Poster 059

5th International Workshop on HIV Drug Resistance and Treatment Strategies, June 4-8, 2001

Association of the Nucleocapsid-p1 (NC-p1) *Gag* Cleavage Site Alanine to Valine Mutation with Mutational Patterns in HIV Protease Producing Protease Inhibitor Resistance

D Kempf, M King, S Brun, J Sylte, T Marsh, K Stewart, E Sun; Abbott Laboratories, Abbott Park, IL USA

ABSTRACT

Background: Mutations in the *gag* cleavage sites for HIV protease (Pr) have been reported to occur along with mutations in the enzyme during the development of resistance to protease inhibitors (PIs). In this study, the incidence of these mutations and their association with specific mutations in HIV Pr in isolates from patients failing PI therapy has been investigated.

Methods: Genotype was determined by population sequencing. Sequence data were available for two *gag* cleavage sites (NC-p1 and p1-p6). Susceptibility to PIs was determined using single cycle recombinant assays.

Results: The Pr and *gag* cleavage site sequences from 117 patients were evaluated. The number of Pr mutations associated with PI resistance ranged from 0 to 10. The A431V *gag* mutation (the S2-subsite of the NC-p1 cleavage site) was found in 35/117 sequences, and was statistically significantly associated ($p<0.01$) with the presence of Pr mutations at positions 10, 24, 46, 54 and 82. All but two (33/35) isolates with the A431V *gag* mutation had a primary Pr mutation at position 82, 84 and/or 90. Within the 36 patients whose isolates contained 3 or more Pr mutations at positions 10, 24, 46, 54 or 82, median viral load was higher in those without the A431V *gag* mutation (4.82 log copies/mL, n=16) than in those with the A431V *gag* mutation (3.93 log copies/mL, n=20, $p=0.027$). Virologic response in these PI-experienced patients to subsequent treatment with lopinavir/ritonavir plus NNRTI/NRTI therapy was not associated with the presence of the A431V *gag* mutation. No significant association was observed between mutations at the p1-p6 cleavage site and Pr mutations.

Conclusions: In 117 isolates from PI-experienced patients, the A431V *gag* cleavage site mutation was associated with the presence of mutations in HIV protease. However, in this study, the presence of the cleavage site mutation did not appear to significantly impact response to subsequent PI therapy.

INTRODUCTION

Cleavage of up to 12 different sequences in the *gag* and *gag-pol* polyproteins of HIV by HIV protease is essential for proper viral assembly and the production of new, infectious virions. Several studies have noted the mutation of amino acids within the HIV protease substrate sequences that occur coincident with mutations in the protease homodimeric protein upon drug selection *in vitro* (Doyon, 1996; Carrillo, 1998) and *in vivo* (Zhang, 1997; Cote, 2001; Catamancio, 2001). In a recent study, mutations at three *gag* cleavage sites (p2-NC, NC-p1, and p1-p7) were found more commonly in patients treated with protease inhibitor (PI) therapy than in matched controls (Cote, 2001). *In vitro*, the presence of a mutation at the NC-p1 and p1-p7 cleavage sites has been shown to partially restore the replicative capacity of viral clones containing multiple mutations in protease (Doyon, 1996; Mammano, 1998; Carrillo, 1998).

The predominant mutation in the NC-p1 cleavage site sequence is the P2-alanine to valine mutation (A431V, Figure 1). In the p1-p7 cleavage site sequence, mutation of the P1'-leucine is most frequently observed (L449F/P/V). The NC-p1 (A431V) mutation, in analyses of relatively small numbers of isolates, has been linked to the presence of the M46I/L mutation in protease. However, the correlation of other protease mutations with *gag* cleavage site mutations has not been reported. In this study, we examined the association of mutations at the NC-p1 and p1-p6 cleavage sites with mutations in protease in 117 viral isolates from patients failing either single or multiple protease inhibitor therapy.

