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In Vitro Development of Resistance to Human Immunodeficiency Virus Protease Inhibitor GW640385

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Development of in vitro resistance to GW640385, a new human immunodeficiency virus type 1 protease inhibitor, was studied. Variants characterized included one with <4-fold resistance and amino acid substitutions Q58E/A71V (protease) and P452K (Gag) and one with >50-fold resistance and amino acid substitutions L10F/G16E/E21K/A28S/M46I/F53L/A71V (protease) and L449F/P453T (Gag). The A28S substitution substantially reduced replication capacity.

The development of resistance during failure of highly active antiretroviral therapy represents a major therapeutic challenge to the long-term suppression of human immunodeficiency virus (HIV) (2). Often substantial levels of cross-resistance render subsequent treatment options less effective than initial treatment regimens, and transmitted resistance can compromise the effectiveness of first-line therapy (6, 13). Consequently, therapies aiming at new antiviral targets (e.g., integrase and CCR5) and new therapies within existing classes with superior potency against resistant viruses are urgently needed (11, 15).

GW640385 is an HIV-1 protease inhibitor (PI) with improved potency against clinical isolates resistant to many of the currently licensed HIV-1 PIs (17). To determine potential resistance mutations that may be observed in the clinical setting, HIV-1_{HXB2} was passaged in the presence of increasing concentrations of GW640385 (4). Historically, in vitro passage of HIV-1 with PIs has provided some correlation with mutations subsequently observed in the clinic (12, 18), but sometimes important mutations observed in vivo have not been selected in vitro (1). Differences between in vitro and in vivo data may be attributed to stochastic effects, founder effects, or codon bias in viral stocks. In addition, mutations conferring low-level resistance have not always been observed in vitro, possibly because the selection pressure rapidly transitioned above concentrations that would select low-level resistance (9). Virus was therefore passaged using either increasingly high concentrations of GW640385 (high-pressure passage; 0.5 to 120 nM) or using lower incremental increases (low-pressure passage; 0.5 to 5 nM). Clonal sequence analysis was employed to identify minority species and linkage between mutations. Site-directed mutant (SDM) viruses were constructed, and the sensitivity of virus to GW640385 was determined.

For the initial passage, final concentrations of 0.02-, 0.1-, 1-, 2-, 10-, 50-, and 200-fold the 50% inhibitory concentration value for GW640385 were used. MT4 cells were infected with HIV-1_{HXB2} (100 50% tissue culture infective doses [$TCID_{50}$]/2 × 10^6 cells). Samples of cell supernatant were collected at 2- to 4-day intervals, monitored for reverse transcriptase activity, and harvested when activity exceeded >125,000 cpm/30 μl (16). Viral RNA was extracted from harvested virus, reverse transcribed, and amplified by PCR to produce a DNA fragment containing the HIV-1 protease gene and the Gag cleavage sites (CS) p7/p1 and p1/p6 (7, 8). The nucleotide sequence was determined using ABI 3700 technology. SDMs were introduced into HIV-1_{HXB2} using the QuickChange kit (Stratagene), and recombinant virus was generated (14). Susceptibility assays for all the SDMs were carried out at least twice.

For the low-selection passage, virus was grown in GW640385 over 15 passages for a total of 105 days, to a maximum concentration of 5 nM. The protease amino acid substitutions Q58E and A71V and CS amino acid substitution R452K were selected in the majority of clones (Fig. 1). SDM virus containing these single, double, and triple protease and CS amino acid substitutions had slightly reduced susceptibility to GW640385: Q58E (fold resistance [FR], 2.42 ± 0.07), A71V (FR, 2.03 ± 0.11), Q58E/A71V (FR, 2.37 ± 0.12), A71V/R452K (FR, 2.43 ± 0.23), and Q58E/A71V/R452K (FR, 2.82 ± 0.11). The triple variant when analyzed in further experiments was not cross-resistant to other PIs tested (Table 1). The amino acid substitutions A71V and R452K were also identified during clonal analysis of the control passage in the absence of drug. Analysis using a GlaxoSmithKline database showed that the Q58E and A71V substitutions were present at increased incidence in PI-experienced populations but were not associated with high-level PI resistance. Analysis of the sensitivity to GW640385 of clinical isolates with multiple substitutions (10–11) including Q58E and A71V showed only limited shifts in susceptibility (FR, 2.8- to 4.8-fold). SDM virus with the observed natural variant V82I alone (FR, 1.03 ± 0.1) and with the double variant V82I/A71V (FR, 2.55 ± 0.68) showed that this substitution had little or no effect on resistance to GW640385.

