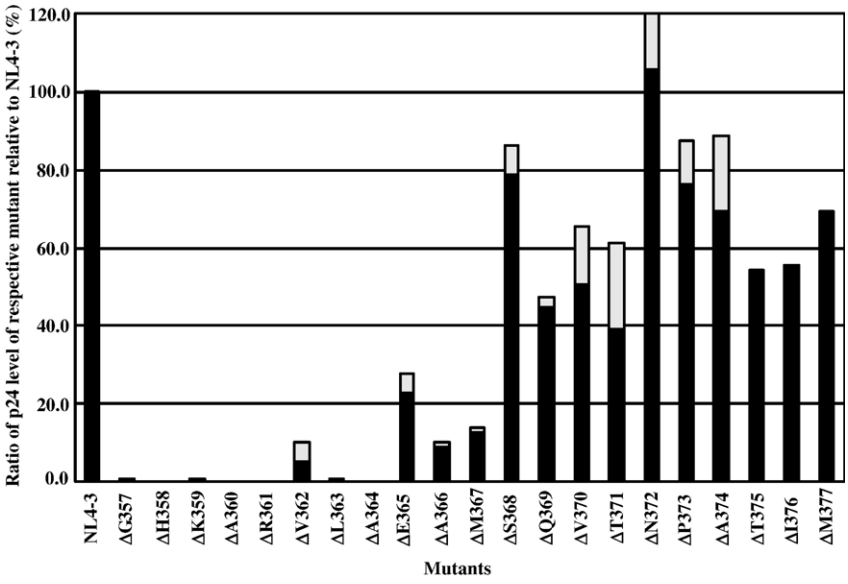

Fig. 2. Analysis of virus production from HeLa cells transfected with the parental NL4-3 and single residue point deletion constructs spanning the CA-SP1 cleavage domain. HeLa cells were transfected with equal amounts of the parental NL4-3 or single residue point deletion mutant proviral DNA. After 48 h, virions were pelleted from the transfected culture supernatant and were analyzed for p24. The level of virus production by NL4-3 was set at 100%. Virus production for each point deletion mutant was normalized by comparison with NL4-3. These data represent an average of at least four independent experiments. Experimental variability indicated by standard deviation bars (gray).

ΔA^{366}, ΔM^{367}, ΔS^{368}, ΔQ^{369}, ΔV^{370}, ΔT^{371}, ΔN^{372}, ΔP^{373}, ΔA^{374}, ΔT^{375}, ΔI^{376} and ΔM^{377}) produced significant amounts of virus relative to the parental NL4-3 (Fig. 2). Virus production by these mutants varied over a range of approximately 1 log and appeared to track as a function of the proximity of the deletion to the CA-SP1 proteolytic cleavage site. For example, SP1 mutants with deletions at residues proximal to the cleavage site, E^{365}, A^{366}, and M^{367}, were most affected, giving <30% of the level of virus observed with the parental NL4-3. This result is similar to those previously reported by Accola et al. (1998) and Liang et al. (2002), and supports their conclusion that the triple residue motif $E^{365}/A^{366}/M^{367}$ within the N-terminus of SP1 is critical to viral particle assembly and budding.

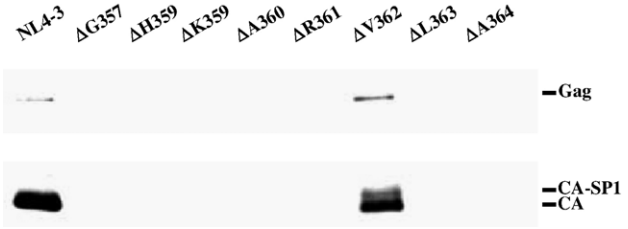

Fig. 3. Effect of point deletions (G^{357}, H^{358}, K^{359}, A^{360}, R^{361}, V^{362}, L^{363}, or A^{364}) on production of extracellular virus. HeLa cells were transfected with equal amounts of pNL4-3 or point deletion mutant proviral DNA as indicated. After 48 h, equal volumes of supernatant from each culture were collected and subjected to ultracentrifugation to pellet virus particles. Virus from each of these point deletion mutants, in parallel with the parental NL4-3, was analyzed by SDS-PAGE and Western blot as described in Materials and methods. Full-length Gag precursor (p55), CA-SP1 intermediate (p25), and mature CA (p24) are indicated by arrows. The data presented in this figure are representative of at least three independent experiments.