Figure 1. NC-p1 and p1-p6 *Gag* Cleavage Sites

		P4	P3	P2	P1	P1'	P2'	P3'	P4'
NC-p1	Wild type	– E –	Q –	A	– N	– F	– L	– G	– K –
	A431V Mutant	– E –	Q –	V	– N	– F	– L	– G	– K –
p1-p6	Wild type	– P –	G –	N –	F	– L	– Q –	S –	R –
	L449P Mutant	– P –	G –	N –	F	– P	– Q –	S –	R –

Cleavage Site

METHODS

- Plasma samples were taken from 117 single and multiple PI-experienced patients entering one of two lopinavir/ritonavir (lopinavir/r) phase I/II clinical trials.
- Genotype was determined by population sequencing at either Virco or ViroLogic, and was available for the entire protease gene and two *gag* cleavage sites (NC-p1 and p1-p6).
- Other baseline covariates were available from the clinical database for each clinical study. HIV RNA (400 copies/mL cutoff) was measured using Roche Amplicor. HIV RNA (50 copies/mL cutoff) was measured using either the experimental Abbott LCx method (single PI-experienced patients) or the Roche Ultrasensitive method (multiple PI-experienced patients).
- The association of the presence of the A431V NC-p1 cleavage site mutation with phenotypic susceptibility to protease inhibitors was analyzed using the Wilcoxon rank-sum test. The association of specific mutations in protease with the A431V mutation was analyzed using Fisher's exact test. Multivariate analyses of the association of protease mutations with the A431V mutation were performed using logistic regression with both forward selection and backward elimination variable selection methods.

RESULTS

Characterization of Viral Isolates

- Samples were available from 61 single and 56 multiple PI-experienced patients (Table 1). PI experience was limited primarily to indinavir (IDV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV).

Table 1. Treatment History

	Single PI-Experienced	Multiple PI-Experienced
Number of isolates	61	56
Median plasma HIV RNA (log copies/mL)	4.10	4.52
Median number of prior NRTIs	2	4
Median number of prior PIs	1	3
Identity of prior PIs*		
• Indinavir	43%	86%
• Nelfinavir	36%	57%
• Ritonavir	5%	77%
• Saquinavir	15%	71%

* For multiple PI-experienced patients, prior PIs were tallied irrespective of dose and simultaneous/sequential PI treatment.

- With the exception of mutations at positions 30 and 88, the prevalence of the more commonly observed mutations in protease (positions 10, 20, 24, 36, 46, 53, 54, 63, 71, 73, 77, 82, 84 and 90) was a median of 104% higher in the isolates from multiple PI-experienced patients than from single PI-experienced patients (Figure 2).
- The prevalence of the NC-p1 *gag* mutation was 29% higher in the isolates from multiple PI-experienced patients (Table 2). There was no difference in the prevalence of the p1-p7 *gag* mutation.
- In single PI-experienced patients, the NC-p1 *gag* mutation appeared in a higher proportion of isolates from patients failing IDV (11/26) or RTV (2/3) therapy than isolates failing NFV (2/22) or SQV (1/9) therapy. The p1-p6 mutation appeared approximately equally among isolates from patients failing IDV (4/26), NFV (4/22) and SQV (2/9).

Figure 2. Prevalence of Protease, NC-p1 *Gag* and p1-p6 *Gag* Mutations

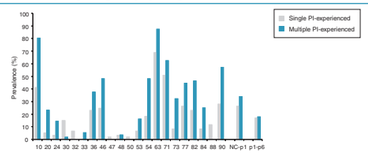


Table 2. Prevalence of *Gag* Mutations

	Single PI-Experienced	Multiple PI-Experienced
Number of isolates	61	56
Number of isolates with <4-fold reduced susceptibility to at least one prior PI*	38 (68%)	44 (79%)
Median number of PI mutations†	3	7
Number of isolates with A431V NC-p1 cleavage site mutation	16 (26%)	19 (34%)
Number of isolates with L449P/V/F p1-p6 cleavage site mutation	10 (16%)	10 (18%)

* Phenotypic characterization available for only 56/61 isolates from single PI-experienced patients.
† PI mutations defined as the following: L10F, I, R or V; K20M or R; L24I; D30N; V32I; L33F; M36I; M46I or L; I47V; G48V; I50V; F53L; I54L, T or V; L63P; A71I, L, V or T; G73A, S or T; V77I; V82A, F, S or T; I84V; N88D and L90M.

Correlation of *Gag* Cleavage Site Mutations with Reduced Susceptibility to Protease Inhibitors

- Isolates containing the NC-p1 cleavage site mutation displayed a significantly greater degree of reduced susceptibility to the PI class (Table 3). In contrast, the median fold susceptibility of viruses either containing or lacking the p1-p6 cleavage site mutation was not different.

