HIV-1 protease inhibitor mutations affect the development of HIV-1 resistance to the maturation inhibitor bevirimat

Axel Fun¹, Noortje M van Maarseveen¹, Jana Pokorná², Renée EM Maas¹, Pauline J Schipper¹, Jan Konvalinka² and Monique Nijhuis¹*

Abstract

Background: Maturation inhibitors are an experimental class of antiretrovirals that inhibit Human Immunodeficiency Virus (HIV) particle maturation, the structural rearrangement required to form infectious virus particles. This rearrangement is triggered by the ordered cleavage of the precursor Gag polyproteins into their functional counterparts by the viral enzyme protease. In contrast to protease inhibitors, maturation inhibitors impede particle maturation by targeting the substrate of protease (Gag) instead of the protease enzyme itself. Direct cross-resistance between protease and maturation inhibitors may seem unlikely, but the co-evolution of protease and its substrate, Gag, during protease inhibitor therapy, could potentially affect future maturation inhibitor therapy. Previous studies showed that there might also be an effect of protease inhibitor resistance mutations on the development of maturation inhibitor resistance, but the exact mechanism remains unclear. We used wild-type and protease inhibitor resistant viruses to determine the impact of protease inhibitor resistance mutations on the development of maturation inhibitor resistance.

Results: Our resistance selection studies demonstrated that the resistance profiles for the maturation inhibitor bevirimat are more diverse for viruses with a mutated protease compared to viruses with a wild-type protease. Viral replication did not appear to be a major factor during emergence of bevirimat resistance. In all in vitro selections, one of four mutations was selected: Gag V362I, A364V, S368N or V370A. The impact of these mutations on maturation inhibitor resistance and viral replication was analyzed in different protease backgrounds. The data suggest that the protease background affects development of HIV-1 resistance to bevirimat and the replication profiles of bevirimat-selected HIV-1. The protease-dependent bevirimat resistance and replication levels can be explained by differences in CA/p2 cleavage processing by the different proteases.

Conclusions: These findings highlight the complicated interactions between the viral protease and its substrate. By providing a better understanding of these interactions, we aim to help guide the development of second generation maturation inhibitors.

Background

Maturation is an essential step in the life-cycle of human immunodeficiency virus type 1 (HIV-1). It is the transition of the immature, non-infectious virus particle to the mature and infectious virion and is triggered by the proteolytic cleavage of the precursor Gag (Pr55Gag) and GagPol (Pr160GagPol) polyproteins by the viral enzyme protease. Gag is cleaved into the structural proteins matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7), p6 and two small spacer peptides (p1 and p2). This protease-mediated cleavage elicits the structural rearrangement that results in the dense conical core, characteristic of infectious HIV-1 particles.

Since immature particles are non-infectious, particle maturation is an excellent target for antiretroviral drugs. Protease inhibitors (PI) successfully inhibit viral replication by targeting the enzyme responsible for maturation and have played a major role in antiviral therapy since
their introduction in 1995. So far, nine different PIs have been approved for clinical use. However, a high degree of cross-resistance between protease inhibitors limits the utility of these inhibitors if PI resistance emerges.

Maturation inhibitors are a new class of antiretrovirals that also impede particle maturation but do so by targeting the substrate of protease (Gag) instead of the protease enzyme itself. Therefore, direct cross-resistance between PIs and maturation inhibitors may seem unlikely. However during PI treatment, co-evolution of the viral protease and its substrate Gag is common, which may have an effect on the subsequent utility of maturation inhibitors [1-5]. Several maturation inhibitors are or have been in development including; bevirimat (BVM, Panacos PA-457, Myriad MPC-4326); PA1050040, which is a second generation maturation inhibitor from Panacos [6], based on bevirimat; two maturation inhibitors from Myriad Pharmaceuticals, Vivecon (MPC-9055) [7,8] and MPI-461359 [9]; PF-46396 [10] from Pfizer and several capsid assembly inhibitors including CAP-1 [11], CAI[12], and BI-257, BI-627 and BI-720 from Boehringer-Ingeheim[13]. Bevirimat was the first of these maturation inhibitors to go into clinical trials and inhibits HIV-1 replication by specifically blocking cleavage of CA from p2, one of the final (rate-limiting) steps in the Gag processing cascade. Incomplete processing of CA from CA-p2 (p25) results in unsuccessful particle maturation and, therefore, non-infectious virions [14]. The CA/p2 cleavage site (CS) has been identified as the bevirimat target region by Western-blotting and in vitro resistance selection studies [14,15]. Nonetheless, the mechanism of action of bevirimat is still poorly understood as the actual binding site of bevirimat has not been identified. Recently, it has been shown that, besides sterically blocking the CA/p2 junction, bevirimat may have a stabilizing effect on the immature Gag lattice. This indicates that bevirimat binds during assembly and must be incorporated to inhibit maturation, which offers an explanation why bevirimat is unable to prevent cleavage of free Gag in solution[16].