A particularly interesting result was that the majority of the SP1 point deletion mutants displayed a normal or near normal Gag processing phenotype with the near normal phenotype characterized by a slight accumulation of Gag processing intermediates such as $P41^{Gag}$ (Fig. 4). It should also be noted that while mutants with deletions at I^{376} or M^{377} (residues flanking the SP1-NC cleavage site) not unexpectedly exhibited an SP1-NC cleavage defect as evidenced by the significant accumulation of Western blot bands corresponding in size to CA-SP1-NC-SP2 and CA-SP1-NC (Fig. 4), CA-SP1 cleavage did not appear to be affected. Our results indicate that deletion of any single residue within SP1, with the exception of the 1st residue (A^{364}), does not appear to disrupt CA-SP1 processing, or significantly affect virus production in the context of the HeLa cell transfections. Due to the effect of point deletions within the C-terminal domain of CA or at the N-terminus of SP1 on virus particle production or Gag processing, virus incorporating these changes could not be used in assays to characterize the determinants of PA-457 activity.

Replication properties of non-defective CA-SP1 point deletion variants

To determine the replication properties of the non-defective CA-SP1 point deletion constructs, equal quantities of the respective proviral DNAs were transfected into Jurkat cells, and virus replication was monitored by regular measurements of extracellular p24 over a 21-day post-transfection period. The data in Fig. 5 show that these point deletion mutants exhibit one of three replication phenotypes; (i) fully competent (akin to the parental NL4-3), (ii) intermediate, and (iii) defective. Fully replication-competent phenotypes included mutants with a

deletion starting at residue V^{370} and extending through T^{375} downstream from the CA-SP1 cleavage site. Mutants containing a deletion at residues S^{368}, Q^{369}, or I^{376} exhibited reduced replication kinetics and were classified as replication-intermediate. In contrast to these first two groups, viruses containing a deletion at any of the CA-SP1 cleavage-site-proximal residues ($E^{365}/A^{366}/M^{367}$), or with a deletion at the C-terminus of the SP1 domain (M^{377}), produced only background levels of extracellular p24, indicating a lack of viral replication during the 21-day observation period.

