# Mutations at the Bottom of the Phe43 Cavity Are Responsible for Cross-Resistance to NBD Analogues

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## Introduction

CD4 mimic small compounds (CD4MCs), NBD-556 [1] and its analogues, inhibit the gp120-CD4 interaction and can also induce conformational changes in the gp120 architecture [1-9] thereby exposing masked epitopes of neutralizing antibodies on the Env protein [3-5,7-9]. Recently, some groups have reported novel potent NBD analogues [9]. We designed and synthesized five new NBD analogues (YIR compounds). In this study, we characterized CD4MC-resistant viruses induced by in vitro selection to these novel NBD analogues.

## Methods

Resistant variants were induced by five CD4MCs (NBD-556 [1], YYA-021 [3], JRC-II-191 [2], HAR-171 [5] or HAR-431 [5]) using the primary KP-5P virus (subtype B, R5) in PM1 cells (FIG. 1). We constructed infectious KP-5P clones with CD4MC-resistant mutation following in vitro selection. The susceptibility of the infectious clones to the novel CD4MCs (YIR-327, YIR-329, YIR-438, YIR-501, YIR-504, DMJ-I-228 [9] and DMJ-II-121 [9]) and other entry inhibitors (Maraviroc, Cenicriviroc, RPR103611 and IC9564) was tested in a TZM-bl assay.

## Results

Resistance against five NBD analogues, NBD-556, YYA-021, HAR-171, JRC-II-191 and HAR-431, was associated with V255M, T375I or M426I substitutions that line the Phe43 cavity of gp120 (FIG. 2). Two of three mutated residues, V255M and T375I, are located at the bottom of the Phe43 cavity, while M426I is at the edge of the cavity (FIG. 3). Clones V255M or T375I were highly resistant against the five NBD analogues (FIG. 4 and TABLE 1). These mutated clones were cross-resistant to all our novel CD4MCs, YIR-327, YIR-329, YIR-438, YIR-501 and YIR-504, and also to DMJ-I-228, DMJ-II-121 (FIG. 4 and TABLE 1). On the other hand, the clone with M426I was more resistant than those with V255M and T375I mutations to sCD4 but not as resistant to the CD4MCs tested, because the Phe43 residue of sCD4 is located at a shallow position in the cavity compared to the CD4MCs (FIG. 3). However, the mutated clones retained wild type sensitivity to other entry inhibitors (Maraviroc, Cenicriviroc, IC9564 and RPR103611) (FIG. 4 and TABLE 1). These results suggest that two mutations at the bottom of Phe43 cavity are critically important not only for binding but also broad resistance to CD4MCs.

## Conclusions

The mutations V255M and T375I at the bottom of Phe43 cavity can induce broad and potent cross-resistance to NBD analogues. These data provide additional knowledge for synthesizing novel NBD analogues with a high genetic barrier.

## References

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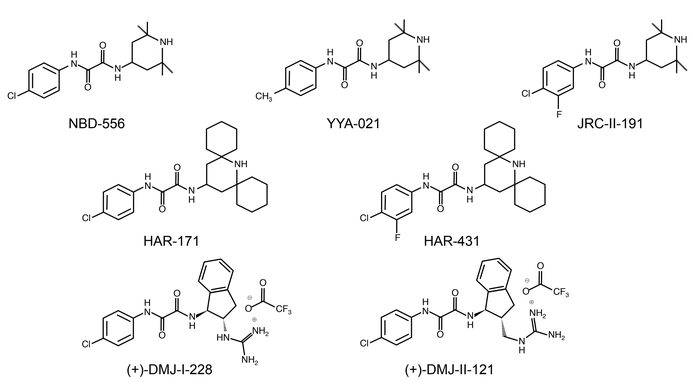



**FIG. 1. Cheical structures of CD4MCs (NBD-556 [1], YYA-021 [3], JRC-II-191 [2], HAR-171 [5], HAR-431 [5], (+)-DMJ-I-228 [9] and (+)-DMJ-II-121 [9]).**