Initial in vitro selection studies identified bevirimat resistance mutations in the CA/p2 cleavage site at Gag positions 358, 363, 364 and 366 [15]. Phase 2b clinical studies demonstrated that baseline polymorphisms slightly downstream of the CA/p2 cleavage site (Gag aa 369, 370 and 371, known as the QVT-motif) also confer resistance [17,18].

We previously showed that bevirimat resistance mutations are more frequently observed in PI resistant but bevirimat naïve HIV-1 isolates, compared to PI and bevirimat treatment naïve isolates; and this was mainly attributed to an accumulation of mutations in the QVT-motif [19]. This study also showed that mutations associated with bevirimat resistance were detected more frequently in HIV-1 isolates with three or more PI resistance mutations than in those with less than three PI resistance mutations. Conversely, Adamson and colleagues suggested that mutations in the viral protease affecting viral replication may delay the selection of maturation inhibitor resistance [20].

To better understand the effect of PI therapy on viral susceptibility to maturation inhibitors, we set up a maturation inhibitor model system. We performed multiple in vitro selection studies with ten different viruses that contained PI resistance mutations in the viral protease and/or Gag CS and that displayed a broad range of replication capacities (RC). Subsequently, we conducted a detailed analysis of the identified resistance mutations. The data in this paper clearly demonstrate that PI resistance mutations alter the resistance profiles for the maturation inhibitor bevirimat. We also show that the protease background determines the level of maturation inhibitor resistance and viral replication.

Results

In vitro selections

To assess the impact of different PI resistant backgrounds on selection of bevirimat resistance, we performed multiple in vitro resistance selection studies with a set of ten different viruses. Two wild-type viruses (HXB2 and NL4-3), two viruses that harbored PI resistance mutations in the viral protease (Table 1) were studied. The broad range of replication capacities of these viruses (Figure 1) allowed us to investigate the impact of RC on selection of bevirimat resistance.

During the in vitro selection experiments, there were no major differences in the rate of virus propagation in the presence of bevirimat between wild-type viruses and viruses with PI resistance mutations in the viral protease. Regardless of their RC, all viruses reached full-blown cytopathic effect (CPE) in a comparable number of days during each serial passage. However, independent of their RC, viruses with NC/p1 CS mutations (without mutations in protease), showed delayed propagation. The delay was primarily the result of a relatively long first passage, with subsequent passages being similar in duration to those of the other viruses (see Additional file 1).

After five serial passages to a final concentration of 240 nM bevirimat, RNA was isolated from all viruses and full Gag and protease genes sequenced. In all cultures, mutations in or near the CA/p2 cleavage site were found clearly supporting the hypothesis that this is the main target region for bevirimat (Tables 2 and 3). Gag
mutations outside this region were found only in a small number of isolates and appeared to be random. The protease gene was completely conserved in all viruses (data not shown).

Viruses with wild-type proteases (HXB2, NL4-3 and NC/p1 variants) selected for Gag mutation A364V in 26 of 28 cultures, with additional mutations observed in 4 of these 26: two cultures had V362I+A364V; one had A366V+A364V and another one V370A+A364V (Table 2). These combinations of mutations were thought to represent separate populations, with no viruses harboring two CA/p2 mutations on one genome. This was confirmed by clonal analysis of one culture containing multiple mutations (culture HXB2 #8; V362I+A364V, data not shown). In the two cultures where A364V was not selected, mutations V362I and V370A were observed respectively.