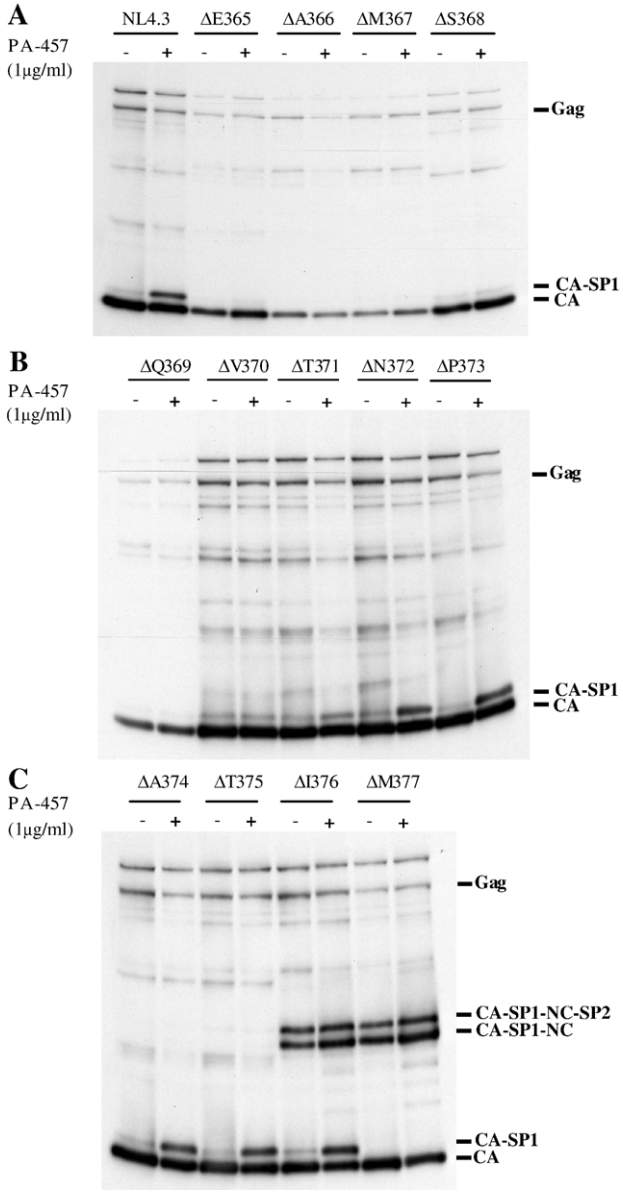

Fig. 4. Western blot analysis of the effect of PA-457 at 1 μg/ml on the conversion of the capsid precursor, CA-SP1, to mature capsid protein (A–C). The virus for Western blotting was obtained using a constant volume of cell culture supernatant. This resulted in some variability in the intensity of the viral protein bands due to differences among the point deletion mutants in the level of virus production. Gag precursor (p55), the Gag intermediates (CA-SP1-NC or CA-SP1-NC-SP1), the CA-SP1 intermediate (p25), and mature CA (p24) are indicated by arrows. The data presented in this figure are representative of at least four independent experiments. Additional experiments for mutant viruses ΔA366 and ΔM367 in which the amount of Gag protein loaded onto the gel was normalized to the NL4-3 control gave identical results to those presented above (data not shown).

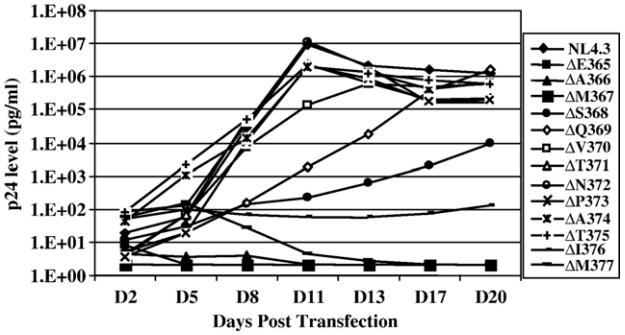

Fig. 5. Replication kinetics of the parental NL4-3 and single residue point deletion mutant viruses in Jurkat cells. Jurkat cells were transfected with equal amounts of the parental pNL4-3 or single residue point deletion mutant proviral DNA and cultured for 3 weeks. Viral replication was monitored regularly by p24 antigen capture ELISA. The data presented in this figure are representative of at least three independent experiments.

We next determined the extent to which the replication phenotypes observed with this panel of point deletion variants correlated with viral particle production. Interestingly, we did not observe a strict correlation between replication competence and virus production. This conclusion assumes that the HeLa cells utilized in the experiment represented in Fig. 2 and the Jurkat cells used to generate data shown in Fig. 5 behave similarly with respect to the replication of these mutant viruses. For example, the ΔS^{368} mutant produced an approximately equal amount of virus as mutants ΔP^{373} and ΔA^{374}; however, ΔS^{368} exhibited an intermediate replication phenotype whereas ΔP^{373} and ΔA^{374} replicated as well as the parental NL4-3. From this, we conclude that the observed variations in viral particle production do not fully explain the replication phenotypes. Rather, these results suggested that viruses produced by the replication-defective mutants (ΔE^{365}, ΔA^{366}, ΔM^{367} and ΔM^{377}) were non-infectious while viral particles derived from the replication-intermediate mutants (ΔS^{368}, ΔQ^{369} and ΔI^{376}) were attenuated and less infectious. Mechanisms by which the introduced changes impact virus infectivity warrant further investigation.

Sensitivity of Gag SP1 point deletion constructs to PA-457

The non-defective Gag SP1 point deletion mutants were characterized for sensitivity to PA-457 in studies that measured the effect of the compound on p25 processing (CA-SP1 cleavage). In these experiments, HeLa cells were transfected in parallel with the parental NL4-3 or SP1 variant proviral DNA in the presence of PA-457 at a concentration of 1 μg/ml or DMSO control. At 48 h post-transfection, virus was harvested from the cell culture supernatant and the Gag processing profile for each virus was analyzed and compared to Gag processing in the absence of compound (DMSO control) (Fig. 4).