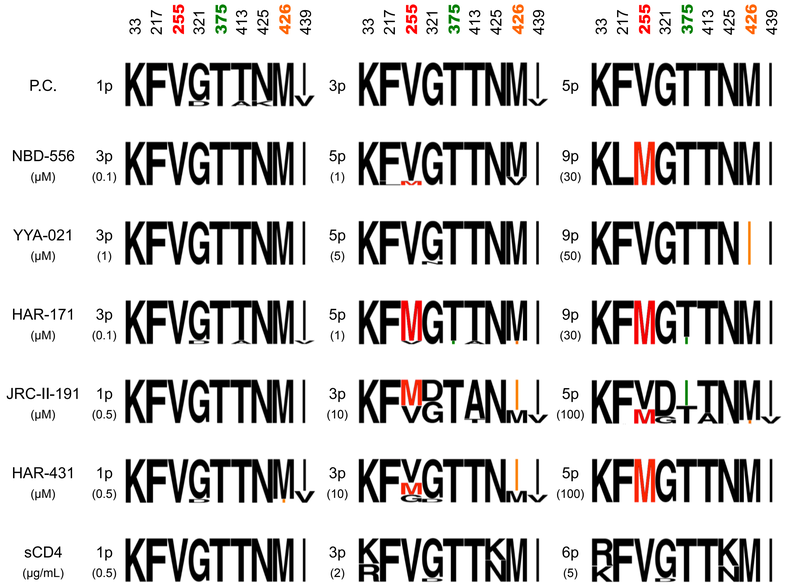



**FIG. 2. Time course of emergence of CD4MC-resistant mutants using the primary isolate KP-5P.** The KP-5P primary isolate (subtype B, R5) was serially passaged in the presence of CD4MCs. The amino acid sequence of the gp120 coding region from clones derived from 3 different passages is indicated. The condition of passage experiments are referred to as "9p (30)", where "9p" stands for passage number, and "(30)" is the concentration of CD4MC in the selection culture. V255M, T375I, and M426I substitutions are indicated in red, green, and tangerine, respectively. The sequence logo was constructed from the alignment of the gp120 amino acid sequence of each variant. The letter size is proportional to the percentage of the total number of clones tested. The WebLogo was generated using WebLogo version 3.

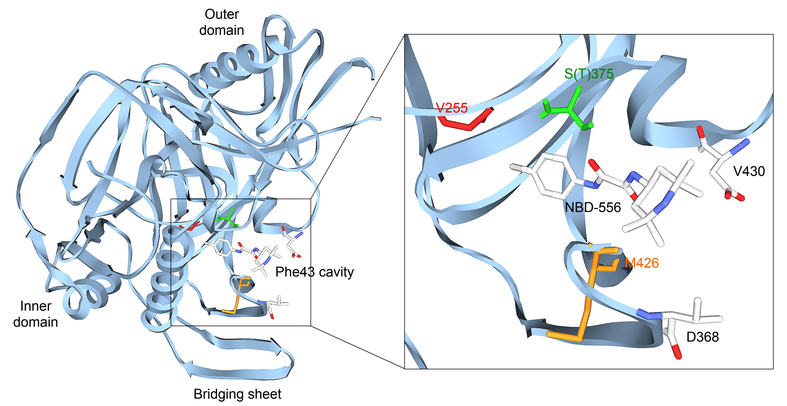



**FIG. 3. The locations of substitutions induced by in vitro selection with five CD4MCs in the Phe43 cavity.** The crystal structures of NBD-556 in complex with HIV-1 gp120 (C1086) was retrieved from the PDB (entry 3TGS) [6]. The side chains of the mutated residues that appeared during *in vitro* selection with NBD-556, YYA-021, JRC-II-191, HAR-171 or HAR-431 are shown in red (M255), green (T375), and tangerine (M426).