In contrast to viruses with WT proteases, viruses with PI resistant proteases (PR-1-PR-6) showed a more diverse resistance pattern (Table 3) with a significantly higher prevalence of mutations at position 362, 368 and in the QVT-motif (V370A/L and T371N, Table 4).

In summary, we identified 8 bevirimat resistance mutations at 7 different codons: V362I, L363M, A364V, A366V, S368N, V370A/L and T371N. Most of these mutations have been selected during in vitro selections.

HXB2 and NL4-3 are subtype B reference viruses. Mutations are as compared to HXB2. All amino acid differences in the viral protease are listed. All protease inhibitor (PI) resistance mutations, as defined by the International AIDS Society[37] are in bold. In addition, mutations in the NC/p1 cleavage site are listed. The CA/p2 cleavage site of the ten viruses was identical. PR-1 is clone 460.2 from Nijhuis et al. [21] and PR-2 through PR-6 are the B6 clones from Maarseveen et al. [30]. PR-3 - PR-6 are site directed mutants created from PR-2. In each of these clones one PI resistance mutation was reverted to wild-type, PR-3 - PR-6 lack PI resistance mutation 46I, 54V, 82A and 90M respectively. The NC/p1 variants only differ from HXB2 at the positions indicated in the table. The level of PI resistance was determined for these viruses against lopinavir (LPV) and atazanavir (ATV). PI resistance is expressed as fold change in EC50 compared to HXB2.

Table 1 Characteristics of the ten viruses that were used for the in vitro selection experiments

| HIV-1 variant | Mutations compared to HXB2 | PI resistance |
|---------------|---------------------------|---------------|
|               | Gag           | Protease     | LPV | ATV |
| HXB2          | -             | -            | -   | -   |
| NL4-3         | -             | 3I-37N       | 1   | 1   |
| PR-1          | 3I-20R-35D    | 36I-54V-63P-71V-82T | 12.2 | 5.6 |
| PR-2          | 431V          | 3I-10I-13V-35D-36I-54V-55R-75K-62V-63P-71T-82A-90M-93L-95F | 15.1 | 7.8 |
| PR-3          | 431V          | 3I-10I-13V-35D-36I-54V-55R-75K-62V-63P-71T-82A-90M-93L-95F | >120 | >120 |
| PR-4          | 431V          | 3I-10I-13V-35D-36I-54V-55R-75K-62V-63P-71T-82A-90M-93L-95F | 8.1  | 11.6|
| PR-5          | 431V          | 3I-10I-13V-35D-36I-54V-55R-75K-62V-63P-71T-82A-90M-93L-95F | 10.8 | 8.9 |
| PR-6          | 431V          | 3I-10I-13V-35D-36I-54V-55R-75K-62V-63P-71T-82A-93L-95F | 19.6 | 5.9 |
| NC/p1         | 431V          | -            | 2.6 | 1.3 |
| NC/p1         | 436E-437T     | -            | 4.7 | 3.3 |

HXB2 and NL4-3 are subtype B reference viruses. Mutations are as compared to HXB2. All amino acid differences in the viral protease are listed. All protease inhibitor (PI) resistance mutations, as defined by the International AIDS Society[37] are in bold. In addition, mutations in the NC/p1 cleavage site are listed. The CA/p2 cleavage site of the ten viruses was identical. PR-1 is clone 460.2 from Nijhuis et al. [21] and PR-2 through PR-6 are the B6 clones from Maarseveen et al. [30]. PR-3 - PR-6 are site directed mutants created from PR-2. In each of these clones one PI resistance mutation was reverted to wild-type, PR-3 - PR-6 lack PI resistance mutation 46I, 54V, 82A and 90M respectively. The NC/p1 variants only differ from HXB2 at the positions indicated in the table. The level of PI resistance was determined for these viruses against lopinavir (LPV) and atazanavir (ATV). PI resistance is expressed as fold change in EC50 compared to HXB2.

Figure 1 Replication capacity of the ten viruses that were used for the in vitro selection experiments. Replication capacities (RC) were determined by culturing the viruses in SupT1 cells in absence of inhibitor and monitoring p24 production[28]. Error bars indicate the standard deviation. Replication of NL4-3 is comparable to that of HXB2 (not shown).
in previous studies, or have already been associated in vivo with reduced bevirimat susceptibility, except for the mutation at position 368, which was considered a potential new resistance mutation. In all 58 isolates at least one of the following mutations was found: Gag V362I, A364V, S368N or V370A/L.

