Predictive values of the human immunodeficiency virus phenotype and genotype and of amprenavir and lopinavir inhibitory quotients in heavily pretreated patients on a ritonavir-boosted dual-protease-inhibitor regimen.

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Revised version 2

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25 pages, 2 figures, 2 tables

Running title: amprenavir and lopinavir/ritonavir inhibitory quotient

This study was presented in part at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy; September 14-17, 2003; Chicago. Abstract H-1998

Abstract

The inhibitory quotient (IQ) of human immunodeficiency virus (HIV) protease inhibitors (PI) that is the ratio of drug concentration to viral susceptibility is considered to be predictive of the virological response. We used several approaches to calculate the IQs of amprenavir and lopinavir in a subset of heavily pretreated patients participating in the ANRS-104 trial, then compared their potential for predicting changes in the plasma HIV-RNA level. Thirty-seven patients were randomly assigned to receive either amprenavir (600mg BID) or lopinavir (400mg BID) plus ritonavir (100 or 200mg BID) for two weeks, before combining the two PIs. The IC_{90} was measured using a recombinant assay with or without additional human serum ($IC_{90+serum}$). Total and unbound PIs concentrations in plasma were measured. Univariate linear regression was used to estimate the relation between the change in viral load and the IC_{90} or IQ values. The amprenavir phenotypic IQ values were very similar when measured with the standard and protein binding-adjusted IC_{90}. No relationship was found between the viral load decline and the lopinavir IQ. During combination therapy the amprenavir and lopinavir genotypic IQ values were predictive of the viral response at week 6 ($p=0.03$). The number of protease mutations ($<$ or ≥ 5) was related to the virological response throughout the study. These findings suggest that the combined genotypic IQ and the number of protease mutations are the best predictors of virological response. High amprenavir and lopinavir concentrations in these patients might explain why plasma concentrations and the phenotypic IQ have poor predictive value.

Introduction

The virological efficacy of protease inhibitors (PI) in patients infected by the Human Immunodeficiency Virus (HIV) is dependent on both their pharmacodynamic and pharmacokinetic properties. *In vitro* high and sustained plasma (or cell) concentrations are needed to maximally suppress viral replication. The dose administered in HIV-infected patients should provide such concentrations.

The inhibitory quotient (IQ), defined as the ratio of the trough drug concentration to viral susceptibility expressed as an inhibitory concentration (ideally the IC_{90}), has been used to estimate the antiviral potency of PIs *in vivo* (24). This parameter has also been proposed to optimize the dosing regimen of treatment-experienced patients (12, 20). Although there is a strong theoretical rationale for using the inhibitory quotient, the practical value of this parameter is controversial. Firstly, there are few prospective studies of the relationship between IQ and virological response (22). Secondly, there is no consensus method for calculating this parameter (1, 24, 27). Standard calculations estimate IQ from both the plasma drug concentration and virus susceptibility. However, several pharmacokinetic and virologic issues remain unsolved. In most pharmacokinetic studies the total drug concentration in plasma is measured, whereas the active component is the free (protein-unbound) fraction. Furthermore, the addition of human albumin to the cell culture medium increases the IC_{90} in vitro. In summary, either the total concentration or the protein-adjusted concentration, and either the standard or the serum-adjusted IC_{90}, can be used to calculate the IQ. More recently, a "genotypic inhibitory quotient" was proposed, as the ratio of the plasma concentration to the number of mutations on the viral protease gene. Indeed, genotypic resistance assays can be performed rapidly and are less costly than phenotypic resistance assays (11). Both genotypic and phenotypic IQs are predictive of changes in the HIV

RNA level in treatment-experienced patients (5, 9, 11, 17, 22, 23, 31), but only one study of the predictive value of the IQ is available in patients treated with two ritonavir-boosted PIs (8).

We aimed at estimating the relationship between IQs of amprenavir and lopinavir and virological response after 2, 6 and 26 weeks of treatment in a group of heavily pretreated patients who participated in the ANRS-104 trial. Several methods for calculating IQs were compared with the viral phenotype and genotype for their ability to predict changes in plasma viral load.

