

# 736 In vitro induction of human immunodeficiency virus type 1 variants resistant to a low-molecular CD4 mimic compound, *N*-(4-Chlorophenyl)-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide (NBD-556)

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## Introduction

*N*-(4-Chlorophenyl)-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide, NBD-556 (Fig.1) is a low-molecular weight compound that reportedly block the interaction between the HIV-1 gp120 and its receptor, CD4 (Table 1). The thermodynamic signature of NBD-556 was similar to that observed for binding of sCD4 to gp120. In this study, we induced HIV-1 variants escape from NBD-556 and rsCD4 in vitro.

## Methods

To investigate whether the effect of binding affinity of anti-gp120 MAbs to Env with NBD-556 is similar to sCD4, we compared FACS profiles of each MAbs binding to Env expressing cell surface. To select HIV-1 variants resistant to NBD-556 and sCD4 in vitro, we exposed PM1-CCR5 cells to HIV-1<sub>LAI</sub> and the virus was serially passaged in the presence of increasing concentrations of NBD-556 or rsCD4, up to 50  $\mu$ M and 20  $\mu$ g/ml, respectively. We determined the amino acid sequence of the gp120-encoding region of the HIV-1<sub>LAI</sub> escape mutant cultured with NBD-556 or sCD4. The multi-drug-effect analysis of Chou et al. was used to analyze the effects of combinations of NBD-556 with anti-gp120 MAbs.

## Results

**Flow cytometric analysis.** In FACS analysis, the profile of binding of anti-envelope (CD4 induced and V3) MAbs to NBD-556-pretreated Env expressing cell surface was completely similar to those of rsCD4-pretreated (Fig.2).

**Selection of a KD-247 escape variant.** At passage 21 in the culture where HIV-1<sub>IIIb</sub> was propagating in the presence of NBD-556 (50  $\mu$ M), two amino acid substitutions, Ser to Asn at position 375 (S375N, 11 of 11 clones) in C3 and Ala to Thr at position 433 (A433T, 4 of 11 clones) in C4 were identified. On the other hand, in the selection with sCD4, 6 mutations (P212, V255E, N280K, S375N and G380R) were appeared through the passage. At passage 5, three substitutions, V255E (5 of 10 clones), G380R (1 of 10 clones) and G431E (2 of 10 clones), were remained in 20  $\mu$ g/ml of sCD4. These two profiles of mutations in the selections of NBD-556 and sCD4 were very similar in a three-dimensional position (Fig.3-5).

**Sensitivities of luciferase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistant envelope mutations to NBD-556, sCD4 and MAbs.** As shown in Fig. 6-B, all resistant clones (IIIb-S375N, V255E and A-433T) were completely resistant to NBD-556 up to 20  $\mu$ M. We also examined the sensitivities of the pseudotyped clones to CD4bs MAb b12 and anti-CD4 MAb RPA-T4 by a single-round replication assay (Fig. 6-C, D). All mutant viruses showed almost the same neutralization sensitivity as wild type virus against b12 and RPA-T4. As shown in Fig. 2, CD4i MAb 4C11 bound to the wild-type JR-FL Env pretreated NBD-556 as well as sCD4. As expectedly, NBD-556-pretreated IIIb was more sensitive to 4C11 than no treatment virus (Fig. 7). We also examined the sensitivities of the resistant pseudotyped clones to 4C11. All mutant viruses were completely resistant to 4C11 with or without NBD-556 pretreatment. This result suggests that CD4 and NBD-556 resistant mutations in the gp120 also hide the epitope for antibody against the CD4-induced epitope.

**Highly synergistic interactions of KD-247 combined with NBD-556.** As shown in Table 2, all the CI values for KD-247 with the NBD-556 were <0.5 against JR-FL at all the inhibitory concentrations tested.