### Impact of PI resistance mutations on bevirimat resistance and viral replication

To characterize the different bevirimat resistance profiles observed in viruses with wild-type and PI resistant proteases, we investigated the impact of the four most frequently selected mutations on bevirimat susceptibility.

| Wild-type proteases | CA | p2 |
|---------------------|----|----|
|                     | 359| 360| 361| 362| 363| 364| 365| 366| 367| 368| 369| 370| 371 |
| HXB2 aa             | K  | A  | R  | V  | L  | A  | E  | A  | M  | S  | Q  | V  | T  |
| WT                  | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
| HXB2 n = 10         | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
| WT                  | -  | -  | -  | -  | -  | A/V| -  | -  | -  | -  | -  | -  | -  |
| NL4-3 n = 10        | -  | -  | -  | -  | -  | A/V| -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
| NC/p1 431V n = 4    | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
| NC/p1 436E-437T n = 4| -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |
|                     | -  | -  | -  | -  | -  | V  | -  | -  | -  | -  | -  | -  | -  |

Schematic representation of the amino acid changes appearing in the CA/p2 region during bevirimat in vitro selection experiments with wild-type HIV-1 or NC/p1 mutants. In vitro selections with wild-type viruses (HXB2 and NL4-3) were performed 10 times, the NC/p1 variants 5 times (n = 4 because one culture was discontinued for each virus). Mutations that previously have been identified in vitro as bevirimat resistance mutations are indicated in bold. The QVT-polymorphisms that are associated with a reduced response to bevirimat in vivo are underlined. The actual CA/p2 cleavage site is between amino acids 363 and 364.
and viral replication in different genetic backgrounds. Therefore we introduced Gag mutations V362I, A364V, S368N or V370A by site-directed mutagenesis in the background of HXB2, PR-1 and PR-2. The bevirimat susceptibility and the relative replication capacity of these 12 viruses were determined.

In wild-type HXB2, mutations A364V and V370A conferred the highest level of resistance (Table 5). Both mutations resulted in a complete lack of inhibition even at 3000 nM (> 150-fold reduced susceptibility to bevirimat, Table 5 and Additional file 2, panels A and B). Mutations V362I and S368N resulted in low-level

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Table 3 Mutations selected in viruses with PI resistant proteases during the bevirimat in vitro selections

| Gag position | 359 | 360 | 361 | 362 | 363 | 364 | 365 | 366 | 367 | 368 | 369 | 370 | 371 |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| HXB2 aa      | K   | A   | R   | V   | L   | A   | E   | A   | M   | S   | Q   | V   | T   |
| PR-1 n=5     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PR-2 n=5     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PR-3 n=5     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PR-4 n=5     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PR-5 n=5     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PR-6 n=5     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |     |     |     |     |     |     |     |     |     |     |     |     |     |

Schematic representation of the amino acid changes appearing in the CA/p2 region after bevirimat in vitro selection experiments with the PR-1 - PR-6 mutants. In vitro selections with the protease mutants were performed 5 times. Mutations that previously have been identified in vitro as bevirimat resistance mutations are indicated in bold. The QVT-polymorphisms that are associated with a reduced response to bevirimat in vivo are underlined. Previously unknown mutations are printed in italic type. The actual CA/p2 cleavage site is between amino acids 363 and 364.
levels conferred by bevirimat resistance mutations in different genetic backgrounds, we performed a biochemical analysis of the specific cleavage efficiencies. The effect of mutations V362I and A364V on CA/p2 processing was analyzed in the background of HXB2 and PR-2 proteases. Nonapeptides representing the WT CA/p2 cleavage site, or containing bevirimat resistance mutation V362I or A364V, were processed with either the HXB2 or the PR-2 protease enzyme. Although the absolute cleavage efficiency of PR-2 was lower compared to HXB2, the relative increase in processing caused by