For the high-selection passage, virus was grown in GW640385 over 14 passages for a total of 217 days to a maximum concentration of 120 nM. The protease amino acid substitutions that predominated (Fig. 2) were constructed as single and double SDMs. Analysis for sensitivity to GW640385 showed only small shifts in susceptibility: for L10F, FR of 1.1; for G16E, FR of 1.59 ± 0.03; for A28S, not done; for M46I, FR of

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Passage         10         20         30         40         50         60         70         80         90          449 452 453 Fraction
No.    ---------+----------+----------+----------+----------+----------+----------+----------+----------+---------- ----------- of clones
HXB2   PQVTLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMSLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF .    .   .
P1     .......... .......... .......... .......... .......... .......... .......... .......... .......... ......... .    .   .   Population
P1     .......... .......... .......... .......... .......... .......... .......... .......... .......... ......... .    .   .   17/29
P1     .......... .....R.... .......... .......... .......... .......... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .........G .......... .......... .......... .......... .......... .......... ......... .    K   .   1/29
P1     .......... .......... .......... .......... .......... ......G... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......... .......... .......... ....R..... .......... .......... .......... ......... .    .   .   1/29
P1     ....F..... .......... .......... .......... .......... .......... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......... .......... .......... .........E .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......E.. .......... .......... .......... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......... .......... .......... .......... .......... .T........ .......... ......... .    .   .   1/29
P1     .......... .......... .......... .......... .......E.. .......... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......... .....I.... .......... .......... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......... ......G... .....L.... .......... .......... .......... .......... ......... .    .   .   1/29
P1     .......... .......... .......... ......G... .......... ........H. .......... .......... .......... ......... .    .   .   1/29

P5     .......... .......... .......... .......... .......... .......... .......... V......... .......... ......... .   k/r  .   Population
P5     .......... .......... .......... .......... .......... .......... .......... V......... .......... ......... .    K   .   6/25
P5     .......... .......... .......... .......... .......... .......... .......... V......... .......... ......... .    .   .   6/25
P5     .......... .......... .......... .......... .......... .......... .......... V......... .A........ ......... .    K   .   1/25
P5     .......... .......... .......... .......... .......... ......S... .......... V......... .......... ......... .    K   .   1/25
P5     .......... .....E.... .......... .......... .......... .......... .......... V......... .......... ......... .    .   .   3/25
P5     ........L. .......... .......... .......... .......... .......... .......... VV........ .......... ......... .    .   .   1/25
P5     .......... .......... .......... .......... .......... .......... .......... VV........ .......... ......... .    K   .   1/25
P5     .......... .......... .......... .......... .......... ...M...... .......... .......... .......... ......... .    .   .   1/25
P5     .......... .........R .......... .......... .......... .......... .......... .......... .......... ......... .    .   .   1/25
P5     .......... .......... .......... .......... .......... .......... .......... .......... .......... ......... .    K   .   1/25
P5     .......... .......... .......... .......... .......... .......... .......... .......... .......... ......... .    .   .   3/25

P10    .......... .......... .......... .......... .......... .......... .......... V......... .......... .......L. .   k/r  .   Population
P10    .......... .......... .......... .......... .......... .......... .......... V......... .......... ......... .    K   .   8/24
P10    .......... .......... .......... .......... .......... .......... .......... V......... .......... ......... .    .   .   4/24
P10    .......... .......... .......... .......... G......... .......... .......... V......... .......... ......... .    .   .   1/24
P10    .......... .......... .......... .......... .......... .......... .......... VM........ .......... ......... .    .   .   1/24
P10    .......... .......... .......... .......... .......... .......... .......... V.....I... .......... ......... .    .   .   1/24
P10    .......... .......... .......... .......... .......... .......... .......... V......... .I........ ......... .    K   .   1/24
P10    .......... .......... .......... .......... .......... .......... .......... V..A...... .......... ......... .    K   .   1/24
P10    .......... ........P. .......... .......... .......... .......E.. .......... V......... .......... ......... .    K   .   1/24
P10    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... .    .   .   2/24
P10    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... .    K   .   4/24