Materials and methods

Study design and study population

The ANRS 104 study was a prospective, randomized, open-label, multicenter trial involving patients with CD4 < 500/mm^3 and plasma HIV RNA > 10 000 copies/mL after receiving successive antiretroviral treatments including at least two PIs and one non nucleoside reverse transcriptase inhibitor (NNRTI). The main objective of this trial was to compare the clinical efficacy and tolerability of a combination of amprenavir and lopinavir/ritonavir in treatment-experienced patients (26). The study was divided into two periods. For the first two weeks (period 1), patients were randomized to receive, in addition to their ongoing nucleoside reverse transcriptase inhibitors (NRTI), 1) lopinavir/ritonavir (400/100mg BID) or lopinavir/ritonavir (400/100 mg BID) + ritonavir (100 mg BID), 2) amprenavir (600 mg BID) + ritonavir (100 mg BID), or amprenavir (600 mg BID) + ritonavir (200 mg BID). From week 3 to week 26 (period 2), all the patients received amprenavir + lopinavir/ritonavir, with or without an additional boost of ritonavir 100 mg BID. The NRTIs were optimized on the basis of viral genotyping results and previous antiretroviral exposure. The genotype was interpreted for each inhibitor by using the 2001 update of the French National Agency for AIDS Research (ANRS) algorithm. Patients were recruited from 16 French clinical AIDS units. All patients signed an Ethics Committee-approved

informed consent form. Patients were instructed to take their medication in the morning and evening, with a light meal. Physical examinations, CD4 and CD8 cell counts, hematologic and clinical chemistry measurements were performed at each study visit (weeks -2, 0, 2, 4 and 6, then monthly for the subsequent 20 weeks). Blood samples were also drawn, at weeks –2, 0, 2, 6, and 26, for plasma HIV-1 RNA, viral genotyping and phenotyping and drug assays in plasma.

Laboratory measurements

Virological parameters

Plasma HIV-1 RNA was assayed locally at weeks –2, 0, 2, 4, 6, 14 and 26, by using the Roche Amplicor HIV-1 Monitor kit (Roche, France; limit of quantitation (LOQ) 200 copies/mL) or the Quantiplex® HIV-RNA 3.0 assay (Bayer diagnostics, France; LOQ 50 copies/mL).

HIV-1 protease genotyping and phenotyping

Viral genotyping was performed at weeks -2, 2, 6 and 26, based on direct sequencing of the HIV-1 protease coding region, using the consensus technique of the ANRS AC11 Resistance Group or the TruGene HIV-1 genotyping kit (Visible Genetics, Bayer). The genotype was taken into account only if a complete protease sequence (amino acids 1-99) was obtained. Protease sequences from each patient were examined for the presence of mutations associated with protease resistance at 21 relevant codons (11): 10, 20, 24, 30, 32, 33, 36, 46, 47, 48, 50, 53, 54, 63, 71, 73, 77, 82, 84, 88 and 90 (The Stanford HIV drug resistance database, 2004. http://hivdb.stanford.edu/). The genotype for lopinavir/ritonavir and amprenavir/ritonavir was interpreted by using the 2006 update of the French National Agency for AIDS Research (ANRS) algorithm.

Resistance phenotyping was performed at screening (week-2) in all the patients and at week 26 in those patients who failed drug regimen and had plasma HIV-RNA above 50 copies/mL (20/37

patients), using a recombinant virus assay (Phenoscript^{R}, Viralliance) (25). Results were expressed as the PI concentration inhibiting virus spread by 90% (IC_{90}), in a standard method without added human serum (IC_{90}). In a subgroup of 12 patients who started their study regimen with amprenavir, the amprenavir IC_{90} was also determined after adding 40% human serum to the growth medium which contains 10% fetal bovine serum in order to reach a total protein concentration close to that found in human plasma ($IC_{90+serum}$).

Drug assays in plasma

Blood samples were drawn at week 2 and week 6, just before the scheduled drug intake (Cmin). The total and unbound amprenavir and total lopinavir Cmin values were measured by HPLC with separation on a C18 column after liquid/liquid extraction of alkaline plasma and UV detection as described elsewhere (2, 26, 29). Bound and unbound amprenavir were separated by ultrafiltration using Centrifree® devices (Amicon, YM-300 filter system, Millipore Corp., Bedford, Massachussets, USA). Amprenavir was then measured in the ultrafiltrate as previously described. The overall day-do-day coefficient of variation was below 12 % (2). All concentrations were expressed in ng/mL or µmol/L for inhibitory quotient calculations.