Table 5 Impact of PI resistance mutations on bevirimat resistance

| Virus         | Fold resistance bevirimat |
|---------------|---------------------------|
|               | - | V362I | A364V | S368N | V370A |
| HXB2          | - | 2.8   | > 150 | 6.6   | > 150 |
| PR-1 (20R-36I-54V-63P-71I-82T) | 0.6 | 2.1   | > 150 | 6.0   | > 150 |
| PR-2 (431V-10I-13V-36I-46I-54V-63P-71T-82A-90M-93L) | 2.1 | > 150 | > 150 | > 150 |
Figure 2 Impact of bevirimat resistance mutations on viral replication in different genetic backgrounds. Viruses were cultured in SupT1 cells in absence of inhibitor and p24 production was monitored for 14 days. All viruses were tested in duplicate. Error bars indicate the standard deviation. Replication curves of (A) the HXB2 site-directed mutants, (B) the PR-1 mutants and (C) the PR-2 mutants.
adding mutation V362I or A364V was approximately 50% greater for the PR-2 protease than for the HXB2 protease (Table 6). For both proteases, processing of the peptide with mutation A364V was one order of magnitude faster compared to V362I.

Discussion

Maturation inhibitors are an experimental class of antiretrovirals that prevent HIV-1 replication by targeting the structural proteins essential for particle maturation and thus formation of infectious virions. The target region for maturation inhibitors, Gag, is the same as the natural substrate of the viral protease. Co-evolution of protease and Gag during PI therapy[1,5,21-23] may, therefore, have consequences for the subsequent use of maturation inhibitors. We used bevirimat in a model system to study the impact of PI therapy on the development of resistance against maturation inhibitors. To date, no direct cross-resistance between PIs and bevirimat has been observed[14,20,24], but it is conceivable that reduced viral replication, as often caused by PI resistance mutations, influences the emergence of bevirimat resistance. Therefore, we wanted to include the effect of viral replication capacity on development of maturation inhibitor resistance in our studies. We chose viruses PR-1 through PR-6 for our resistance selection studies because of their broad range of replication capacities.

During in vitro selection, there were only small differences in rates of virus propagation in the consecutive passages between the wild-type and PR-1 - PR-6 viruses. We did not find a clear correlation between the rate of selection for bevirimat resistance and the viral replication capacity. The differences within the individual cultures from a particular molecular clone were often larger than the differences between the averages of the various clones. However, the viruses with mutations only in the NC/p1 cleavage site appeared to have a delayed emergence of bevirimat resistance. This delay cannot be explained by viral replication capacity since this was comparable for the 431V mutant and wild-type virus. A possible explanation is that altering both rate-limiting cleavage sites (CA/p2 and NC/p1) without having an adapted protease is unfavorable for the virus.

In all our in vitro selection cultures, mutations were selected in or slightly downstream from the CA/p2 cleavage site. We showed that PI resistance mutations have a substantial impact on the selection of bevirimat resistance: the resistance profiles were remarkably different for viruses with PI resistant proteases compared to wild-type proteases. Mutation A364V occurred most frequently and was associated with a completely resistant phenotype in all three protease backgrounds (HXB2, PR-1 and PR-2). This mutation had no effect on replication in an HXB2 background, which might explain the almost exclusive selection of A364V by viruses with a wild-type protease. We also observed selection of multiple QVT-mutations. Although these mutations are known to cause bevirimat resistance, until recently they had only been found as naturally occurring polymorphisms in clinical isolates demonstrating reduced bevirimat susceptibility[17,18,25]. Knapp and colleagues showed selection of QVT-mutations in a different experimental setup in which they used mixed, clinically derived gag-protease recombinant HIV-1 samples to select for bevirimat resistance[26]. We have now shown that QVT-mutations can also be selected by clonal strains and wild-type virus. However, they are much more often selected by viruses with a mutated protease, which is in line with our previous in vivo observations[19]. We also showed this to be the case for mutation V362I, which recently has been identified as a natural polymorphism that confers bevirimat resistance[27]. In addition, we identified a previously unknown bevirimat resistance mutation, S368N. This mutation was not found in any cultures with wild-type proteases, but appeared frequently in cultures with PI resistant proteases.