Table 3. Correlation of *Gag* Mutations with Susceptibility to Protease Inhibitors

	Fold Change in IC_{50}					
	LPV	IDV	NFV	RTV	SQV	APV*
Median with NC-p1 mutation (n=35)	7.4	11.7	16.2	20.9	2.6	3.3
Median without NC-p1 mutation (n=82)	1.2	2.1	7.8	2.5	1.5	1.8
p-value	<0.001	<0.001	0.012	<0.001	0.111	0.128
Median with p1-p6 mutation (n=20)	1.8	4.1	10.8	5.7	1.8	2.2
Median without p1-p6 mutation (n=97)	2.1	6.1	9.5	5.6	1.6	2.2

* Phenotype for APV available for only 59 isolates.

Correlation of *Gag* Cleavage Site Mutations with Mutations in the HIV Protease Gene

- In univariate analyses, five mutations in protease (amino acid positions 10, 24, 46, 54 and 82) were statistically significantly associated ($p<0.01$) with the presence of the NC-p1 A431V mutation (Table 4).
- In contrast, there was no apparent association of any specific protease mutation with the p1-p6 L449F/P/V mutation (data not shown). Nor was there any apparent association between the p1-p6 *gag* mutation and the number of mutations in protease (median number of PI mutations was 5 for the 20 isolates displaying the p1-p6 mutation and for the 97 isolates lacking the mutation).

Table 4. Correlation of *Gag* Mutations with Mutations in Protease

Amino Acid Position	With NC-p1 Mutation (n=35)	Without NC-p1 Mutation (n=82)	p-value
10	28 (80%)	42 (51%)	0.003
20	6 (17%)	10 (12%)	0.559
24	8 (23%)	2 (2%)	<0.001
30	0 (0%)	10 (12%)	0.032
32	1 (3%)	3 (4%)	>0.999
33	0 (0%)	3 (4%)	0.553
36	9 (26%)	26 (32%)	0.660
46	23 (66%)	19 (23%)	<0.001
47	0 (0%)	1 (1%)	>0.999
48	0 (0%)	4 (5%)	0.319
50	0 (0%)	1 (1%)	>0.999
53	3 (9%)	10 (12%)	0.752
54	19 (54%)	19 (23%)	0.002
63	28 (80%)	63 (77%)	0.811
71	20 (57%)	46 (56%)	>0.999
73	10 (29%)	13 (16%)	0.131
77	14 (40%)	27 (33%)	0.528
82	22 (63%)	18 (22%)	<0.001
84	8 (23%)	11 (13%)	0.269
88	0 (0%)	7 (9%)	0.101
90	18 (51%)	31 (38%)	0.220

- Only 2/117 isolates contained the NC-p1 *gag* A431V without a protease mutation at position 46 or 54. All but three of the 35 isolates with the NC-p1 mutation contained a mutation at either position 82 or 90 (two of the remaining three contained mutations at positions 24 and 46).
- In forward selection and backward elimination logistic regression analyses that considered only the 5 mutations associated in univariate analyses, mutations at positions 46, 82 and 24 in protease were independently associated with the presence of the NC-p1 mutation (Model 1, Table 5).
- In separate multivariate analyses that considered all mutations, the following 4 mutations were independently associated with the NC-p1 mutation: 46, 82, 24 and 90 (Model 2, Table 5).

Table 5. Multivariate Analyses Evaluating the Association Between Protease Mutations and the NC-p1 *Gag* Cleavage Site Mutation

	Model 1		Model 2	
Amino Acid Position	Odds Ratio	p-value*	Odds Ratio	p-value*
24	6.02	0.039	11.47	0.009
46	11.62	<0.001	5.46	<0.001
82	7.62	0.006	3.85	0.008
90	n/a	n/a	3.17	0.031

* Final models were identical for the forward selection and backward elimination logistic regression analyses.

- Three distinct patterns of protease mutations were identified among the isolates containing the NC-p1 mutation: 46/90/10, 54/82/10, and 46/82. The majority of isolates (29/35, 83%) fell into one or more of these patterns (Table 6).