As summarized in Fig. 4, these experiments revealed a striking difference in compound sensitivity among the point

deletion mutants. Viruses with a deletion at any position starting with residue E^{365} and extending through V^{370} yielded a PA-457-resistant phenotype as evidenced by a lack of accumulation of the CA-SP1 precursor (p25) in the presence of compound. These data indicate that the N-terminal residues of SP1 are required for PA-457 activity. The observation of a critical role for residue A^{366} in the compound's antiviral activity is consistent with previous resistance selection experiments that identified both A^{364} and A^{366} as determinants of PA-457 activity (Li et al., 2003; unpublished results). In contrast to these PA-457-resistant variants, viruses with a deletion at any position starting with residue T^{371} through I^{376} were sensitive to PA-457 treatment, as evidenced by the significant accumulation of the CA precursor, p25. These results demonstrate that the majority of residues within the C-terminus of Gag SP1 do not play a direct role in the activity of PA-457. Compared to the entire panel of PA-457-sensitive mutants, ΔT^{371} was unique in that it exhibited intermediate sensitivity to compound treatment. We speculate that this intermediate PA-457-sensitive phenotype, identifies the T^{371} residue as transition point within SP1 from the region critical to PA-457 activity to the region not directly involved in the activity of the compound.

As noted previously, deletion of either the I^{376} or M^{377} residue resulted in a clear Gag processing defect in the absence of PA-457. We speculate that this effect on processing results from the critical position of these two residues in flanking the SP1-NC cleavage site. Interestingly, ΔI^{376} was sensitive to PA-457 treatment while ΔM^{377} was not.

The Gag processing experiments specifically measure the effect of PA-457 on CA-SP1 cleavage and the ability of the compound to inhibit the release of mature CA protein. To further characterize the effect of PA-457 on virus replication in the panel of SP1 point deletion mutants, we employed the U87 CD4/CXCR4 cell-based replication assay. As summarized in Table 1, the results of this assay correlate well with those obtained in the Gag processing experiment. For example, parental level PA-457 inhibitory activity was consistently observed in the panel of mutants including ΔT^{371}, ΔN^{372}, ΔP^{373}, ΔA^{374}, and ΔT^{375}, which exhibited PA-457 sensitivity in the Gag processing experiment. Additionally, three point deletion mutants (ΔS^{368}, ΔQ^{369}, and ΔV^{370}) identified as PA-457-resistant in the Gag processing experiment lacked compound sensitivity in the virus replication assay. Together, these results demonstrate a strong correlation between the cell-based replication assay and the Gag processing experiment in characterizing PA-457 activity against HIV-1. These data further support observations regarding the critical role of the N-terminal half of SP1 in PA-457 activity.

Table 1
Summary of the effect of point deletions on PA-457 activity

Virus	Gag processing assay	Cell-based replication assay
NL4-3	+[a]	+
ΔE365	−[b]	NA[c]
ΔA366	−	NA
ΔM367	−	NA
ΔS368	−	−
ΔQ369	−	−
ΔV370	−	−
ΔT371	+	+
ΔN372	+	+
ΔP373	+	+
ΔA374	+	+
ΔT375	+	+
ΔI376	+	NA
ΔM377	−	NA

[a] Full PA-457 activity as determined in Gag processing experiment or in cell-based replication assay.

[b] A lack of PA-457 activity as determined in Gag processing experiment or in cell-based replication assay.

[c] Data could not be generated due to replication defects of these mutants in U87 CD4 CXCR4 cells.

Discussion

PA-457 is a potent HIV drug candidate that acts through a novel mechanism of action to block Gag processing and inhibit virus replication (Li et al., 2003; Zhou et al., 2004b). The primary goal of the work described in this report was to extend our understanding of the viral determinants of PA-457 activity. We chose the CA-SP1 cleavage domain as our main focus because: (i) PA-457 inhibits virus replication by blocking Gag cleavage at this site and (ii) several residues proximal to the CA-SP1 cleavage site have previously been shown to play significant roles in mediating resistance to PA-457(Li et al., 2003; Zhou et al., 2004a, 2004b).

Our experiments generated a number of new observations with respect to the viral determinants of PA-457 activity. Significantly, the determinants of compound activity mapped to a relatively large region of the Gag SP1 domain with each residue within the N-terminal half of SP1 being required for PA-457 activity (Fig. 4 and Table 1). Not unexpectedly, the entire C-terminal half of the SP1 domain, with the exception of residue M^{377}, was not implicated in PA-457 activity (Fig. 4 and Table 1). Surprisingly, the M^{377} residue, furthest removed from the CA-SP1 cleavage site, in some as yet to be determined fashion, affects PA-457 activity. We believe that there are several possible explanations for this latter observation. One possibility involves the effect of the M^{377} deletion on the rate of CA-SP1 cleavage. It has been shown that the rate of CA-SP1 processing is regulated by cleavage at the SP1-NC site with a block to processing at that site resulting in a 20-fold increase in the rate of CA-SP1 cleavage (Pettit et al., 1994). It has also been demonstrated that an M to I substitution at P1 of the SP1-NC cleavage site significantly reduces the level of processing at that site (Pettit et al., 2002). We speculate that the increase in the rate of CA-SP1 processing resulting from the M^{377} deletion (equivalent to an M to I substitution at P1) could affect the ability of PA-457 to disrupt this cleavage event resulting in the PA-457-resistant phenotype. Consistent with this hypothesis, Sakalian et al. (2006) recently described one example of a mutation-induced increase in the rate of CA-SP1 processing that seemed to correlate with a reduction in PA-457 activity. An alternative explanation involves the direct interaction of the M^{377} residue with PA-457 and the effect of the deletion of this residue on PA-457 binding and activity. However, as reported