P13    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... .    K   .   Population
P13    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... .    K   .   15/29
P13    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... .    .   .   7/29
P13    .......... .......... .......... .......... .........T .......E.. .......... V......... .......... ......... X    X   X   1/29
P13    .......... .......... .......... .......... .......... .......EH. .......... V......... .......... ......... .    K   .   1/29
P13    .......... .......... .......... .......... .......... .......E.. .......... V......... ..S....... ......... X    X   X   1/29
P13    .......... .......... .......... .......... .......... .......E.. ...V...... V......... .I........ ......... X    X   X   1/29
P13    .......... .......... .......... .......... .......... .......E.. .......... V......... .I........ ......... .    .   .   1/29
P13    .......... .......... .......... .......... .......... .......E.. .......... V......... .I........ ......... .    K   .   2/29

P14    .......... .......... .......... .......... .......... .......E.. .......... V......... .I........ ......... .    K   .   Population
P14    .......... .......... .......... .......... .......... .......E.. .......... V......... .I........ ......... .    .   .   2/21
P14    .......... .......... .......... .......... .......... .......E.. .......... V......... .I........ ......... .    K   .   3/21
P14    .......... .......... .......... .......... .......... ...T...E.. .......... V...S..... .I........ ......... .    .   .   1/21
P14    .......... .......... .......... ......N... .......... .......E.. .......... V......... .I........ ......... .    K   .   1/21
P14    .......... .......... .......... .......... .......... ....E..E.. .......... V......... .I........ ......... .    K   .   1/21
P14    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... .    K   .   10/21
P14    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... F    N   Q   1/21*
P14    .....R.... .......... .......... .......... .......... .......E.. .......... V......... .......... ......... F    A   N   1/21#
P14    .......... .......... .......... .......... .......... .......E.. .......... V......... .......... .....A... .    K   .   1/21
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FIG. 1. Amino acid PRO (1 to 99) and CS (449, 452, and 453) sequences of clones derived from HIV-1_{HXB2} passaged under low-pressure selection of GW640385. Two clones (* and #) had a two-nucleotide insertion and a one-nucleotide deletion, respectively, resulting in a frame shift. X, undetermined amino acid. HIV-1_{HXB2} was passaged in the presence of GW640385 at the following concentrations: passage 1 (P1), 0.5 nM; P5, 1 nM; P10, 2 nM; P13, 3 nM; and P14, 5 nM.

0.83 ± 0.35; for A71V, FR of 2.03 ± 0.11; for M46I/A71V, FR of 1.47 ± 0.35; and with CS substitutions, for L449F, FR of 0.81 ± 0.28, and for P453T, FR of 1.42 ± 0.37. SDM viruses containing A28S failed to replicate sufficiently for evaluation. The A28 residue is located in the protease enzyme active site close to the critical aspartate triad. The amino acid substitution A28S has previously been selected with TMC-126 in an HIV-1_{NL4-3} background and dramatically reduced viral replication (20). The A28S substitution resulted in a greater than 1,500-fold decrease in k_{cat}/k_m for peptide substrates (5). Database analysis showed that a substitution at A28 rarely (0.9% after three PIs were received) occurs following treatment with multiple PIs (19).

It is feasible that the A28S substitution requires an additional substitution in the CS coding region and other substitutions within protease to increase viral fitness and replicative capacity, as observed previously with other PIs (8). However,

TABLE 1. Cross-resistance of variants from the high- and low-selection analysis[a]

PI	Resistance of variant			
	L10F/G16E/K20T/A28S/M46I/A71V/L449F/P453T		Q58E/A71V/R452K	
	IC_{50} (nM)	FR	IC_{50} (nM)	FR
GW640385	45 ± 15	>52.5	0.9 ± 0.2	2.4
APV	283 ± 110	8.7	20.9 ± 0.3	0.4
LPV	173 ± 98	15	24.6 ± 4.1	1.0
SQV	22 ± 9	2	15.7 ± 0.8	1.1
NFV	136 ± 17	5	26 ± 22.8	1.0
IDV	597 ± 293	55	56.8 ± 0.6	0.9
ATZ	16 ± 9	1	20.6 ± 1.1	1.4

[a] FR, fold resistance, ratio of IC_{50} of test virus to that of HIV-1 strain HXB2; means and standard deviations for IC_{50}s were calculated from two assays run in parallel for all drugs. APV, amprenavir; LPV, lopinavir; SQV, saquinavir; IDV, indinavir; NFV, nelfinavir; ATZ, atazanavir.