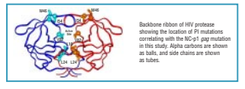
Table 6. Patterns of Protease Mutations in Isolates Containing the NC-p1 *Gag* Mutation

Mutation Pattern	Number of Isolates
46/90/10	7/35 (20%)
54/82/10/46	7/35 (20%)
54/82/10/(no 46)	9/35 (26%)
46/82	6/35 (17%)
Other	6/35 (17%)

Structural Relationship Between the NC-p1 *Gag* Mutation and Protease Mutations at Position 46 and 54

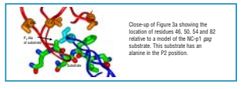
- Of the 5 mutations associated with the NC-p1 *gag* mutation, only residues 46 and 54 lie in relative proximity to the P2 subsite of the HIV protease active site, which binds the A431V mutant side chain (Figures 3a and 3b). Residue 82 comprises part of the P1 and P3 subsites, and is distal from the P2 subsite.
- The terminal methyl group of the Ile-54 side chain that is lost upon mutation to I54V is in close van der Waals contact with the Ile-50 side chain (Figures 4a and 4b). The Ile-50 side chain in turn forms part of the cavity that defines the protease P2 subsite (and thus directly contacts the inhibitor). Crystal structures of inhibitor complexes with multiply-mutant HIV protease containing the I54V mutation (Munshi, 2000) demonstrate a significant shift in the Ile-50 side chain, compared to wild-type, that enlarges the volume of the P2 subsite. The NC-p1 substrate sequence contains an alanine at the S2 position, which is smaller than the residue at that site in any other documented HIV protease substrate. In the NC-p1 mutant, the Ala-431 side chain becomes larger by two methyl groups by substitution of valine.
- In one plausible mechanistic scenario, the increase in the volume of the P2-subsite due to the I54V mutation lowers the efficiency for cleavage of the wild-type NC-p1 substrate. The NC-p1 substrate compensates for reduced enzyme kinetics (and lower viral fitness) by mutating the S2 alanine to valine (Figures 5 and 6) thereby creating a larger side chain to fill the void in the P2 subsite.
- There is no evidence from crystal structures for an influence of the M46I/L mutation on the size of the P2 subsite. However, the M46I/L mutation (as well as the I54V mutation) is likely to affect flap dynamics (and thus alter fitness) during substrate cleavage (see Poster 069).

Figure 3a.



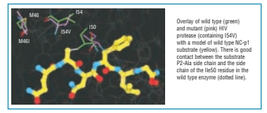
Backbone ribbon of HIV protease showing the location of PI mutations correlating with the NC-p1 *gag* mutation in this study. Alpha carbons are shown as balls, and side chains are shown as tubes.

Figure 3b.



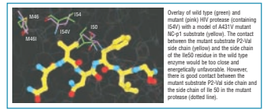
Close-up of Figure 3a showing the location of residues 46, 50, 54 and 82 relative to a model of the NC-p1 *gag* substrate. This substrate has an alanine in the P2 position.

Figure 4a.



Overlay of wild type (green) and mutant (pink) HIV protease (containing I54V) with a model of A431V mutant NC-p1 substrate (yellow). There is good contact between the substrate P2-Val side chain and the side chain of the Ile50 residue in the wild type enzyme (dotted line).

Figure 4b.



Overlay of wild type (green) and mutant (pink) HIV protease (containing I54V) with a model of A431V mutant NC-p1 substrate (yellow). The contact between the mutant substrate P2-Val side chain and the side chain of Ile 50 in the mutant protease (dotted line).

Exploration of the Effect of the NC-p1 *Gag* Mutation in Patients with Multiple PI Mutations

- To further explore possible effects of the NC-p1 *gag* mutation, the subset of 36 isolates with at least three of the five mutations in protease associated with the *gag* mutation in univariate analyses (positions 10, 24, 46, 54 and 82) were examined. The majority (26/36) of these isolates were taken from multiple PI-experienced patients, and the NC-p1 A431V *gag* mutation was present in 20/36 isolates.
- The median number of PI mutations was not different between the isolates either containing or lacking the NC-p1 *gag* mutation (7 vs. 7.5, respectively). The median phenotypic susceptibility of the isolates lacking the NC-p1 mutation to the five PIs tested (LPV, IDV, NFV, RTV and SQV) was slightly lower (higher fold IC_{50} compared to wt) than the median susceptibility of the isolates containing the NC-p1 mutation, although the differences were not statistically significant (Table 7).
- The median viral load of the 20 isolates containing the NC-p1 mutation was statistically significantly lower than the median viral load of the 16 isolates lacking the NC-p1 mutation. ($p<0.03$)