Our results indicate that the protease background determines the level of resistance and the impact on replication. When introduced into the PR-2 background, mutations V362I and S368N result in much higher levels of resistance than in backgrounds HXB2 or PR-1. High levels of bevirimat resistance for mutation V362I have also been observed in other genetic backgrounds [27]. A possible explanation for these observations is the difference in cleavage efficiencies of the Gag substrate by the viral protease. It has previously been reported that the level of bevirimat resistance is reduced in a PI resistant virus with a reduced Gag processing efficiency [20]. We show that processing of the CA/p2 cleavage site is accelerated by the presence of a bevirimat

| Substrate | Relative substrate conversion |
|-----------|-----------------------------|
|           | HXB2 | PR-2 | (PR-2/HXB2) |
| WT        | 1    | 1    | -          |
| V362I     | 0.87 | 1.3  | 1.49       |
| A364V     | 7.6  | 11   | 1.45       |

Comparison of the CA/p2 processing efficiencies of the HXB2 and PR-2 proteases. Three different nonapeptides representing the CA/p2 cleavage site were cleaved with either the HXB2 or the PR-2 protease: 1. wild-type (WT) KARVL\_\_AEANLe-NH\_2; 2. (V362I) KARVL\_\_AEANLe-NH\_2; and 3. (A364V) KARVL\_\_AEANLe-NH\_2. The bevirimat resistance mutations are underlined and the arrow indicates the actual junction. The cleavage efficiency of the WT substrate was set to 1, conversion of substrates with V362I or A364V was measured relative to the conversion of the WT substrate. The test was performed in triplicate.
resistance mutation, which is likely to augment bevirimat resistance, parallel to what is observed for PI resistance[4,5]. Both proteases that were tested (HXB2 and PR-2) processed substrate with mutation A364V one order of magnitude more effectively than substrate with a V362I mutation. This might explain the high levels of bevirimat resistance conferred by A364V in all backgrounds. Furthermore, the relative increase in substrate conversion is greater in the context of the PR-2 protease compared to the HXB2 protease and we hypothesize that this relative increase in CA/p2 processing contributes to the enhanced bevirimat resistance levels observed for the PR-2GagV362I and PR-2GagS368N viruses.

Conclusions
Like most new drug classes, maturation inhibitors are likely to be introduced as part of salvage therapy. The majority of patients requiring new therapeutic options will be infected with viruses that harbor multiple resistance mutations, most likely including PI resistance mutations. Therefore, it is essential to understand the consequences of prior treatment with PIs for the use of maturation inhibitors. Our data show that predicting treatment responses for maturation inhibitors might not be straightforward and that the complex interactions between protease and Gag have to be taken into account.

The development of new and more potent maturation inhibitors should therefore aim to overcome the issues encountered by the current drugs with virus containing the baseline polymorphisms found in the C-terminal Gag region (QVT-mutations) and, ideally, new maturation inhibitors would exhibit synergy with protease inhibitors. They should capitalize on the reduced processing often caused by PI resistance mutations in such a way that there is added value from the use of a maturation inhibitor in salvage therapy for PI experienced patients.

Methods
Viral and cell culture
Cells
293T cells were maintained in DMEM with L-glutamine (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Zwijndrecht, The Netherlands) and 10 μg/ml gentamicin (Invitrogen, Breda, The Netherlands). SupT1 and MT-2 cells were maintained in RPMI 1640 with L-glutamine (Lonza) supplemented with 10% FBS and 10 μg/ml gentamicin.

Recombinant virus panel
We selected a panel of ten different viruses for in vitro resistance selection studies (Table 1). Two wild-type viruses (HXB2 and NL4-3), six recombinant viruses with PI resistance mutations in the viral protease (PR-1 - PR-6) and two recombinant viruses without mutations in the viral protease but with PI resistance associated mutations in the Gag NC/p1 cleavage site (NC/p1 431V and NC/p1 436E-437T). PR-1 and PR-2 were gag-protease recombinant viruses from patient isolates that had acquired resistance mutations during long-term PI therapy and have different resistance profiles and replication capacities. PR-1 was selected because it displayed wild-type replication kinetics despite the presence of multiple PI resistance mutations. In contrast, like most PI resistant isolates, PR-2 had a slightly defective replication compared to wild-type (Figures 1 and 2C). PR-3 through to PR-6 are site directed mutants created from PR-2 in which in each of these clones one PI resistance mutation was reverted to wild-type. This resulted in dramatic changes in RC (Figure 1) while the mutations were very stable; they remained present and no additional protease mutations were acquired during long term culture in T cells in the absence of PIs [28]. These findings were consistent with other studies that described a significant effect on RC of a single mutation in the viral protease[29-31]. The NC/p1 variants had divergent replications capacities and both conferred low-level PI resistance in the absence of mutations in the viral protease. Fold changes against the commonly used PIs lopinavir and atazanavir are given in Table 1 for all variants.

