Contribution of Gag and protease to variation in susceptibility to protease inhibitors between different strains of subtype B human immunodeficiency virus type 1

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Recent reports have shown that human immunodeficiency virus type 1 (HIV-1) Gag can directly affect susceptibility to protease inhibitors (PIs) in the absence of known resistance mutations in protease. Inclusion of co-evolved Gag alongside protease in phenotypic drug susceptibility assays can alter PI susceptibility in comparison with protease with a WT Gag. Using a single-replication-cycle assay encompassing full-length Gag together with protease we demonstrated significant variation in PI susceptibility between a number of PI-naïve subtype B viruses. Six publicly available subtype B molecular clones, namely HXB2, NL4-3, SF2, YU2, JRFL and 89.6, displayed up to nine-fold reduced PI susceptibility in comparison with the assay reference strain. For two molecular clones, YU2 and JRFL, Gag contributed solely to the observed reduction in susceptibility, with the N-terminal region of Gag contributing significantly. Gag and protease from treatment-naïve, patient-derived viruses also demonstrated significant variation in susceptibility, with up to a 17-fold reduction to atazanavir in comparison with the assay reference strain. In contrast to the molecular clones, protease was the main determinant of the reduced susceptibility. Common polymorphisms in protease, including I13V, L63P and A71T, were shown to contribute to this reduction in PI susceptibility, in the absence of major resistance mutations. This study demonstrated significant variation in PI susceptibility of treatment-naïve patient viruses, and provided further evidence of the independent role of Gag, the protease substrate and in particular the N-terminus of Gag in PI susceptibility. It also highlighted the importance of considering co-evolved Gag and protease when assessing PI susceptibility.

INTRODUCTION

The introduction of highly active antiretroviral therapy (HAART) to treat human immunodeficiency virus (HIV) has improved greatly the prognosis of HIV-infected individuals. HAART usually comprises two nucleoside reverse transcriptase inhibitors (NRTIs) together with either a non-nucleos(t)ide reverse transcriptase inhibitor (NNRTI) or a boosted protease inhibitor (bPI). PIs are a potent class of inhibitors, with a recent study demonstrating that use of a PI as the third drug in HAART leads to significantly fewer patients with viral failure than HAART using an NNRTI as the third agent (Gupta et al., 2008). Nonetheless, therapy failure during treatment with PIs remains a significant problem.

Resistance to PIs develops via the accumulation of both major and minor resistance mutations in protease and Gag (Molla et al., 1996). Major mutations confer resistance to PIs by reducing the affinity of the protease to the PI, preventing the PI from blocking the protease active site and leading to protease activity despite the presence of PI. Within protease, major mutations are found in the portion of the sequence that encodes the substrate-binding pocket of the enzyme and also at more distant sites (Condra et al., 1995). Major mutations also reduce the affinity of the protease to the natural substrate, Gag, thus reducing the fitness of the resistant virus (Zennou et al., 1998). Minor mutations may compensate partially for the effect on viral fitness caused by major mutations in protease (Croteau et al., 1997). These are found in both protease and Gag, at cleavage sites as well as other distant sites (Clavel & Mammano, 2010; Gatanaga et al., 2002).

Supplementary methods and six supplementary figures are available with the online version of this paper.

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Whilst the role of Gag mutations in compensating for loss of fitness is well established, several recent studies have shown that Gag mutations can affect directly PI susceptibility, independent of compensating for reduced fitness (Dam et al., 2009; Jinnopat et al., 2009; Nijhuis et al., 2007; Parry et al., 2009). For example, mutations in the cleavage sites in the C-terminus of Gag were shown to affect PI resistance at levels beyond compensating solely for loss of fitness (Maguire et al., 2002; Zhang et al., 1997). Furthermore, recent studies demonstrated a direct effect of particular changes in NC (nucleocapsid)/p1/p6 cleavage sites at the C-terminal end of Gag on PI susceptibility (Parry et al., 2009; Parry et al., 2011). Other studies also showed that changes in CA (capsid) at position 165, within the N-terminus of Gag, affected PI susceptibility (Jinnopat et al., 2009; Kameoka et al., 2010).

In keeping with these data, in vitro studies using phenotypic drug susceptibility assays demonstrated that inclusion of Gag from treatment-naive individuals could also convey reduced susceptibility to PIs. A study using subtype A and C molecular clones and viruses derived from treatment-naive patients showed that inclusion of patient-derived Gag alongside patient-derived protease altered susceptibility in comparison with patient-derived protease with a WT Gag (Gupta et al., 2010). This effect has also been seen with viruses derived from treatment-naive patients infected with subtype CRF01_AE viruses and in an extensively PI-treated patient infected with subtype B virus (Jinnopat et al., 2009; Parry et al., 2009). To date, the effect of the inclusion of co-evolved Gag alongside protease from subtype B viruses derived from treatment-naive patients has not been investigated.