Calculation of IQs

Phenotypic IQs were calculated as the ratio of the plasma Cmin of each PI to the IC_{90} measured at baseline. For amprenavir, the IC_{90} values were determined with or without added protein ($IC_{90+serum}$ or IC_{90}), and both the total and unbound Cmin ($Cmin_u$) values were measured. Two methods to adjust for protein binding were tested, namely the $Cmin_u$ and the $IC_{90+serum}$ (24). Amprenavir IQs were therefore calculated as the ratios of $Cmin_u/IC_{90}$ (IQ_u) and $Cmin/IC_{90+serum}$ (IQ_{serum}).

The genotypic inhibitory quotient (GIQ) of each PI was calculated as the ratio of the plasma Cmin corrected for protein binding ($Cmin_u$) to the baseline number of protease resistance mutations. The following mutations on the viral protease were considered: L10I/F/R/V, K20M/R, L24I, D30N, V32I, L33F, M36I, M46I/L, I47A/V, G48V, I50V, F53L, I54L/T/V, L63P, A71I/L/V/T, G73S, V77I, V82A/F/T/S, A84V, N88D/S, and L90M.

During the second treatment period, in which patients received two ritonavir-boosted PIs, the combined IQs were calculated as the sum of the phenotypic IQu for each PI. $Cmin_u$ was not measured but was calculated as the total Cmin corrected by the average protein binding of amprenavir (10%) (2, 15) and lopinavir and ritonavir (1%) (3, 4). The genotypic combined IQ was calculated as the sum of the ratios of $Cmin_u$/number of protease resistance mutations.

Statistical analysis

The median (range) was used to describe the distribution of amprenavir, lopinavir and ritonavir parameters and for the different IQ calculations. Univariate linear regression was used to estimate the relation between the decline in viral load (difference between baseline and weeks 2, 6 or 26) and the different Cmin, IC90 and IQ values. The higher the proportion of explained variance (r^2) of viral load, the better the model. All statistical tests were run on SAS software (version 8.2; SAS Institute).

Results

Overall, 37 patients were enrolled in the ANRS 104 study. Their baseline characteristics are shown in table 1. Twenty three patients started their study antiretroviral drug regimen with lopinavir/ritonavir (group 1) and 14 patients started with amprenavir (group 2) .

Amprenavir and lopinavir Cmin values are reported in detail elsewhere (26, 29) and were not related to the virologic response. The median unbound amprenavir $Cmin_u$ at week 2 was 177 ng/mL and was not predictive of the virologic response at week 2.

In a subgroup of 12 patients who had phenotypic studies (group 2), the amprenavir IC_{90} was 57.8 ng/mL (8.7-150.9), and increased to 453 ng/mL (33-1105) when measured in the presence of 50% human serum ($IC_{90+serum}$). The $IC_{90+serum}$ was a good predictor of the early virologic response (w2 p=0.006), whereas the IC_{90} was not. Amprenavir IQs adjusted for protein binding (IQu and IQ_{serum}) were rather similar (2.5 and 3.6, respectively). The relationship with viral load decline at week 2 was slightly better with IQ_u than IQ_{serum} (r^2=0.45, p=0.02 versus r^2=0.31, p=0.06), although those results might be explained by an outlier patient (r^2=0.24, p=0.12 versus r^2=0.11, p=0.31 without the outlier patient).

In the 21 patients treated with lopinavir during the first period, the lopinavir IC_{90} was 34.2 ng/mL (0.7-330.4) and was a good predictor of the virologic response at weeks 2, 6 and 26 (r^2=0.37, p=0.003; r^2=0.23, p=0.03; r^2=0.33, p=0.006 respectively).

The number of protease mutations (< or ≥5) was related to the virologic response throughout the study (at week 2, r^2=0.18, p=0.008; at week 6 r^2=0.11, p=0.046; and at week 26 r^2=0.12, p=0.034 - figure 1).

Table 2 shows the virologic responses throughout the study as a function of the amprenavir and lopinavir phenotypic IQ and GIQ values measured at week 2 or week 6. The amprenavir genotypic IQ (corrected amprenavir Cmin at week 2/number of protease mutations at week -2) was related to the virologic response at week 2 (r^2=0.66, p=0.0004) and at week 6 (r^2=0.38, p=0.02). Patients who had a Cmin corrected GIQ above 75 had a median decline in HIV-RNA of 1.23 log10 copies/mL (-2.27 ; -0.97) versus 0.19 log10 copies/mL (-1.07 ; 0.15) in patients with a

GIQ below 75 (p=0.005). In contrast, none of the lopinavir IQs was predictive of antiretroviral activity.