by our group and others (Li et al., 2003; Sakalian et al., 2006; Zhou et al., 2004a, 2004b), viral determinants of PA-457 activity, mapped by several different approaches, cluster to the region flanking the CA-SP1 cleavage site. To date, distal site effects on compound activity have not been reported. Additionally, changing the M^{377} residue to either A or L resulted in a PA-457-sensitive phenotype (unpublished results); strongly suggesting that M^{377} is not directly involved in the interaction with PA-457. A final possible explanation involves the contribution of the M^{377} residue to the formation of necessary PA-457 target structures. We speculate that the deletion of the M^{377} residue could disrupt PA-457 target formation resulting in the observed drug-resistant phenotype.

A lack of functional mutant viruses impeded our efforts to better understand the role of the C-terminal residues of CA in PA-457 sensitivity. However, based on our observations in characterizing the role of the SP1 residues, we would predict that a relatively large number of C-terminal CA residues proximal to the CA-SP1 cleavage site are involved in compound activity. Results from our efforts to select for and characterize PA-457-resistant isolates support this hypothesis. Our most recent *in vitro* drug-resistance selection studies have identified a number of C-terminal CA residues that confer some level of PA-457 resistance. These include the last residue of capsid, CA^{L231} as well as the sixth capsid residue upstream from the CA-SP1 cleavage site, CA^{H226} (unpublished results). We believe that it is likely that these residues play a critical role in mediating the effect of the compound on CA-SP1 processing. To obtain additional information, several other approaches, including the preparation of SIV/HIV Gag CA-SP1 chimeras and an alternative mutagenesis approach involving site-specific substitutions targeting the C-terminus of CA are being pursued in our laboratories.

It should be noted that the Gag residues identified in this study as viral determinants of PA-457 activity overlap a region of the protein predicted to exhibit α-helical secondary structure (Accola et al., 1998; Morellet et al., 2005). It has been proposed that this putative helical structure plays an important role in HIV-1 assembly and budding based on the observation that the introduction of structure-disrupting mutations into this region of Gag results in a defect in virus particle production (Accola et al., 1998; Liang et al., 2002). While the determinants of PA-457 activity map to this putative structural domain, we and others have shown that the compound exhibits no discernible effect on virus assembly and budding (Li et al., 2003; Zhou et al., 2004b). A possible explanation for this observation involves a mechanism by which PA-457 binds to the CA-SP1 region of Gag and disrupts p25 processing without interfering with the function of the putative structural elements. A more complete understanding of the structure and function of this region of Gag in HIV-1 morphogenesis and the mechanism of PA-457 action will be required before more definitive conclusions can be drawn.

The model by which PA-457 exerts its antiviral activity has been proposed in earlier reports in which we speculated that PA-457 bound directly to the CA-SP1 cleavage site in the context of a higher-order Gag structure and disrupted p25 processing by blocking PR access to this site. The results reported here are consistent with this model, and extend our characterization of the PA-457 target to include portions of the CA-SP1 cleavage domain other than the residues immediately flanking the p25 cleavage site. Although our results do not completely rule out other potential PA-457 targets, such as an unidentified cellular factor necessary for Gag processing, we believe the proposed model is most consistent with the previous results reported by our group and others (Li et al., 2003; Sakalian et al., 2006; Zhou et al., 2004a, 2004b, 2005). Ongoing studies to identify and characterize a direct binding site for PA-457 should allow for a more definitive identification of the molecular target for this novel compound and provide a more complete evaluation of our proposed model for PA-457 action.