An assessment of the contribution and variability of full-length Gag to PI susceptibility is required. Furthermore, investigation of the regions of Gag affecting PI susceptibility and the mechanisms by which this can occur is also necessary. To address this current gap in our knowledge, we determined the PI susceptibility of full-length Gag–protease from a range of WT subtype B viruses with no previous PI exposure. Any viruses showing significant variation in susceptibility were further analysed to identify regions within Gag–protease or specific changes that played a role in this variation in susceptibility.

RESULTS

Variation in PI susceptibility of full-length Gag–protease observed between six subtype B molecular clones

The protease sequences of molecular clones pHXB2, pNL4-3, pYU2, pSF2, p8.96 and pJRFL (Fig. S1, available in JGV Online) indicated that none had any major or minor PI resistance mutations, as determined by the Stanford HIVdb Genotypic Resistance Interpretation Algorithm (http://hivdb.stanford.edu/). Full-length gag–protease of the molecular clones was cloned into p8.9NSX+, the Gag-Pol expression vector, creating six vectors designated HXB2gagpro, NL4-3gagpro, YU2gagpro, SF2gagpro, 89.6gagpro and JRFLgagpro. The susceptibility of these six constructs was determined for six PIs: amprenavir (APV), atazanavir (ATV), darunavir (DRV), lopinavir (LPV), saquinavir (SQV) and tipranavir (TPV).

Differences in susceptibility to each of the six PIs were observed for the molecular-clone-derived vectors compared with the reference strain, p8.9NSX+ (Fig. 1). In particular, the Gag–protease of four molecular clones showed significant reductions in susceptibility to three of the PIs (APV, ATV and LPV) to varying degrees in the order: YU2>JRFL>89.6>SF2 (P<0.05). The largest reductions in PI susceptibilities of up to nine-fold were observed for YU2gagpro (for APV, ATV and LPV), JRFLgagpro (for ATV) and 89.6gagpro (for APV). Two of the molecular-clone-derived vectors, HXB2gagpro and NL4-3gagpro, did not display PI susceptibilities that were significantly different from the reference strain for most of the PIs (P<0.05). To verify that the observed variation in PI susceptibilities was not a consequence of variations in vector DNA preparations, we tested additionally pseudovirions derived from two separate DNA preparations of each of the molecular clones, 89.6gagpro and YU2gagpro. This showed no significant differences in PI susceptibilities exhibited by individual DNA preparations from the same clone (P<0.05; Fig. S2).

Contribution of Gag to reduction in PI susceptibility observed for full-length Gag–protease in subtype B molecular clones

Next, we investigated the role of Gag and protease separately in the reduced PI susceptibility observed for pseudovirions expressing full-length Gag–protease derived from molecular clones. Molecular clones YU2, JRFL, SF2 and 89.6 were selected for this further analysis as they exhibited the greatest reductions in susceptibility to the PIs APV, ATV and LPV. To determine the PI susceptibility of the gag and protease genes separately, chimeric vectors containing gag derived from molecular clone and protease from p8.9NSX+, and vice versa, were produced for each of the four molecular clones of interest (Fig. 2a). The position of the Apal site results in the inclusion of part of Gag NC, p1 and p6 in the protease-only chimeric pseudovirus (Fig. 2a).

For the molecular clones JRFL and YU2, the Gag-only chimeric pseudovirions displayed significant reductions in PI susceptibility in comparison with the assay reference strain, whereas the protease-only chimeric viruses showed susceptibilities similar to that exhibited by the assay
ref-er-ence-strain (F-i-g. 2 b, c, Ta-b-le 1). The dif-fer-ence in the fold changes in EC\textsubscript{50} values for the Gag-only and protease-only chimeric psue-duovi-rions were sta-tis-tic-ally significant to APV and ATV for JRFL, and all three PIs for YU2 (\textit{P}<0.05). These data in-dicate that in these two molecular clones, Gag con-ferr-ed solely the re-duced sus-cetibili-ty to the three PIs ob-serve-d for the full-length Gag–pro-tease frag-ment. In con-trast, both the Gag-only and the protease-only chimeric viruses for molecular clones 89.6 and SF2 showed re-duced PI sus-cetibi-lities of up to fourfold (F-i-g. 2 d, e, Ta-b-le 1). Thus, these data in-dicated that both the \textit{gag} and \textit{protease} genes con-tri-buted to the re-duced sus-cetibili-ty of full-length Gag–pro-tease in 89.6 and SF2.

\textbf{Fig. 1.} PI sus-cetibili-ty of full-length Gag–pro-tease from six subtype B molecular clones was de-tected us-ing a single-re-plication-cycle drug sus-cetibili-ty assay. Data dis-played are fold changes in EC\textsubscript{50} values in com-pa-rison with the re-ference strain for each of six PIs: APV, ATV, DRV, LPV, SQV and TPV. *Viruses for which the raw EC\textsubscript{50} values were sta-tis-tically different from that of the reference strain (\textit{P}<0.05).