Table 7. Characteristics of Isolates Containing Three or More Protease Mutations at Positions Associated with the NC-p1 *Gag* Mutation

	Isolates with NC-p1 mutation (n=20)	Isolates without NC-p1 mutation (n=16)	p-value*
Median number of PI mutations	7	7.5	ns
Median fold IC_{50}			
Lopinavir	15.3	18	ns
Indinavir	11.8	21	ns
Nelfinavir	19	31	ns
Ritonavir	47	76.5	ns
Saquinavir	2.9	6.5	ns
Median viral load (log copies/mL)	3.93	4.82	0.027

* ns: not significant

Response of Patients with *Gag* Mutations to Further PI Therapy

- Each of the patients initiated therapy with lopinavir/r, either nevirapine (single PI-experienced) or efavirenz (multiple PI-experienced), and NRTIs.
- Virologic response (Week 48, HIV RNA <400 copies/mL, dropouts as censored) was somewhat lower in those subjects whose baseline isolates contained (20/30, 67%) vs. lacked (60/76, 79%) the NC-p1 *gag* mutation. This small difference was also observed at the 50 copies/mL level and remained when adjusted for the number of PI mutations at baseline, expressed as the baseline lopinavir mutation score (Kempf, 2000). However, the differences were not statistically significant (Table 8).
- There was no apparent difference in virologic response between those patients whose isolates contained (17/19, 89%) or lacked (77/91, 85%) the L449P/F/V p1-p6 mutation.

Table 8. Virologic Response with Respect to Presence of the NC-p1 *Gag* Mutation at Baseline

	No. with <400 copies/mL		No. with <50 copies/mL	
NC-p1 Mutation at Baseline:	Present	Absent	Present	Absent
LPV Mutation Score ≤5	13/17 (76%)	48/58 (83%)	10/17 (59%)	41/56 (73%)
LPV Mutation Score ≥6	7/13 (54%)	12/18 (67%)	7/13 (54%)	12/18 (67%)

CONCLUSIONS

- In isolates from single and multiple PI-experienced patients, the NC-p1 *gag* cleavage site mutation (P2 alanine to valine, A431V) was associated with mutations in protease at positions 10, 24, 46, 54, 82 and 90.
- The majority of isolates with the NC-p1 *gag* mutation display one or more of the following three distinct patterns of protease mutations (positions 46/90/10, 54/82/10 and 46/82).
- Of the protease mutations associated with the NC-p1 *gag* mutation, residues I54 and M46 are most likely to be mechanistically linked, based on three-dimensional structural considerations.
- Patients whose isolates contain three or more of the above 5 mutations, but lack the NC-p1 mutation, had a statistically significantly higher viral load than those with the protease mutations and the NC-p1 mutation, suggesting a role of the NC-p1 in viral fitness *in vivo*. Since the NC-p1 mutation increases replicative capacity *in vitro*, the lower viral load in the presence of the *gag* mutation suggests that the restoration of fitness *in vivo* is incomplete.
- Virologic response to subsequent therapy with LPV/r plus NNRTI/NRTIs was somewhat lower in the presence of the NC-p1 mutation. However, the difference was not statistically significant. The presence of the p1-p6 did not impact the success of subsequent therapy.

REFERENCES

Carrillo A. et al., *J. Virology* **72**, 7532-7541 (1998).
Catamancio S. L. et al., *J. Clin. Microbiology* **39**, 1124-1129 (1124).
Cote H. C. F. et al., *J. Virology* **75**, 589-594 (2001).
Doyon L. et al., *J. Virology* **70**, 3763-3769 (1996).
Larder B. A. et al., 2nd International Workshop on HIV Drug Resistance and Treatment Strategies, Lake Maggiore, Italy (1998), Abstract 23.
Kempf D. et al., 4th International Workshop on HIV Drug Resistance & Treatment Strategies, Sitges, Spain (2000), Abstract 38.
Mammano F. et al., *J. Virology* **72**, 7632-7637 (1998).
Munshi S. et al., *Acta Crystallogr., Sect. D* **56**, 381 (2000).
Zhang Y. M. et al., *Journal of Virology* **71**, 6662-6670 (1997).