Materials and methods

Construction of HIV-1 NL4-3 point deletion mutants

To map the genetic determinants of PA-457 activity, we generated a panel of single residue point deletion mutants spanning the CA-SP1 cleavage domain in the context of the HIV-1 NL4-3 molecular clone (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID). The CA-SP1 sequences for these constructs are shown in Fig. 1 and the numbering of CA-SP1 residues is based on the standard nomenclature system for the Gag polyprotein of the HXB2 virus (Kuiken et al., 2000). DNA mutagenesis was carried out using the PCR–ligation–PCR strategy and other standard molecular cloning techniques as previously described (Ho et al., 1989; Sambrook et al., 1989). Each clone was analyzed to confirm the sequence of the Gag CA-SP1 region.

Cell culture, DNA transfection and infection

HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml Streptomycin, and cells were passaged upon confluence. Jurkat E6-1 cells (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) were cultured in RPMI 1640 medium (Meditech) supplemented with 10% FBS, and 50 μg/ml of gentamicin (Sigma). Jurkat cells were passaged every 2–3 days to maintain a concentration of 10^5–10^6 cells/ml. All plasmid DNAs were prepared using the Qiagen midiprep kit.

HeLa and Jurkat cells were transiently transfected with equal amounts of parental or mutant NL4-3 proviral DNAs using the FuGENE 6 transfection reagent (Roche). HeLa cells were employed to address the effect of the point deletions on virus production and Gag processing. At 48 h post-transfection, both cells and culture supernatant were harvested for analysis (see SDS-PAGE and Western blot sections below). p24 antigen capture ELISA (ZeptoMetrix) was used to measure the level of virus production from HeLa cell transfection. The effect of point deletions on virus replication kinetics was characterized in Jurkat cells where virus replication was monitored by regular measurements of extracellular p24 protein over a 21-day

observation period post-transfection, as described previously (Demirov et al., 2002b).

SDS-PAGE and Western blot

Western blotting was used to characterize the effect of the point deletions on virus production and Gag polyprotein processing. Briefly, at 48 h post-transfection, culture medium containing viral particles was collected and clarified by centrifugation at 4 °C for 20 min at 2000 rpm using a Sorvall RT 6000B centrifuge. Virus-containing supernatants were then concentrated through a 20% sucrose cushion in a microcentrifuge at 4 °C for 120 min at 13,000 rpm and pellets were resuspended in lysis buffer (150 mM Tris–HCl, 5% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), pH 8.0). For analysis of cell lysates, at 48 h post-transfection, cells were washed once with PBS, lysed, and then centrifuged at 4 °C for 5 min at 13,000 rpm to remove nuclear fractions. Viral proteins were separated on a 12% NuPAGE Bis-Tris Gel (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen) followed by blocking in a PBS buffer containing 0.5% Tween and 5% dry milk powder. The membrane was incubated with pooled immunoglobulin from HIV-1-infected patients (HIV-Ig) (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) and hybridized with goat anti-human horseradish peroxidase (Sigma). The immune complex was visualized using an ECL system (Amersham Pharmacia Biotech) according to the instructions provided by the manufacturer.

Effect of PA-457 on Gag processing in point deletion mutants

To address the effect of the point deletions on the ability of PA-457 to inhibit CA-SP1 processing, HeLa cells were transfected with parental HIV-1 pNL4-3 and various point deletion mutant proviral DNAs as described above. PA-457 at a concentration of 1 μg/ml or DMSO (no drug control) was maintained throughout the entire period of the culture and SDS-PAGE/Western blot for analyzing viral proteins derived from these cultures was performed as described above.

In vitro cell-based antiviral activity assay

PA-457 was tested for *in vitro* activity against the point deletion mutations using U87.CD4/CXCR4 cells as targets. Cells were plated in a 96-well flat-bottom plate at a 3×10^3 cells/well one-day prior to use. PA-457 was tested in triplicate at a starting concentration of 0.1 μg/ml, followed by three 10-fold serial dilutions. Next, virus stocks generated by HeLa cell transfections were added to each well. Virus inocula ranged from 25 to 250 $TCID_{50}$/ml. Cultures were maintained on days 1, 3, and 6 post-infection. Maintenance consisted of removing spent medium from each well and adding an equal volume of fresh medium. On days 6 and 8 post-infection, culture supernatant was collected for p24 ELISA analysis according to the manufacture's instructions (ZeptoMetrix). Linear regression was used to determine EC_{50} values.

Acknowledgments

This work was supported, in part, by Public Health Service Grant 5 R44 AI051047-03 to G.P.A. from the National Institutes of Health, National Institute for Allergy and Infectious Disease.

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