\textbf{Fig. 2.} PI sus-cetibili-ty of molecular clone Gag-only and protease-only chimeric viruses. (a) Schematic diagram of chimeric viruses con-tain-ing either Gag (MC\textit{gag}) or protease (MC\textit{pro}) derived from each molecular clone. INT, in-tegran-se; RT, re-ver-ses transcrip-tase. Seg-ments derived from the re-ference are white and seg-ments derived from molecular clones are shaded grey. (b–e) The sus-cetibili-ties to three PIs (APV, ATV and LPV) are dis-played for (b) JRFL, (c) YU2, (d) SF2 and (e) 89.6. *Viruses for which the fold changes in EC\textsubscript{50} values for Gag-only and protease-only chimeric viruses were sta-tis-tically different (\textit{P}<0.05).
were constructed using a unique conserved restriction site, flanking amino acids 240 and 241 of Gag within the CA subunit. Two chimeric vectors for each molecular clone were generated: gagN, containing the N-terminus of Gag and protease derived from four treatment-naïve patients was cloned into p8.9NSX and E102D changes contribute to the reduced susceptibility to the double mutant, suggesting an additive rather than synergistic effect of the two amino acid changes. In addition, a single-cycle replicative capacity assay showed no effect of these changes on the replicative capacity of either molecular clone (Fig. S5). Overall, these data indicated that the R30K and E102D reversions contribute to the reduced susceptibility observed for full-length Gag–protease of JRFL and YU2.

Reduced susceptibility to the three PIs APV, ATV and LPV was observed for the gagN-derived pseudovirions of up to seven- and 16-fold change for JRFL and YU2, respectively (Fig. 3b, c, Table 1). In contrast, the gagC-derived pseudovirions showed susceptibilities at levels similar to the reference strain. For each PI, the change in susceptibility between the gagN and gagC pseudovirions was statistically significant (P<0.05). This demonstrates that for both YU2 and JRFL, the N-terminal portion of Gag encompassing MA and the beginning of CA is largely responsible for the reduced susceptibility observed for full-length Gag. Alignment and comparison of the molecular clone sequences was carried out in order to identify amino acid changes within Gag that could account for the reduced PI susceptibility (Figs S3 and S4). Sequence analysis of the cleavage sites found no previously reported mutations that could account for this variation in susceptibility and only the p2/NC cleavage site, within the C-terminus of Gag, contained sequence variation. Analysis identified only one residue, at position 30, where an amino acid change occurs exclusively in molecular clones YU2 and JRFL, and one at position 102, which is also found in SF2. Both of these changes are within the N-terminal segment of Gag shown to confer reduced susceptibility in these molecular clones. There were also a number of unique changes from the consensus sequence in either JRFL or YU2, and within the N-terminus of Gag these changes were T53S, Q69K, R76T, N47D, R91K for YU2 (Fig. S3).

Further analysis was carried out for YU2 and JRFL, the two molecular clones in which Gag conferred solely reduced susceptibility, in order to identify the specific region of Gag conferring the reduced susceptibility. Chimeric Gag vectors were constructed using a unique SpeI restriction site, flanking amino acids 240 and 241 of Gag within the CA subunit. Two chimeric vectors for each molecular clone were generated: gagN, containing the N-terminus of Gag derived from the molecular clone and the C-terminus of Gag and protease from p8.9NSX+, and gagC, containing the C-terminus of Gag derived from the molecular clone and the N-terminus of Gag and protease from p8.9NSX+ (Fig. 3a).