In vitro selections with wild-type viruses HXB2 and NL4-3 were performed 10 times (10 parallel in vitro selections per virus), all other viruses 5 times. One culture of each NC/p1 variant was discontinued because of inadequate viral replication.

Transfections
Viruses were generated by transfecting 293T cells with 10 μg of plasmid DNA of the molecular clones using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. Cell free virus was harvested 2 days after transfection. Infectious virus titer (TCID50) was determined by end-point dilution assays in MT-2 cells.

In vitro selections
Multiple in vitro selection experiments were started simultaneously for all viruses, 10 times with reference viruses HXB2 and NL4-3 and 5 times with all other viruses in. The in vitro resistance selections were started by infecting 2.0 × 10⁶ SupT1 cells with virus at a multiplicity of infection (MOI) of 0.001. Bevirimat concentration in the initial cultures was 20 nM. Cultures were monitored daily for cytopathic effect (CPE) and twice a week half of the culture was replaced by fresh culture media supplemented with bevirimat. When full-blown CPE was observed, cell free virus was harvested. Subsequent passages were started by infecting 2.0 × 10⁶ SupT1 cells with virus containing supernatant from the
previous passage. Bevirimat concentration was raised in each passage to a final concentration of 240 nM in passage 5. After passage 5, HIV-1 RNA was isolated from all cultures for genotypic analysis.

Genotypic analysis

**Viral RNA extraction, amplification and sequencing**

HIV-1 RNA was extracted using the Nuclisens Isolation kit (BioMérieux, Boxtel, The Netherlands). 100 μl of virus supernatant was added to 900 μl lysis buffer with 40 μl silica beads. After 10 minutes incubation, beads were washed twice in wash buffer, twice in 100% ethanol and once in acetone and subsequently air-dried. RNA was eluted at 56°C with 100 μl of 40 ng/μl poly-A RNA. Full Gag and protease genes were reversed transcribed and amplified in a single-step reaction using the Titan One Tube RT-PCR kit (Roche Diagnostics, Almere, The Netherlands). In viruses HXB2, PR-1 and PR-2, Gag substitutions Site-directed mutagenesis of HIV-1 RNA was isolated from each passage to a final concentration of 240 nM in passage 5. After passage 5, HIV-1 RNA was isolated from all cultures for genotypic analysis.

**Site-directed mutagenesis**

In viruses HXB2, PR-1 and PR-2, Gag substitutions V362I, A364V, S368N and V370A were introduced by amplification using VentR DNA polymerase (New England Biolabs, Ipswich, MA, USA) with primers 5’-Anna and 3’prot-6. Full Gag and protease sequences were obtained using a set of ten primers: GA1 (5’-GAC GCA GGA CTC GCC TGG CT-3’; 688-707), MArev-1 (5’-TGA TGT ACC ATT TGG GCC CCA ATT TT-3’; 2710-2685) were used. The second PCR was carried out with primers 5’-Anna (5’-ACT CGG CTT GCT GAC CGC GC-3’; 696-716) and 3’prot-5 (5’-TGG TTT TAT TTT TTC TTC TGT CAA TGG CCA-3’; 2648-2619). Sequence analysis was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Full Gag and protease sequences were obtained using VentR DNA polymerase (New England Biolabs, Ipswich, MA, USA) with primers 5’-Anna and 3’prot-6 and a third mutagenesis primer: GagV362 (5’-GGC AAG AAT TTT GGC TGA AGC AAT G-3’; 1866-1890), GagA364V (5’-GGC AAG AGT TTT GGT TGA AGC AAT G-3’; 1866-1890), GagS368N (5’-GGC TGA AGC AAT GAA CCA GGT AAC CA-3’; 1878-1903) or GagV370A (5’-GCA ATG AGC CAG GCA ACC AAT TC; 1885-1907).

