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HIV-1 protease mutation 82M contributes to phenotypic resistance to protease inhibitors in subtype G

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Objectives: The purpose of this study was the qualitative and quantitative assessment of the *in vitro* effect of HIV-1 protease (PR) mutation 82M on replication capacity and susceptibility to the eight clinically available PR inhibitors (PIs).

Methods: The 82M substitution was introduced by site-directed mutagenesis in wild-type subtype B and G strains, as well as reverted back to wild-type in a therapy-failing strain. The recombinant viruses were evaluated for their replication capacity and susceptibility to PIs.

Results: The single 82M mutation within a wild-type subtype B or G background did not result in drug resistance. However, the *in vitro* effect of single PR mutations on PI susceptibility is not always distinguishable from wild-type virus, and particular background mutations and polymorphisms are required to detect significant differences in the drug susceptibility profile. Consequently, reverting the 82M mutation back to wild-type (82I) in a subtype G isolate from a patient that failed therapy with multiple other PR mutations did result in significant increases in susceptibility towards indinavir and lopinavir and minor increases in susceptibility towards amprenavir and atazanavir. The presence of the 82M mutation also slightly decreased viral replication, whether it was in the genetic background of subtype B or subtype G.

Conclusions: Our results suggest that 82M has an impact on PI susceptibility and that this effect is not due to a compensatory effect on the replication capacity. Because 82M is not observed as a polymorphism in any subtype, these observations support the inclusion of 82M in drug resistance interpretation systems and PI mutation lists.

Keywords: HIV, drug resistance, PIs

Introduction

The rise of HIV-1 non-B subtypes in Europe and the roll-out of antiviral therapy in the developing world have led to the initiation of studies investigating the impact of HIV-1 genetic variability on *in vitro* and *in vivo* drug susceptibility. Although certain polymorphisms were associated with slightly increased and/or decreased *in vitro* drug susceptibility,[1] several observational studies revealed that HIV-1 patients were as likely to achieve first-line therapy success irrespective of their subtype.[2] However, other studies illustrated the emergence of subtype-specific drug resistance mutations, underscoring the limitations of genotypic drug resistance interpretation systems that failed to include these novel mutations.[3]

A previous study revealed that the novel 82M protease (PR) substitution was significantly linked to subtype G and to indinavir and lopinavir exposure. The aim of this study was to explore its role in HIV-1 subtype G susceptibility towards PR inhibitors (PIs).[4]

Materials and methods

Viral RNA was extracted from plasma samples obtained from HIV-1 subtype G patients. For subtype B, the p83.2 vector was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The

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PR sequences were amplified, cloned and sequenced as previously described.[5] Mutations at PR position 82 were introduced by site-directed mutagenesis. The drug susceptibility and replication capacity of the recombinant viruses were assessed as previously described.[5] Statistical analysis was performed in Microsoft Excel using a two-sided Student's *t*-test and the Bonferroni correction to account for multiple analyses.

Results and discussion

The historical connection between Portugal and some African countries has produced an HIV-1 epidemiological profile that is dominated by subtypes B (41.7%) and G (29.4%). A study that aimed at detecting subtype-specific drug resistance mutations within PR showed differences in the prevalence of amino acids at PR position 82 between naive and treated patients within each subtype (Table 1). Correction for the difference in PI exposure and the inherent amino acid differences between the two subtypes revealed that within the Portuguese dataset the PR 82M substitution was 27 times more likely to occur in subtype G than in subtype B, and that the presence of that particular amino acid substitution was significantly associated with experience of indinavir and/or lopinavir/ritonavir.[4] As early as 1 year after their respective clinical approval, amino acid substitutions at PR position 82 were associated with reduced susceptibility to both drugs.[6,7] Resistance development to indinavir occurred through variable patterns of several substitutions, but all resistant isolates displayed 46I/L and/or 82A/F/T.[6] The latter mutations were also significantly associated with reduced *in vitro* susceptibility to lopinavir.[7] The purpose of this study was to assess qualitatively and quantitatively the *in vitro* effect of 82M on replication capacity and susceptibility to the available PIs.