None of the phenotypic or genotypic IQs of amprenavir or lopinavir (determined with the Cmin corrected for protein binding and measured at week 6) was predictive of the viral load decline. However, a combination of the amprenavir and lopinavir genotypic IQs, whether measured either with the standard equation or adjusted for protein binding, and with or without ritonavir, was predictive of the viral response at week 6 but not at the end of the study as shown on figure 2. Patients who responded at week 6 (plasma HIV-RNA < vs ≥ 10000 copies/mL) had a higher median combined genotypic IQ measured at week 6 (65 vs 29 p=0.01).

Discussion

A significant proportion of antiretroviral-experienced patients never have optimal viral suppression or experience a viral rebound shortly after starting a new treatment. Our population was heavily pretreated with a high proportion of baseline NRTIs genotypic resistance, consequently, the efficacy of the combination results essentially from both PIs. Treatment thus needs to be optimized according to viral susceptibility and the plasma PI concentration. This study provides IQ data for two currently used ritonavir-boosted PIs, amprenavir and lopinavir, when administered alone (first two weeks of the study) or in combination, in heavily pretreated HIV-infected patients. This is one of the few studies to show that the combined IQ can be a useful predictor in patients who receive ritonavir-boosted dual-PI therapy (8).

We compared two methods for calculating the phenotypic IQ, which incorporates protein binding. Amprenavir and lopinavir are highly bound to plasma proteins, and especially alpha1 acid glycoprotein (90% and 98-99% respectively). As only free drug inhibits viral replication, protein binding affects the potency of these two PIs. ICs measured *in vitro* must be adjusted for

protein binding before extrapolation to the clinical setting. Moreover, ICs suffer from variability due to a lack of standardisation of phenotyping methods (14). There are three methods for adjusting the *in vitro* IC for protein binding: multiplication of the IC by the free fraction measured *in vivo*; measurement of the IC in the presence of 50% human serum albumin; and multiplication of the IC by a constant to adjust for assay variations (20), but none of these methods is clinically validated. Our results show that, whatever the equation used to calculate IQ ($Cmin_u$/standard IC_{90} or Cmin/$IC_{90+serum}$), the relationship with viral load decline is very similar. The best correlation was obtained with the IQ_u ($Cmin_u$/IC90). It has previously been demonstrated that measuring and calculating the $Cmin_u$ gives similar results (29). However, it remains to be determined whether these findings can be extrapolated to PIs other than amprenavir.

We found no relationship between lopinavir or amprenavir exposure and the decline in viral load, in agreement with previous studies (6, 7, 13, 16, 30). Amprenavir and lopinavir Cmin values obtained with the ritonavir boost were far higher than the IC_{50} reported for wild-type virus (28) and the IC_{90} measured at screening. This might explain why plasma concentrations were poorly predictive of the decline in plasma HIV RNA. Compliance with medication was not measured in our study; the amprenavir and lopinavir Cmin values suggested that it was maximal at week 2 and week 6 but declined thereafter. Thus, none of the phenotypic IQ values based on concentrations measured at week 6 was predictive of antiviral efficacy.

The genotypic IQ is simpler to measure than the phenotypic IQ. We found that the genotypic IQ of amprenavir was associated with the virologic response at week 2 and week 6 but not at week 26. Furthermore the amprenavir GIQ cut off of 75 could be a useful tool in clinical practice as previously demonstrated by Marcelin et al. (18). Our findings confirm that the genotypic IQ, which incorporates both baseline viral resistance and the level of drug exposure in plasma, is