The full Gag and protease PCR fragments were digested with restriction enzymes BssHII and MluNI. Digested fragments were then cloned into the previously described HXB2 reference vector CP-Wt[33] that also was digested with BssHII and MluNI. PCR product and vector (pHXB2ΔGagPR) were ligated using the Rapid DNA Ligation System (Promega Benelux, Leiden, The Netherlands) and subsequently transformed in competent cells.

**Phenotypic analysis**

**Drug susceptibility analysis**

Drug susceptibility was determined by a multiple cycle cell-killing assay[35]. MT-2 cells (5 × 10⁴ in 200 μl RPMI 10% FBS per well) were plated in 96-well microplates. Sample virus and reference virus were inoculated for five days on a single 96-well plate in the presence of threefold dilutions of bevirimat. Both sample virus and reference virus were inoculated at multiple MOIs to adjust for any differences in viral RC. Fold change (FC) values were calculated by dividing the mean 50% effective concentration (EC₅₀) for a sample virus by that of the HXB2 reference strain. Fold changes are averages of at least two separate experiments.

**Viral replication assay**

For each viral clone the amount of p24 was determined by ELISA (Aalto Bioreagent, Dublin, Ireland). Replication capacity was determined by infecting 2.0 × 10⁶ SupT1 cells (in duplicate) with an equivalent of 100 ng p24 of each virus. After 2 hours of incubation, cells were washed twice with fresh RPMI 1640 medium with L-glutamine and subsequently resuspended in 10 ml RPMI 1640 medium with L-glutamine supplemented with 10% FBS and gentamicin. Cultures were maintained in the absence of inhibitor for fourteen days and once daily 300 μl of cell-free virus supernatant was harvested for p24 analysis.

**CA/p2 processing efficiencies of the HXB2 and PR-2 protease enzymes**

HXB2 and PR-2 proteases were over-expressed in *E. coli* and purified to homogeneity as described previously[36]. Briefly, *E. coli* BL21(DE3)RIL (Novagen, Darmstadt, Germany) were transfected by pET 24a plasmid coding for the corresponding enzyme. The insoluble recombinant protein, accumulated in the form of inclusion bodies, was isolated and solubilized in 67% (v/v) acetic acid. The recombinant proteases were refolded by diluting in a 25-fold excess of water and overnight dialysis against water at 4°C followed by overnight dialysis against 50 mM 2-[(N-morpholino)ethanesulfonic acid (MES) pH 5.8, 10% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% (v/v) 2-mercaptoethanol. The proteases were purified by cation exchange chromatography using MonoS FPLC (Amersham Biosciences,
Utrecht, The Netherlands. Purified enzymes were stored at -70°C. The proteolytic activities of these enzymes were tested with substrates derived from Gag wt, GagV362I and GagA364V, represented by the following nonapeptides: KARVL_{AEANL}e-NH\_2, KARIL\_AEANL\_e-NH\_2, and KARVL\_VEANL\_e-NH\_2 as previously described. Substrates (200 μM) were incubated for 25 min with either HXB2 or PR-2 protease (75 nM) enzyme in 50 mM MES buffer (300 mM NaCl, pH 6.0) at 37°C. The arrow indicates the actual cleavage site. The cleavage reaction was stopped by adding concentrated formic acid. Enzymatic reaction mixtures were resolved in triplicates on a Zorbax SB-C18 reversed phase HPLC column (4.6 × 150 mm, particle size 1.8 μm, Agilent Technologies, USA).

Additional material

Additional file 1: Virus propagation in the presence of increasing bevirimat concentrations. The cumulative number of days until full blown CPE was observed is shown averaged for each variant.

Additional file 2: Impact of protease background on bevirimat resistance. Fold increase in bevirimat EC_{50} caused by single CA/p2 mutations in different protease backgrounds.

Additional file 3: Impact of bevirimat resistance mutations on PI susceptibility. The impact of the bevirimat resistance mutations on PI susceptibility is presented.

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Author details

1Department of Virology, Medical Microbiology, University Medical Center Utrecht, The Netherlands. 2Gilead Sciences and IOCB Research Center, Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic, Prague, Czech Republic.

Authors’ contributions

AF, NMvM and MN conceived and designed the study. AF, NMvM, JP, REMM and PJS performed the experiments. AF, NMvM, JP, REMM, PJS, JK and MN analyzed the data. AF, NMvM, JK and MN wrote the paper. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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