A subtype B and a subtype G strain that could be considered as representative for both subtypes were selected from our repository of reference viruses and clinical isolates (Table 2). The 82M substitution was introduced by site-directed mutagenesis in both backgrounds, whereas the 82A and 82F mutations were elected to be introduced in the subtype B background, because 82A is the most prevalent amino acid change after *in vivo* PI exposure in subtype B and 82F is reported with the highest fold-change in susceptibility towards indinavir (1–3-fold).[8] The recombinant viruses were tested phenotypically, but no significant effect against indinavir and lopinavir was detected, either for subtype B or for subtype G (Table 2). This was not entirely unexpected, since, in contrast to non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance, the *in vitro* effect of single PR mutations on PI susceptibility is not always distinguishable from wild-type virus and not even by standard commercially available assays.[8] Often, particular background mutations and polymorphisms are required to detect significant differences in the drug susceptibility profile. Therefore, the next step in our study was to analyse the effect of the 82M mutation in the presence of major PI mutations.

For that purpose, a sample from a patient who was infected with a subtype G virus and failing PI therapy in the presence of 82M was selected (G_{MT}). Although 82M has also been observed in subtype B isolates,[9] none of the subtype B patients attending the University Hospitals Leuven and only one patient at the Laboratório de Biologia Molecular (Lisbon) displayed this mutation. Unfortunately, no patient sample was available for further analysis.

Drug susceptibility testing revealed that G_{MT} was highly resistant to both indinavir (31-fold) and lopinavir (97-fold) (Table 2).

Table 1. Prevalence of amino acid changes at PR position 82 in PI-naive and PI-treated patients

	Subtype B				Subtype G			
	PI naive		PI treated		PI naive		PI treated	
PR mutation[a]	HIVdb[b]	Portugal[c]	HIVdb[b]	Portugal[c]	HIVdb[b]	Portugal[c]	HIVdb[b]	Portugal[c]
82V	98.3%	97.7%	39.1%	56%	10%	2%	6.4%	5.7%
82I	1.7%	2.3%	1.6%	1.6%	90%	98%	72%	67%
82A	<0.0%	—	29%	33%	—	—	3.3%	3.3%
82T	—	—	3.6%	4.5%	—	—	10%	11%
82F	—	—	2.1%	3.2%	—	—	1.7%	1.9%
82S	<0.0%	—	1.5%	0.5%	—	—	3.3%	3.3%
82C	—	—	0.9%	0.5%	—	—	—	—
82L	—	—	0.3%	—	—	—	—	—
82M	—	—	0.1%	0.2%	—	—	3.3%	7.7%
82H	—	—	<0.0%	—	—	—	—	—
82R	—	—	<0.0%	—	—	—	—	—
82L	<0.0%	—	—	—	—	—	—	—

[a]PR polymorphisms within wild-type subtype B and G strains (82V and 82I, respectively) and PR mutations within PI-treated subtype B and G strains, ordered according to prevalence within subtype B.
[b]At the time of analysis, 13910 PI-naive subtype B, 7074 PI-treated subtype B, 829 PI-naive subtype G and 247 PI-experienced subtype G strains were available within the HIV drug resistance database.[8]
[c]At the time of analysis, 133 PI-naive subtype B, 440 PI-treated subtype B, 151 PI-naive subtype G and 209 PI-experienced subtype G strains were available within the Portuguese database.

Table 2. Genotypic and phenotypic characteristics of HIV-1 subtype B and G recombinant viruses

	PR mutations[a]																			
	L10	I13	K14	I15	K20	E35	M36	R41	M46	I47	F53	I54	I62	L63	C67	H69	T74	**V82**	L89	L90
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**—**	—	—
B-82M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**M**	—	—
B-82A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**A**	—	—
B-82F	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**F**	—	—
G_{CON}	—	V	—	—	I	—	I	K	—	—	—	—	—	—	S	K	—	**I**	M	—
G	—	V	—	—	I	—	I	K	—	—	—	—	—	P	S	K	—	**I**	M	—
G-82M	—	V	—	—	I	—	I	K	—	—	—	—	—	P	S	K	—	**M**	M	—
G_{MT}	V	A	R	V	I	D	I	K	I	V	L	V	V	P	Y	K	A	**M**	I	M
G_{MT}-82I	V	A	R	V	I	D	I	K	I	V	L	V	V	P	Y	K	A	**I**	I	M