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               10         20         30         40         50         60         70         80         90              449 452 453  Fraction
      ---------+----------+----------+----------+----------+----------+----------+----------+----------+---------   -----------  of clones
HXB2  PQVTLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMSLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF    L   R   P
P2    XXXXX..... .......... .......... .......... .......... .......... .......... .......... .......... .........    .   .   .   Population
P2    .......... .......... .......... .......... .......... .......... .......... .......... .......... .........    .   .   .   4/6
P2    .......... .......... .......... .......... .......... .......... .......... .......... .......... .........    .   .   T   1/6
P2    .......... .......... .......... .......... .......... .......... .......... V......... .......... .........    .   .   .   1/6

P3    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   .   Population
P3    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   T   7/25
P3    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   .   3/25
P3    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    R   .   T   1/25
P3    .......... .....E.... .......... .......... .......... .......... .......... .......... .......... .........    .   .   .   3/25
P3    .......... .....E.... .......... .......... .......... .......... .......... .......... .......... .........    .   .   T   2/25
P3    .......... .......... .......... .......... .......... .......... .......... .......... .......... .........    .   .   T   2/25
P3    .......... .......... .......... .......... .......... .......... .......... .......... .......... .........    .   .   .   1/25
P3    .......... ....T..... .......... ...G...... .......... .......... .......... .......... .......... .........    .   K   .   1/25

P4    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   .   Population
P4    .......... .....E.... .......... .......... .......... .......... .......... .......... .......... .........    .   .   .   12/55
P4    .......... .....E.... .......... .......... .......... .......... .......... .......... .......... .........    .   .   T   1/55
P4    .......... .....E.... .......... .......... .........V .......... .......... .......... .......... .........    .   .   T   2/55
P4    .......... .S........ .......S.. .......... .......... .......... .......... .......... .......... .........    .   .   T   1/55
P4    .......... .......... .......S.. .......... .......... .......... .......... .......... .......... .........    .   .   .   6/55
P4    .......... .....E.... .......S.. .......... .......... .......... .......... .......... .......... .........    .   .   T   1/55
P4    .......... .....E.... .......S.. .......... .......... .......... .......... .......... .......... .........    .   .   .   1/55
P4    .......... .....E.... .......... .......... .........V .......... .......... .......... .I........ .........    .   .   T   1/55
P4    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   T   4/55
P4    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   .   6/55
P4    .......... .....E.... .......... .......... .......... .......... .......... .......... .I........ .........    .   .   I   1/55
P4    .......... .....E.... .......... .......... .......... .......... .......... V......... .I........ .........    .   .   .   1/55
P4    .......... .....E.... .......... .......... .......... .......... .......... V......... .I........ .........    .   .   T   1/55
P4    .......... ....M..... .......S.. .......... .....L.... .......... .......... .......... .I........ .........    .   .   .   1/55
P4    .......... .......... .......S.. .......... .......... .......... .......... .......... .I........ .........    .   .   .   2/55
P4    .......... .......... .......S.G .......... .......... .......... .......... V......... ..                       .   .   T   1/55
P4    .......... .....E.... .......S.. .......... .......... .......... .......... .......... .I........ .........    .   .   .   1/55
P4    .......... .....E.... .......S.. .......... .......... .......... .......... V..A...... .I........ .........    .   .   T   1/55
P4    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    .   .   T   1/55

P5    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .I........ .........    .   .   T   Population
P5    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .I........ .........    .   .   T   17/35
P5    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .I........ .........    .   .   I   1/35
P5    .......... .......... .......S.. .......... .......... .......... .......... V......... .I........ .........    .   .   T   1/35
P5    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    .   .   T   9/35
P5    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    .   G   T   4/35

P6    .......... .....E.... .......S.. .......... K......... .......... .......... V......... .I........ .........    .   .   T   Population
P6    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .I........ .........    .   .   T   4/9
P6    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    .   .   T   3/9
P6    .......... .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    F   .   T   1/9

P9    .......... .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   Population
P9    .......... .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   4/6

P10   .......... .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   Population
P10   .........F .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    F   .   T   1/20
P10   .........P .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   1/20
P10   .......... .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   2/20
P10   .......... .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   13/20