Table 1. PI susceptibility of chimeric viruses derived from molecular clones

<table>
<thead>
<tr>
<th>Vector</th>
<th>APV Fold change in EC_{50} ± SEM</th>
<th>ATV Fold change in EC_{50} ± SEM</th>
<th>LPV Fold change in EC_{50} ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRFLgagpro</td>
<td>5.31 ± 1.27</td>
<td>9.04 ± 1.91</td>
<td>5.21 ± 1.69</td>
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<tr>
<td>JRFLgag</td>
<td>10.88 ± 1.28</td>
<td>7.21 ± 0.67</td>
<td>7.36 ± 0.50</td>
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<tr>
<td>JRFLpro</td>
<td>0.82 ± 0.07</td>
<td>0.91 ± 0.42</td>
<td>0.72 ± 0.24</td>
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<tr>
<td>JRFLgagN</td>
<td>6.75 ± 0.68</td>
<td>7.01 ± 0.47</td>
<td>7.41 ± 0.94</td>
</tr>
<tr>
<td>JRFLgagC</td>
<td>0.78 ± 0.12</td>
<td>0.87 ± 0.09</td>
<td>0.44 ± 0.09</td>
</tr>
<tr>
<td>P-value (gag and pro)</td>
<td>0.0007</td>
<td>0.0007</td>
<td>0.0004</td>
</tr>
<tr>
<td>YU2gagpro</td>
<td>9.08 ± 0.24</td>
<td>9.00 ± 1.61</td>
<td>8.60 ± 1.26</td>
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<tr>
<td>YU2gag</td>
<td>14.05 ± 2.02</td>
<td>7.75 ± 0.91</td>
<td>6.95 ± 1.12</td>
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<tr>
<td>YU2pro</td>
<td>0.67 ± 0.03</td>
<td>3.82 ± 0.63</td>
<td>1.27 ± 0.63</td>
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<tr>
<td>P-value (gag and pro)</td>
<td>0.0014</td>
<td>0.0002</td>
<td>0.0018</td>
</tr>
<tr>
<td>YU2gagN</td>
<td>5.54 ± 0.55</td>
<td>7.08 ± 0.41</td>
<td>16.87 ± 3.39</td>
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<tr>
<td>YU2gagC</td>
<td>0.95 ± 0.21</td>
<td>0.97 ± 0.09</td>
<td>1.84 ± 0.46</td>
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<tr>
<td>P-value (gagN and gagC)</td>
<td>0.0015</td>
<td>0.0001</td>
<td>0.0118</td>
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<tr>
<td>SF2gagpro</td>
<td>4.02 ± 0.79</td>
<td>6.24 ± 0.82</td>
<td>5.64 ± 0.44</td>
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<tr>
<td>SF2gag</td>
<td>2.84 ± 1.20</td>
<td>1.94 ± 0.51</td>
<td>2.79 ± 0.44</td>
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<td>SF2pro</td>
<td>4.01 ± 0.38</td>
<td>1.81 ± 0.20</td>
<td>0.98 ± 0.29</td>
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<tr>
<td>P-value (gag and pro)</td>
<td>0.2017</td>
<td>0.4131</td>
<td>0.0129</td>
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<tr>
<td>89,gagpro</td>
<td>8.55 ± 1.33</td>
<td>4.48 ± 0.17</td>
<td>4.46 ± 1.03</td>
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<tr>
<td>89,gag</td>
<td>3.97 ± 0.72</td>
<td>4.39 ± 0.93</td>
<td>3.82 ± 0.44</td>
</tr>
<tr>
<td>89,pro</td>
<td>3.04 ± 0.18</td>
<td>2.23 ± 0.76</td>
<td>1.61 ± 0.35</td>
</tr>
<tr>
<td>P-value (gag and pro)</td>
<td>0.1053</td>
<td>0.0735</td>
<td>0.068</td>
</tr>
</tbody>
</table>

P-values derived from two-sample t-tests are given.

Role of the N-terminus of Gag in reduced susceptibility exhibited by JRFL and YU2 molecular clones

Further analysis was carried out for YU2 and JRFL, the two molecular clones in which Gag conferred solely reduced susceptibility, in order to identify the specific region of Gag conferring the reduced susceptibility. Chimeric Gag vectors were constructed using a unique SpeI restriction site, flanking amino acids 240 and 241 of Gag within the CA subunit. Two chimeric vectors for each molecular clone were generated: gagN, containing the N-terminus of Gag derived from the molecular clone and the C-terminus of Gag and protease from p8.9NSX+, and gagC, containing the C-terminus of Gag derived from the molecular clone and the N-terminus of Gag and protease from p8.9NSX+ (Fig. 3a).

Variation in PI susceptibility of full-length Gag–protease from treatment-naïve HIV-1-infected patients

Having demonstrated variation in susceptibility of full-length Gag–protease in molecular clones, the variation in PI susceptibility of viruses isolated directly from HIV-1-infected individuals was investigated. Full-length Gag–protease derived from four treatment-naïve patients was cloned into p8.9NSX+ and the PI susceptibility determined. Population sequencing and clonal analysis were carried out to determine the majority variant present in each patient. A majority variant could only be determined

In general, reversion of at least one of the amino acid positions increased the susceptibility of the virus to all three PIs. The difference in susceptibility between MGagpro and the mutant viruses was statistically significant for JRFL in susceptibility to APV and YU2 in susceptibility to ATV and LPV (P<0.05). For either clone, the independent effects of the single mutants on PI susceptibility were comparable with that of the double mutant, suggesting an additive rather than synergistic effect of the two amino acid changes. In addition, a single-cycle replicative capacity assay showed no effect of these changes on the replicative capacity of either molecular clone (Fig. S5). Overall, these data indicated that the R30K and E102D reversions contribute to the reduced susceptibility observed for full-length Gag–protease of JRFL and YU2.
for patient 1, whereas for patients 2, 3 and 4 the Gag sequences were more heterogeneous. Therefore, for patients 2, 3 and 4, the majority variants were selected as those with the least number of non-conserved amino acid changes from the amino acid sequence determined by population-based sequencing. This led to the selection of two viral variants each for patients 2 and 3 that were equally similar to the population sequence, and a single variant for patient 4.

Fig. 5 shows the variation in PI susceptibility of pseudovirions containing Gag–protease derived from treatment-naïve patient viruses. Gag–protease from patients 2 and 4 conferred reduced susceptibility to all PIs tested relative to the reference strain, and the reductions were far greater than shown for viruses derived from patients 1 and 3. This reduced susceptibility was most significant for APV, ATV, LPV and TPV ($P<0.01$), but was particularly pronounced for ATV where reductions of up to 17- and 16-fold in susceptibility were seen for patients 2 and 4, respectively. Of note, in patients 2 and 3 where two variants were tested, both showed broadly similar susceptibility levels to individual PIs.