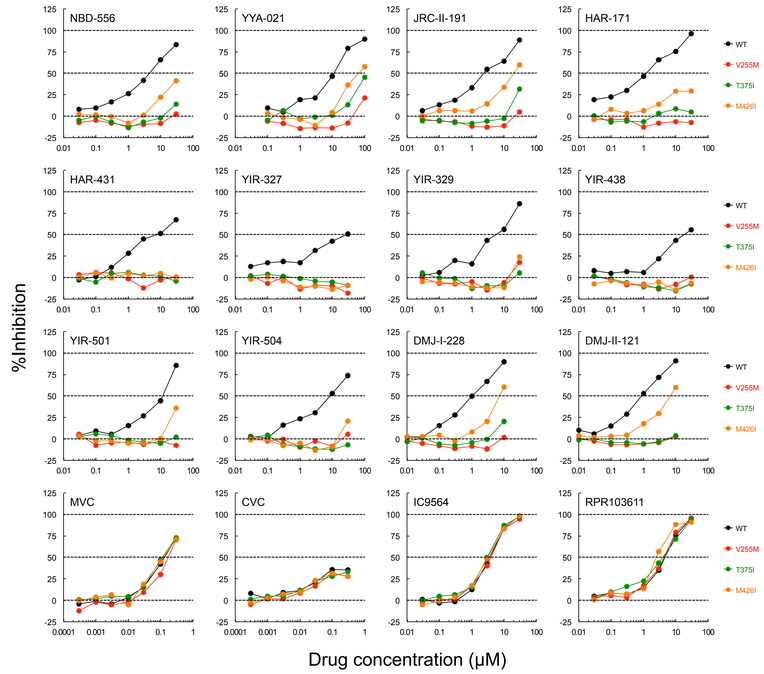



**FIG. 4. Susceptibility of HIV-1 with alterations in the gp120 to CD4MCs and other entry inhibitors.** TZM-bl cells ($1x10^4$) were exposed to 100$TCID_{50}$ of NL4-3/KP-5Penv virus in the presence of various concentrations of NBD-556, YYA-021, JRC-II-191, HAR-171, HAR-431, YIR-327, YIR-329, YIR-438, YIR-501, YIR-504, DMJ-I-228, DMJ-II-121, Maraviroc (MVC), Cenicriviroc (CVC), IC9564 and RPR103611 in 96-well flat-bottom micro culture plates, and incubated for 48h. The $IC_{50}$ value was determined by using a chemiluminescent assay for β-galactosidase detection (TABLE 1).

**TABLE 1. The $IC_{50}$ of CD4MCs and entry inhibitors against molecular clones.**

| | WT | V255M | T375I | M426I |
|---|---|---|---|---|
| NBD-556 | 4.6 | >30 (>7) | >30 (>7) | >30 (>7) |
| YYA-021 | 11 | >100 (>9) | >100 (>9) | 65 (6) |
| JRC-II-191 | 2.4 | >30 (>13) | >30 (>13) | 20 (8) |
| HAR-171 | 1.2 | >30 (>25) | >30 (>25) | >30 (>25) |
| HAR-431 | 7.6 | >30 (>4) | >30 (>4) | >30 (>4) |
| YIR-327 | 28 | >30 (>1) | >30 (>1) | >30 (>1) |
| YIR-329 | 5.5 | >30 (>6) | >30 (>6) | >30 (>6) |
| YIR-438 | 18 | >30 (>2) | >30 (>2) | >30 (>2) |
| YIR-501 | 12 | >30 (>3) | >30 (>3) | >30 (>3) |
| YIR-504 | 8.5 | >30 (>4) | >30 (>4) | >30 (>4) |
| DMJ-I-228 | 1.0 | >10 (>10) | >10 (>10) | 7.3 (8) |
| DMJ-II-121 | 0.86 | >10 (>12) | >10 (>12) | 6.8 (8) |
| MVC | 0.13 | 0.17 (1) | 0.11 (1) | 0.12 (1) |
| CVC | >0.30 | >0.30 | >0.30 | >0.30 |
| IC9564 | 3.6 | 4.0 (1) | 3.0 (1) | 3.2 (1) |
| RPR103611 | 4.7 | 4.4 (1) | 4.0 (1) | 2.5 (1) |

Numbers indicate concentrations to reach the $IC_{50}$ in the TZM-bl assay (FIG. 4). > indicates that the $IC_{50}$ for a given virus was not reached at the concentration tested and is highlighted in red. $IC_{50}$ that is >5-fold compared with WT are highlighted in tangerine.

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