229 superior to drug exposure alone in predicting the virologic response to a salvage regimen (18).
230 However, lopinavir IQs did not correlate with virologic efficacy, and our data do not support
231 those reported elsewhere (5, 9, 16). This is probably because the lopinavir Cmin values in our
232 patients were sufficiently high not to restrict efficacy (13), whereas lopinavir concentrations were
233 lower in other studies (5, 9). In these latter studies, the Cmin was determined as part of routine
234 therapeutic drug monitoring or observational studies, settings in which compliance is important
235 for overall exposure (5, 9, 16). One limitation of therapeutic drug monitoring is the wide intra-
236 patient variability of trough concentrations, as recently demonstrated by Nettles et al.(21) and
237 Goujard et al. (10). Poor compliance and an effect of food may be involved in this variability. We
238 acknowledge that one limitation of our study is that compliance was not measured. However,
239 comparison of trough concentrations measured at weeks 6, 14 and 26 clearly shows that
240 compliance is decreasing from week 6, where concentrations of lopinavir and amprenavir were in
241 the expected range in all patients, in contrast to weeks 14 and 26 where 7 and 6 patients
242 respectively, had concentrations below the limit of quantification of the assay (26).
243 Interestingly, the combined GIQ was predictive of the virologic response at week 6, as in the
244 GigHAART trial (8). This suggests that a combination of two PIs has a strong antiviral effect that
245 might overcome resistance in these strongly pretreated patients. As expected, ritonavir did not
246 participate in the drug effect, as the concentrations used to boost PIs are too low (even though
247 some patients received 200 mg BID). The combined IQ of amprenavir+lopinavir+ritonavir was
248 close to the combined IQ of amprenavir+lopinavir and had the same predictive potential.
249 However, the predictive value of this parameter disappeared at the end of the study, for several
250 possible reasons: in particular, the Cmin tends to fall, as a negative initial interaction between the
251 two PIs and a decreased compliance tends to reduce their concentrations and exposure levels;
252 second, the viral resistance profile is continually evolving (19). Further studies are needed to

253 determine which viral mutations have the biggest impact on the genotypic IQ, and how these
254 mutations can be taken into account in the calculations (30).
255 The combined genotypic IQ did not have better predictive value than the number of mutations. In
256 these highly pretreated patients with high PI Cmin values, the number of PI resistance mutations
257 is a major determinant of virologic outcome (5, 13, 16) and, in our study, was the only factor
258 predictive of the virologic response at week 26. Finally, as previously demonstrated (26), patients
259 with more than five protease resistance mutations or a lopinavir mutation score (13,14) higher
260 than 5 at baseline had a significantly poorer virological response than other patients (p=0.04 and
261 p=0.006, respectively).
262 Thus, this study suggests that when treatment compliance is optimal and high PI concentrations
263 are achieved, the viral genotype is the best predictor of virologic outcome.

Acknowledgments

This work was supported by ANRS (French National Agency for Research on AIDS and viral hepatitis), Paris, France. We thank Céline Boucherie who assisted us in the statistical analysis and the preparation of the revised manuscript.

Appendix

Puzzle1 Study Group: The following institutions and investigators participated in the Puzzle 1 ANRS 104 Study: W. Rozenbaum, L. Naït-Ighil, T.H. Nguyen, L. Slama, PM Girard, Hôpital Rothschild, Paris; J.M. Molina, D. Sereni, N. Colin de Verdière, C. Lascoux-Combes, C. Pintado, D. Ponscarme, F. Prevoteau de Clary, M. Tourneur, Hôpital Saint-Louis, Paris; M. Bentata, L. Guillevin, O. Launay, R. Mansouri, F. Rouges, Hôpital Avicenne, Bobigny; M. Kazatchkine, A. Aouba, M. Azizi, J.N. Fiessinger, P. Le Houssine, Hôpital Européen Georges Pompidou, Paris; D. Sicard, C. Bernasconi, D. Salmon, B. Silbermann, Hôpital Cochin, Paris; J.P. Cassuto, C. Ceppi, Dr Poiree, Hôpital de l'Archet, Nice; G. Raguin, M. Merad, Hôpital de la Croix Saint Simon, Paris; J.F. Delfraissy, C. Goujard, Y. Quertainmont, Hôpital de Bicêtre, Le Kremlin-Bicêtre; C. Perronne, P. de Truchis, Hôpital Raymond Poincaré, Garches; B. Dupont, J.L. Bresson, I. Calatroni, Hôpital Necker, Paris; F. Raffi, J.L. Esnault, S. Leautez, Hôtel Dieu, Nantes.

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400 antiretroviral-experienced HIV-infected patients. J Med Virol **77:**460-4.