	Drug susceptibility[b]									Replication capacity[c]	
	PI								NRTI	*k*	R^2
	AMP	ATV	DRV	IDV	LPV	NFV	SQV	TPV	3TC		
B-82M	—	—	—	1.1 ± 0.5	1.0 ± 0.6	—	—	—	—	1.30	0.95
B-82A	—	—	—	0.9 ± 0.3	1.1 ± 0.4	—	—	—	—	1.35	0.99
B-82F	—	—	—	0.7 ± 0.2	0.7 ± 0.3	—	—	—	—	0.95	0.89
G-82M	—	—	—	1.5 ± 0.2	0.9 ± 0.3	—	—	—	—	1.01	0.97
G_{MT}	17.3 ± 3.5	13.4 ± 3.3	3.3 ± 1.0	30.7 ± 12.7	96.6 ± 17.3	18.9 ± 7.5	4.6 ± 1.0	4.3 ± 1.0	1.1 ± 0.3	1.45	0.99
G_{MT}-82I	**7.7 ± 2.2**	**6.1 ± 0.3**	1.7 ± 0.3	**6.9 ± 3.4**	**38.4 ± 12.7**	13.1 ± 4.4	**8.5 ± 2.1**	2.6 ± 1.2	0.8 ± 0.2	1.71	0.98

[a]PR mutations within the wild-type (B and G) and mutant viruses (B-82M, B-82A, B-82F, G-82M, G_{MT} and G_{MT}-82I), relative to the consensus sequence for subtype B, as obtained from HIV databases (http://www.hiv.lanl.gov). For comparison, the consensus sequence for subtype G (G_{CON}) is also displayed. Mutations in bold are the subject of the study. The frequencies of the G_{MT} background polymorphisms and mutations in other subtype G isolates containing the 82M mutation and present in the HIV drug resistance database are: 10V (38%), 13A (38%), 14R (38%), 15V (50%), 20I (88%), 35D (75%), 36I (88%), 43K (75%), 46I (50%), 47V (0%), 53L (25%), 54V (88%), 62V (38%), 63P (50%), 67Y (0%), 69K (100%), 74A (0%), 89I (75%) and 90M (63%).[8]

[b]Drug susceptibility of recombinant viruses towards PIs (AMP, amprenavir; ATV, atazanavir; DRV, darunavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; SQV, saquinavir; TPV, tipranavir) and the NRTI lamivudine (3TC), expressed as mean fold-change and standard deviation. Values were obtained from at least three independent experiments, each performed in triplicate.[5] A *P* value was calculated for the difference of fold-change between the site-directed mutant and the corresponding original virus strain (B-82M, B-82A, B-82F versus B, G-82M versus G and G_{MT}-82I versus G_{MT}). Values with $P < 0.005$ are displayed in bold.

[c]For wild-type subtype B, $k = 1.70$ and $R^2 = 0.99$. For wild-type subtype G, $k = 1.48$ and $R^2 = 0.98$. The percentage of infected cells was measured every day and normalized to the percentage of infected cells at day 0 (day 0 is 72 h after initial infection, aim of 0.1% infected cells). The curves were fitted exponentially and the resulting *k* values ($y = e^{kt}$) were used as a measure of replication capacity. Data are the means of at least three independent experiments, each performed in triplicate.[5]

Reverting the 82M mutation back to 82I, the consensus wild-type amino acid in subtype G, resulted in a significant increase in susceptibility to both PIs (Table 2). Nevertheless, the recombinant virus remained resistant to indinavir (7-fold) and lopinavir (38-fold). Concurrently, the G_{MT} and G_{MT}-82I recombinant viruses were studied for susceptibility to all other clinically available PIs, as particular amino acid changes at that position are associated with *in vitro* cross-resistance to all PIs but saquinavir.[10] The G_{MT} recombinant displayed wild-type susceptibility towards lamivudine and variable reductions in susceptibility towards amprenavir (17-fold), atazanavir (13-fold), darunavir (3-fold), nelfinavir (19-fold), saquinavir (5-fold) and tipranavir (4-fold). Unlike for indinavir and lopinavir, the reversion of 82M to 82I resulted in: (i) a <2-fold reduction in resistance levels, suggesting a minor role for 82M in resistance towards amprenavir and atazanavir; and (ii) no significant resistance reduction for darunavir, nelfinavir and tipranavir. In accordance with a previously reported beneficial effect of some substitutions at the 82 position, namely 82F/L, on saquinavir susceptibility,[10] we also observed a slightly lower fold-change in resistance towards saquinavir in G_{MT}, when compared with G_{MT}-82I.