P11   .......... .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   Population
P11   .......... .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   8/24
P11   ......R... .......... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   1/24
P11   .........F .....E.... .......S.. .......... .......... .......... .......... V......... .......... .........    F   .   T   1/24
P11   .........F .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   3/24
P11   .......... I....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   1/24
P11   .......... .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   4/24

P12   .......... .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   Population
P12   .......... .....E.... K......S.. .......... .....I.... .......... .......... .......... .......... .........    F   .   T   1/24
P12   .......... .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   7/24
P12   .......... .....E.... .......S.. .......... .....I.... .......... .......... V....V.... .......... .........    F   .   T   1/24
P12   .........F .....E.... .......S.. .......... .....I.... ........C. .......... V......... .......... .........    F   .   T   1/24
P12   .........F .....E.... .......S.. .......... .....I.... .......... .......... .......... .......... .........    F   .   T   2/24
P12   .........F .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   8/24

P14   .........F .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   Population
P14   .......... .....E.... K......S.. .......... .....I.... .......... .......... .......... .......... .........    F   .   T   1/23
P14   .......... .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   1/23
P14   .........F .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   12/23
P14   .........F .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   3/23
P14   .........F .....E.... K......S.. .......... .....I.... .......... .......... .......... .......... .........    F   .   T   1/23

P15   .........F .....E.... .......S.. .......... .....I.... ..L....... .......... V......... .......... .........    F   .   T   Population
P15   .......... .....V.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   1/19
P15   .........F .....E.... K......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   1/19
P15   .........F .....E.... .......S.. .......... .....I.... .......... .......... V......... .......... .........    F   .   T   7/19
P15   .........F .....E.... .......S.. .......... .....I.... ..L....... .......... V......... .......... .........    F   .   T   4/19
P15   .........F .....E.... .......S.. .......... .....I.... ..L....... ..F....... V......... .......... .........    F   .   T   1/19
P15   .........F .....E.... .......S.. .......... .....I.... .......... .......... .......... .......... .........    F   .   T   1/19
P15   .........F .....E.... .......S.. .......... K....I.... ..L....... .......... V......... .......... .........    F   .   T   1/19
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FIG. 2. Amino acid PRO (1 to 99) and CS (449, 452, and 453) sequences of clones derived from HIV-1$_{HXB2}$ passaged under high-pressure selection of GW640385. HIV-1$_{HXB2}$ was passaged in the presence of GW640385 at the following concentrations: passage 2 (P2), 0.5 nM; P4, 2 nM; P5, 4 nM; P6, 5 nM; P9, 40 nM; P10, 40 nM; P11, 60 nM; P12, 80 nM; P14, 100 nM; and P15, 120 nM.

additional amino acid substitutions in the protease/CS only marginally increased growth (1.8 $\log_{10}$ $TCID_{50}$) of the A28S-containing SDM virus (G16E/K20T/A28S/M46I/A71V/L449F/P453T). Addition of the L10F amino acid substitution, which was observed at late passages, to a clone extracted from passage 9 caused a further small increase in virus growth (2.5 $\log_{10}$ $TCID_{50}$), which enabled phenotypic analysis to be carried out. Sensitivity assays showed high-level resistance to GW640385 (>52.5-fold) and cross-resistance to some PIs tested (Table 1). The poor growth of the A28S-containing virus extracted from in vitro passage may indicate that upstream mutations facilitate viral replication, as has been reported (3, 10). A repeat of the high-selection passage with GW640385 and HIV-1_{HXB2} again selected the A28S amino acid substitution, despite the effect of this mutation on the replicative capacity of the virus.

In conclusion, using two in vitro selection strategies, two alternative resistance pathways were identified. Low-pressure selection gave rise to three protease amino acid substitutions (Q58E, A71V, and V82I) and one CS substitution (R452K) that conferred low-level resistance. In contrast, during high-pressure passage, seven protease amino acid substitutions (L10F, G16E, E21K, A28S, M46I, F53L, and A71V) and two CS substitutions (L449F and P453T) were selected. Virus containing the A28S substitution replicated extremely poorly, but in the presence of other protease substitutions, high-level resistance to GW640385 was detected. These observations are consistent with high-level drug resistance developing at a replicative cost to the virus and highlight the complex balance between resistance, drug pressure, and replicative capacity.

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