**Contribution of Gag and protease to reduction in PI susceptibility observed for full-length Gag–protease in subtype B clinical viruses**

Pseudovirions derived from patients 2 and 4, in which significant reductions in PI susceptibility of Gag–protease were observed, were selected for further analysis. For patient 2, variant 1 was selected for further analysis as it showed the highest fold change in EC$_{50}$ values for APV, ATV, LPV and TPV ($P<0.01$), but was particularly pronounced for ATV where reductions of up to 17- and 16-fold in susceptibility were seen for patients 2 and 4, respectively. Of note, in patients 2 and 3 where two variants were tested, both showed broadly similar susceptibility levels to individual PIs.

**Fig. 3. PI susceptibility of chimeric viruses derived from the N-terminal and C-terminal ends of molecular-clone-derived Gag.** (a) Schematic diagram of chimeric viruses created containing either the N-terminus or C-terminus from each molecular clone. Segments derived from the reference strain are white and segments derived from the molecular clones are shaded grey. (b, c) The susceptibilities to three PIs (APV, ATV and LPV) are displayed for (b) JRFL and (c) YU2. *Viruses for which the fold changes in EC$_{50}$ values for Gag-only and protease-only chimeric viruses were statistically different ($P<0.05$).

**Fig. 4. PI susceptibility of molecular-clone-derived viruses containing reversion of two changes in the N-terminus of Gag.** (a) Schematic diagram of viruses containing reversions to the consensus B sequence of positions 30 and 102 of Gag, marked with a circle. (b, c) The susceptibilities to three PIs (APV, ATV and LPV) are displayed for (b) JRFL and (c) YU2. *Viruses for which the fold changes in EC$_{50}$ value were statistically different from that of MCgagpro ($P<0.05$).
The chimeric pseudovirions containing only protease from patient 2 and 4 viruses displayed reduced ATV susceptibilities of up to 10- and 14-fold for patient 2 and 4, respectively. Conversely, the Gag-only chimeric pseudovirions from both patients showed PI susceptibilities similar to the reference strain. The differences in the fold changes in EC50 values for the Gag-only and protease-only chimeric viruses were statistically significant for APV and ATV for viruses from both patients (P<0.05). These data showed that in both patients 2 and 4 the protease gene, and not the gag gene, is solely responsible for the reduced susceptibility observed for full-length Gag–protease. This is contrary to the effect observed with the molecular clones in this study, where the gag gene was shown to contribute significantly to the reduced PI susceptibility of full-length Gag–protease.

Although the analysis of protease sequences of the viruses from patients 2 and 4 using the Stanford HIVdb Genotypic Resistance Interpretation Algorithm showed no major PI resistance mutations, it indicated the presence of two minor resistance mutations (A71T and L63P) and a polymorphism (I13V) in both patients (Fig. S1). In addition, there were a number of other amino acid changes from the consensus subtype B protease in each of the patients: I15V, P39E, D60E, I72V, V771, I93L for patient 2 and K14R, E35D, N37T, R41K, I62V for patient 4. In contrast, viruses from patients 1 and 3 did not contain the changes A71T, I13V and L63P in combination, with only the L63P mutation present in patient 3. However, patients 1 and 3 shared minor resistance mutations L10I and L33I, and each had a number of other additional polymorphisms in protease: G17E, L19I, E35D, M36I, L63T, E65D, I72T in patient 1 and T12S, N37C, I62V, H69Q, I93L in patient 3.

**Effect of amino acid changes in protease on PI susceptibility of treatment-naive, patient-derived viruses**

We examined the role of the three changes in protease present in both patients 2 and 4 in the reduction in PI susceptibility exhibited by the two patient-derived viruses. The three protease positions were sequentially reverted to the consensus subtype B amino acid using site-directed mutagenesis, generating three vectors for each patient: IM (T71A), 2M (T71A and V13I) and 3M (T71A, V13I and P63L) (Fig. 7a). A71T was chosen to be studied alone as it was reported to be a minor resistance mutation to ATV, indinavir, LPV, nelfinavir and SQV (Johnson et al., 2011). Polymorphism I13V was reverted next as it was less common that L63P, which is a common polymorphism considered a minor resistance mutation to LPV only. In addition, the three protease changes were introduced into the p8.9NSX+ reference strain, p8.9_SDM. The susceptibilities of the three vectors for each patient and the p8.9_SDM vector were then tested.

Overall, reversion of protease positions 13, 63 and 71 to the consensus subtype B increased the susceptibility of the pseudovirus towards that of the reference strain (Fig. 7b, c). This effect was supported statistically for patient-2-derived pseudovirions to ATV. However, for some PIs reversion of A71T conferred a further reduction in PI susceptibility (for patient 2 to APV; for patient 4 to ATV and LPV). In addition, for APV reversion to WT did not appear to increase the susceptibility of the viruses towards that of the reference strain; in fact, reversion resulted in a virus significantly less susceptible for patient-2-derived viruses. Fig. 7(d) demonstrates that introduction of the three protease changes resulted in a small reduction in the susceptibility of the reference strain, of up to threefold for ATV. Taken together, the data provided evidence that the minor resistance mutations and polymorphism present in the protease from patients 2 and 4 contributed to the reduced susceptibility observed.