Finally, a replication capacity assay was performed to assess the qualitative nature of the impact of 82M on the virus' fitness in the absence of drug (Table 2). In each of the three independent experiments, the presence of the 82M mutation slightly decreased the virus' capacity to replicate, whether it was present in the genetic background of wild-type subtype B, wild-type subtype G or mutant subtype G; however, statistical significance was not reached. This suggests that the higher resistance level is not due to the compensatory role of 82M on replication capacity, but has to be due to a direct effect on drug activity, which could hypothetically be attributed to its position within the active site of PR.

The propensity of subtype G to acquire the 82M mutation more frequently than subtype B after PI exposure is likely due to the differences in wild-type codon use at position 82 in PR. For subtype B, the consensus is GTC, encoding for valine, and for subtype G it is ATC, encoding for isoleucine (Table 1). This makes the genetic barrier smaller in subtype G, as only one mutation is required to achieve a methionine (ATC to ATG), in contrast with the two mutations needed in subtype B (GTC to ATG). This might also explain why the alanine substitution is 10 times less frequent in subtype G as opposed to in subtype B (GTC to GCC), since it requires two mutations (ATC to GCC). The codon ATC at PR position 82 is also the consensus in several circulating recombinant forms containing subtype G within the PR region (e.g. CRF06-cpx, CRF14_BG, CRF20_BG, CRF23_BG, CRF24_BG, CRF25_cpx and CRF37_cpx) and it is also detected in some strains from all other pure subtypes. This can explain, although at a low frequency, the selection of 82M in PI-treated patients infected with subtypes other than subtype G.[9]

Although our phenotypic assay is not clinically validated and cut-offs cannot be extrapolated between different assays, it is noteworthy that the here observed resistance levels for G_{MT} and G_{MT}-82I were all well above the biological cut-offs of Virco-TYPE HIV-1 and Phenosense.[8] For the drugs for which clinical cut-offs are available, G_{MT} scored above the lower clinical cut-off for saquinavir/ritonavir and tipranavir/ritonavir, and above the upper clinical cut-off for amprenavir/ritonavir, indinavir/ritonavir and lopinavir/ritonavir. Reversion to 82I led to potentially clinically relevant changes for amprenavir/ritonavir, indinavir/ritonavir and lopinavir/ritonavir, as their fold-changes dropped below their respective upper clinical cut-off values.

When the G_{MT} sequence was interpreted using the genotypic drug resistance interpretation systems in the HIV drug resistance database (ANRS 2011.05, HIVdb v6.0.11 and Rega v8.0.2), all drugs were scored resistant except for: (i) darunavir/ritonavir, which received a susceptible score in ANRS and HIVdB and an intermediate resistant score in Rega; and (ii) tipranavir/ritonavir, which received an intermediate score in ANRS and HIVdb. We noticed that 82M was not included in the ANRS system, but was included in all but one PI rule in the HIVdb and Rega systems. In both of the latter systems, the weight was the highest for indinavir and lowest (zero) for darunavir. Nevertheless, the reversion to 82I resulted in only a resistant-to-intermediate change for atazanavir/ritonavir and lopinavir/ritonavir in HIVdb, and for tipranavir/ritonavir in Rega.

In conclusion, the PR mutation 82M has been observed in clinical practice for some time, but this is the first time that it has been studied *in vitro*. We have shown that this mutation, when present in a subtype G strain and within the background of other PI mutations, significantly reduces the susceptibility towards indinavir and lopinavir, and slightly towards other PIs. According to our results, this effect on drug susceptibility is not due to a compensatory effect on the replication capacity. Because 82M is not observed as a polymorphism in any subtype, these observations support the inclusion of 82M in drug resistance interpretation systems and PI mutation lists.

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Transparency declarations

None to declare.

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