401 **TABLE 1: Baseline characteristics of the patients participating in the ANRS 104 Study.**
402 **Patients from group 1 started with amprenavir/ritonavir for the first 2 weeks and patients**
403 **from group 2 started with lopinavir/r**

Parameters	Group 1, amprenavir n=14	Group 2, lopinavir n=23
Median age, years (range)	47 (32-53)	41 (27-65)
Males, n (%)	12 (86)	21 (91)
CDC clinical stage,	n (%)	n (%)
A	6 (42)	10 (43)
B	4 (29)	3 (13)
C	4 (29)	10 (43)
Median CD4+ cells/mm^3 (range)	195 (65-385)	185 (3-509)
Median HIV1-RNA, log_{10} copies/mL (range)	4.9 (3.6-5.7)	4.6 (3.8-5.6)
Median number of previous antiretrovirals (range)	7.5 (4-12)	7.5 (4-12)
Median number of antiretrovirals taken prior to inclusion and still in use at inclusion (range)	9.5 (7-13)	10 (8-13)
Genotypic resistance	n (%)	n (%)
Amprenavir/r	7 (50)	9 (56)
Lopinavir/r	7 (50)	7 (30)
Median (range) number of protease mutations	7.0 (1.0-9.0)	7.0 (1.0-10.0)
Median (range) number of reverse transcriptase mutations	6.5 (0-11.0)	7.0 (0.0-11.0)
Median (range) phenotypic resistance index		

Amprenavir	2.8 (0.5-24.3)	2.5 (0.5-19.5)
Lopinavir	8.7 (0.3-84.0)	10.7 (0.2-95.3)

404

405 **TABLE 2: Relationships between the viral load decline at weeks 2, 6 and 26 and the**
406 **amprenavir, lopinavir and ritonavir inhibitory quotients (univariate analysis)**

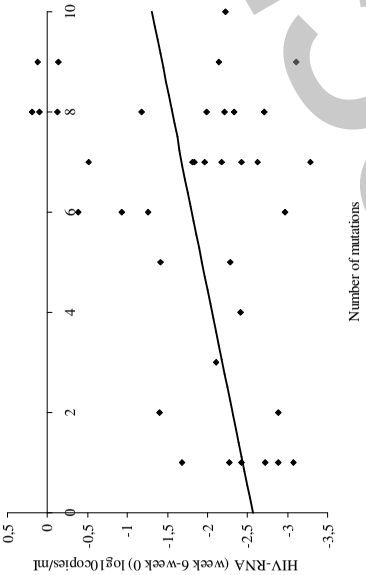
Inhibitory quotients		Week	Number of patients	Median (min-max)	p		
					Week 2	Week 6	Week 26
Amprenavir	IQsd	2	13	4.3 (0.62-25.9)	*0.04*	0.24	0.70
		6	13	1.8 (0.20-11.7)		0.80	0.26
	GIQ	2	14	81.2 (17.9-291.1)	*0.0004*	*0.02*	0.16
		6	14	26.4 (9.8-69.1)		0.56	0.59
Lopinavir	IQsd	2	21	4.0 (0.16-153.3)	0.97	0.65	0.66
		6	21	2.0 (0.15-157.5)		0.70	0.80
	GIQ	2	23	23.9 (6.6-177.6)	0.37	0.20	0.20
		6	23	17.8 (3.6-182.5)		0.15	0.36
Ritonavir	IQsd	2	34	0.01 (0.0004-0.5)	0.55	0.18	0.28
		6	34	0.005 (0.0004-0.8)		0.34	0.87
	GIQ	2	37	1.7 (0.12-19.1)	0.18	0.10	0.12
		6	37	1.3 (0.12-20.4)		0.16	0.45
Amprenavir+ Lopinavir	IQsd	6	34	4.9 (0.36-166.5)		0.34	0.71
	GIQ	6	37	46.9 (15.9-543.4)		*0.03*	0.15
Amprenavir+ Lopinavir+ Ritonavir	IQsd	6	34	4.9 (0.37-167.3)		0.34	0.71
	GIQ	6	37	48.8 (16.0-549.3)		*0.03*	0.15

407 IQsd: standard inhibitory quotient: trough concentration (Cmin) at week 2 or 6/IC90 standard at

408 week -2, GIQ: genotypic inhibitory quotient: corrected Cmin (calculated protein unbound $Cmin_u$)

409 at week 2 or 6/number of protease mutations at week –2.

Figure 1: relationship between viral load decline at week 26 and the number of protease mutations at screening (r^2 0.13, p=0.03).



1 Figure 2: relationship between viral load decline at week 6 and combined (lopinavir and

2 amprenavir) genotypic inhibitory quotient at week 6 (r^2= 0.12, p=0.03).