**DISCUSSION**

We have demonstrated significant variation in PI susceptibility of up to 17-fold in comparison with the reference strain. The differences in PI susceptibilities of up to threefold for patient-2-derived viruses were statistically significant for APV and ATV for viruses from both patients (P<0.05). These data showed that in both patients 2 and 4 the protease gene, and not the gag gene, is solely responsible for the reduced susceptibility observed for full-length Gag–protease. This is contrary to the effect observed with the molecular clones in this study, where the gag gene was shown to contribute significantly to the reduced PI susceptibility of full-length Gag–protease.
INTRT

For two molecular clones, JRFL and YU2, the reduced susceptibility of full-length Gag–protease from WT subtype B HIV-1 viruses. The susceptibilities of four molecular clones, YU2, SF2, JRFL and 89.6, were shown to be reduced compared to the reference strain for full-length Gag–protease from viruses derived from each patient. Segments derived from the reference strain are white and segments derived from patient-derived viruses are shaded grey. INT, integrase; RT, reverse transcriptase. (b, c) The susceptibilities to four PIs (APV, ATV, LPV and TPV) are shown for (b) patient-2- and (c) patient-4-derived viruses. *Viruses for which the fold changes in EC50 values for Gag-only and protease-only chimeric viruses were statistically different (P<0.05).

Fig. 6. PI susceptibility of patient-derived Gag-only and protease-only chimeric viruses. (a) Schematic diagram of chimeric viruses containing either Gag or protease from viruses derived from each patient. Segments derived from the reference strain are white and segments derived from patient-derived viruses are shaded grey. INT, integrase; RT, reverse transcriptase. (b, c) The susceptibilities to four PIs (APV, ATV, LPV and TPV) are shown for (b) patient-2- and (c) patient-4-derived viruses. *Viruses for which the fold changes in EC50 values for Gag-only and protease-only chimeric viruses were statistically different (P<0.05).

strain for full-length Gag–protease from subtype B viruses that have not been exposed previously to PIs. To our knowledge, this is the first demonstration of variation in PI susceptibility of full-length Gag–protease from WT subtype B HIV-1 viruses. The susceptibilities of four molecular clones, YU2, SF2, JRFL and 89.6, were shown to be reduced significantly in comparison with the reference strain and we demonstrate that this is caused, at least in part, by Gag. For two molecular clones, JRFL and YU2, the reduced susceptibility can be attributed solely to the gag gene (a region encompassing MA, CA, p2 and partial NC) and use of Gag chimeric viruses demonstrated that the N-terminal end of Gag (encompassing MA and the N-terminus of CA), and not the C-terminus, conferred this reduced susceptibility. Standard phenotypic or genotypic testing that includes only protease would not have captured this observed reduction in susceptibility. This is in keeping with other studies that have shown the role of Gag in PI susceptibility for subtype A and C molecular clones (Gupta et al., 2010), subtype A and CRF01_AE viruses from treatment-naïve HIV-1-infected patients (Gupta et al., 2010; Jinnopat et al., 2009), subtype B from treatment-experienced HIV-1-infected patients (Dam et al., 2009; Parry et al., 2009), and from PI-resistant viruses created by in vitro passage in the presence of PI (Nijhuis et al., 2007). The exclusion of the NC/p1/p6 cleavage sites from the Gag-only chimeric virus further emphasizes the role of other regions within Gag in PI susceptibility, as mutations in the NC/p1/p6 that affect PI susceptibility have already been described (Dam et al., 2009; Nijhuis et al., 2007). To date, two other studies have implicated the N-terminus of Gag in contributing to reduced susceptibility in an extensively treated patient infected with HIV-1 subtype B and a treatment-naïve patient infected with subtype CRF01_AE HIV-1 (Jinnopat et al., 2009; Parry et al., 2009).

The assay reference strain, p8.9NSX+ , is derived from HXB2 up to the SpeI site in Gag and from NL4-3 from the SpeI site onwards. Hence, the little variation in susceptibility observed for these molecular clones was expected. However, both HXB2 and NL4-3 displayed reduced susceptibility in comparison with the reference strain to APV. This further emphasizes the importance of considering full-length Gag when investigating PI susceptibility as in the absence of the co-evolved N-terminus of Gag the susceptibility of both HXB2 and NL4-3 was reduced. This provides evidence of an effect of separating co-evolved Gag and protease even in closely related viruses.

Amino acid alignment and site-directed mutagenesis showed the involvement of Gag amino acid positions 30 and 102 in PI susceptibility. Amino acid changes at these positions have not been previously reported to affect susceptibility to PIs. The exact mechanism by which changes at position 30 and 102 of Gag may affect PI susceptibility is unknown. However, positions surrounding amino acid 30 have been implicated in correct targeting of Gag to the plasma membrane (Ono & Freed, 2004). These amino acid changes are not uncommon, with lysine (K) at position 30 in 27.9 % of group M viruses and glutamic acid (E) at position 102 in 17 % (http://www.hiv.lanl.gov/). However, these positions do not confer solely the observed reduction in susceptibility, indicating that additional unique changes identified in the N-terminus of Gag of YU2 and JRFL must also contribute independently or in combination to the observed reduction in PI susceptibility (Fig. S3).

We demonstrate significant variation in PI susceptibility of full-length Gag–protease from viruses derived from two treatment-naïve patients, with reduced susceptibilities to four of the PIs tested: APV, ATV, LPV and TPV. The extent...
of the reduction in susceptibility was particularly pronounced for ATV, with patient-2-derived viruses showing up to 17-fold reduction in EC₅₀ in comparison with the reference strain. This is of particular importance given the role of ATV as a first-line PI (London HIV Consortium, 2011).

Further experiments have shown that protease is the sole contributor to the reduced PI susceptibilities for patients 2 and 4. This finding was contrary to our observations of the molecular clones, where Gag was shown to contribute at least in part to the reduced susceptibility. It is not clear why the observations were different between the molecular-clone and patient-derived viruses, and although we have not shown evidence of a role of Gag in PI susceptibility of patient-derived viruses, a number of other studies have (Gupta et al., 2010; Jinnopat et al., 2009). Molecular clones YU2 and JRFL, in which reduced susceptibility was conferred solely by Gag, were both derived directly from brain tissue. JRFL was subjected to limited cell culture, but YU2 was not subjected to any tissue culture and the remaining molecular clones were subjected to more extensive cell culture before their derivation (Koyanagi et al., 1987; Li et al., 1991). It is possible that tissue culture adaptation resulted in changes in Gag that are not present in JRFL and YU2, explaining the difference in susceptibility between these two molecular clones and the other four. However, this does not explain the difference in the role of Gag in susceptibility between our patient-derived virus and molecular clones. It is also possible that variants in which Gag conveyed reduced susceptibility were present within the quasi-species within a patient, but were not detected by clonal analysis. Another possibility is that changes in Gag occur to favour replication in the central nervous system, from where both JRFL and YU2 were isolated.

Three amino acid changes in protease were present in both patient-2- and patient-4-derived viruses, A71T, I13V and L63P, and we show that these changes contribute in part to the observed reduction in susceptibility. The Stanford HIV/db Genotypic Resistance Interpretation Algorithm predicted that these patient viruses were fully susceptible to all PIs in this study, hence their inclusion. Our data indicate that the presence of a combination of minor resistance mutations and polymorphisms in protease (here, I13V, L63P and A71T) can convey reduced susceptibility to

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**Fig. 7.** Effect of protease changes on PI susceptibility in viruses derived from two patients. (a) Schematic representation of the three vectors created for each patient using site-directed mutagenesis. (b, c) The susceptibilities to four PIs (APV, ATV, LPV and TPV) are shown for (b) patient 2 and (c) patient 4. *Viruses for which the fold changes in EC₅₀ values were statistically different from that of Ptgagpro (P<0.05). (d) Schematic of p8.9_SD, the p8.9NSX + reference strain with the three protease changes introduced, and its susceptibility to four PIs.
PIs in the absence of major PI resistance mutations. Each of these changes are present in subtype B treatment-naïve viruses, I13V in 15.1%, L63P in 60.2% and A71T in 7.2%. Of these, L63P is found at higher frequency in treated patients, providing further evidence of a role in PI susceptibility (Dolling et al., 2013). The mechanism by which these protease positions may affect PI susceptibility is unknown, as computer modelling showed that they are not located near the active site of the protease enzyme (Fig. S6).

A previous study used statistical analyses to predict a small fold change in EC$_{50}$ values to LPV when I13V, L63P and A71T were found together with other changes, but the study did not examine the specific effect of these three amino acids alone nor were the three other PIs studied here considered (Kempf et al., 2008). In addition, two studies have been carried out that demonstrate the role of these three protease changes with an increased rate of PI resistance development during passage in vitro in the presence of drug (Lisovsky et al., 2010; Vergne et al., 2006). In vivo, a number of studies have reported an association between these protease changes and reduced rates of virological response in patients when present at baseline (Baxter et al., 2006; Johnson et al., 2005; Marcelin et al., 2007; Pellegrin et al., 2006, 2008). This indicates that the precise mechanisms of development of resistance to PIs and the role of protease polymorphisms remain to be elucidated fully.

This study provides further evidence for the co-evolution of Gag and protease, and the importance of considering them together when studying PI susceptibility. For example, our data show a greater reduction in susceptibility to APV for Gag-only chimeric viruses than for full-length Gag–protease virus for JRFL (Figs 1 and 2b). In addition, the protease-only chimeric virus for patient 4 had a significant reduction in susceptibility to APV in comparison with that of Gag–protease (Figs 5 and 6c). Commercial phenotypic PI susceptibility assays include only protease and a short length of the C-terminus of Gag from the patient (the portion of Gag included is p6, which overlaps with the protease in the Gag–Pol transframe region), with the rest of the HIV genome coming from the test vector (Hertogs et al., 1998; Petropoulos et al., 2000). However, our data demonstrate that the N-terminus of Gag is an important region in the determination of PI susceptibility. Since our study included a relatively small number of viruses, further investigation is required to determine whether inclusion of co-evolved Gag alongside protease in phenotypic assays is necessary.

Our data demonstrate that both Gag and protease contribute to variation in PI susceptibility of treatment-naïve viruses of subtype B, highlighting the importance of considering Gag and protease together when assessing PI susceptibility, and indicating that the precise mechanisms determining PI susceptibility and resistances are yet to be fully elucidated.

**METHODS**

**HIV-1 molecular clones and clinical samples.** The following six subtype B molecular clones were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program: pYU2, pSF2, pJRFL, p89.6, pHXB2 and pNL4-3. Plasma samples from HIV-1-infected patients were obtained from the HIV drug resistance genotypic testing service at Public Health England – Colindale. Anonymized samples from new diagnoses were selected based on viral load, subtype (B), treatment history (naïve) and absence of major drug resistance mutations.

**Resistance test vectors.** This study utilized a previously described HIV Gag–Pol expression vector, p8.9NSX+ (Parry et al., 2009). Full-length gag–protease was cloned from the molecular clones into p8.9NSX+ using primers GagNot+ (5'-CGCGGCGCCGCAAGGAGAGATGGGTGCG-3') and ProXhoR2 (5'-CTGGTGACTGCTCCGAGRGGACTRATKGG-3') to introduce NofI and Xhol restriction sites. The gag–protease PCR fragment was directly cloned into p8.9NSX+ , digested with NofI and Xhol, using the Rapid DNA Dephos and Ligation kit (Roche).

For patient samples, viral RNA was extracted manually from plasma samples using the QIAamp UltraSens Virus kit (Qiagen). Following RNA denaturation, cDNA synthesis was carried out using the antisense primer ProOutR (5'-TTGGGGCATCATTCTCAGG-3'), RNaseOut (Invitrogen) and SuperScript III enzyme (Invitrogen). Nested PCRs to amplify the gag–protease region with Expand High Fidelity PCR system (Roche) utilized the following primers in the first round: sense primer BKT03 (5'-GGCGAGACTGCGGTTG-3') and antisense primer ProOutR (5'-TTGGGGCATCATTCTCAGG-3'), and GagNot+ and ProXhoR2 in the second round. PCR products were then TA-cloned into the pGEM-T vector (Promega). The clones were individually and bulk sequenced to determine the majority Gag variant. The majority variants were then cloned into the p8.9NSX+ vector using NofI and Xhol restriction sites, as described above.

Chimeric gag–protease p8.9NSX+ vectors were created by swapping gag and protease gene segments using the unique Sphl and AapI sites, flanking amino acids 240 and 241 (CA) and amino acids 406 and 407 (NC), respectively. Since the AapI site is present towards the end of the gag gene, a small portion of gag is included in the protease fragment – part of NC, p1 and p6. Site-directed mutagenesis was performed using the Quik-Change Lightning Site-Directed Mutagenesis kit (Stratagene).

**PI susceptibility.** PI susceptibility was determined using a single-replication-cycle phenotypic drug susceptibility assay described previously (Gupta et al., 2010; Parry et al., 2009). Briefly, 293T cells were co-transfected with the p8.9NSX+–derived viral test vector, pMDG expressing vesicular stomatitis virus envelope glycoprotein (VSV-g), and pCSELFW expressing the firefly luciferase reporter gene and containing HIV-1 packaging signal. Cells were harvested 16 h post-transfection and seeded with serial dilutions of PI. After 24 h, harvested pseudovirus was used to infect fresh 293T cells and the infectivity was monitored by measuring luciferase activity 48 h after infection using Steady-Glo (Promega). From these data, drug concentrations required to inhibit 50% of viral infection (EC$_{50}$) were determined by linear regression and data were expressed as fold change in EC$_{50}$ values compared with the reference virus (p8.9NSX+ ). For each experiment, infection of cells in the absence of PI was carried out to account for variation in transfection efficiency and replicative capacity between test viruses, and the luciferase activity of virus in the absence of cells was determined to account for changes in background luminescence. Assays were performed in triplicate for each virus construct.

**Statistical analyses.** Unpaired, two sample t-tests were carried out on raw EC$_{50}$ or fold-change EC$_{50}$ data to test for significant differences.
Gag mutations strongly contribute to HIV-1 resistance to compensating for fitness loss. Protease inhibitors in highly drug-experienced patients besides multiple protease inhibitors. Isolates from patients treated with antiretroviral drugs. Phenotypic resistance to inhibitors of protease and reverse transcriptase. Resistance database (UKHDRD) and the UK Collaborative HIV Cohort Study (UK CHIC) (2013).

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REFERENCE