Fig.1 Structure of NBD-556

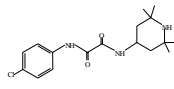


Table 1. Inhibitory activity of sCD4 and NBD-556 on infection of laboratory adapted and primary HIV-1 strains

Virus strain	IC <sub>50</sub>	
	sCD4 ( $\mu$ g/ml)	NBD-556 ( $\mu$ M)
Laboratory adapted		
<b>X4</b>		
IIIb	0.46	8.1
Dual 89.6(-)	0.93	10.0
<b>RS</b>		
R4L	1.5	>30
SF162	3.1	>30
JR-FL	4.1	>30
YU2	4.7	>30
Primary isolates		
<b>RS</b>		
MTA	0.2	3.4
IT01	0.2	5.3
Y15	1.4	>30
MNA	3.8	12.9
M15	6.5	>30

\*NBD-556, CC<sub>50</sub>=170  $\mu$ M (PM1-CCR5 cells)

<sup>a</sup>PM1-CCR5 cells (2x10<sup>6</sup>) were exposed to 100 TCID<sub>50</sub> of each virus and then cultured in the presence of various concentrations of sCD4 or NBD-556. The IC<sub>50</sub> values were determined using the MTT assay on day 7 of culture. All assays were conducted in duplicate. The values shown are representative of two or three separate experiments.

Fig.2 Comparison of antibody binding to cell surface-expressed with sCD4 or NBD-556

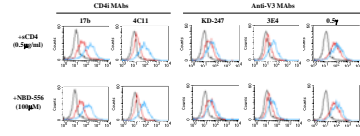


Fig.2 JR-FL chronically infected PM1 cells were pre-incubated with or without rsCD4 (0.5  $\mu$ g/ml) or NBD-556 (100  $\mu$ M) for 15 min, and then incubated with each anti-HIV-1 MAb (17b, 4C11, KD-247, 3E4 and 0.5y) at 4 °C for 30 min. The cells were washed with PBS, and FITC-conjugated goat anti-mouse IgG antibody was used for antibody-staining.

Table 2. Combination indices (CI) for KD-247 or T-140 and sCD4 or NBD-556 against JR-FL and IIIb

Combination	Virus	CI values at different IC <sup>a</sup>		
		IC <sub>10</sub>	IC <sub>25</sub>	IC <sub>50</sub>
KD-247+sCD4	JR-FL	0.313	0.266	0.227
KD-247+NBD-556	JR-FL	0.174	0.043	0.011
T-140+sCD4	IIIb	0.705	0.528	0.400
T-140+NBD-556	IIIb	0.786	0.713	0.655

<sup>a</sup>The multiple-drug-effect analysis of Chou and colleagues was used to analyze the effects of the drugs in combination. IC, inhibitory concentration. CI<0.9, synergy; 0.9<CI<1.1, additivity; CI>1.1, antagonism.

Fig.3 Crystal structures of HIV-1 gp120 complexed to sCD4 and docking simulation of NBD-556



Fig.3 Docking simulations were performed by FlexSIS module of SYBYL 7.1 (Tripos, St. Louis). The atomic coordinates of the crystal structure (1R22) were retrieved from Protein Data Bank (PDB) (entry 1R22). The active site was defined as all amino acids within 6.5 Å proximity of the Phe 43 of co-crystallized CD4.

Fig.4 Isolation of the NBD-556 and sCD4-resistant mutant from IIIb in vitro

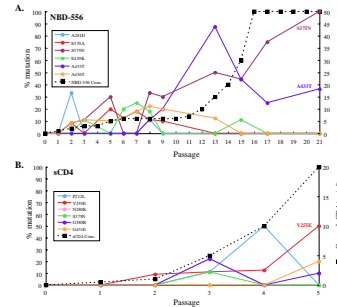


Fig.4 For the selection of NBD-556 (A) and sCD4 (B) escape virus, IIIb was treated with various concentrations of NBD-556 or sCD4 and then infected to PM1/CCR5 cells.

Fig.5 Putative binding site of sCD4 and NBD-556

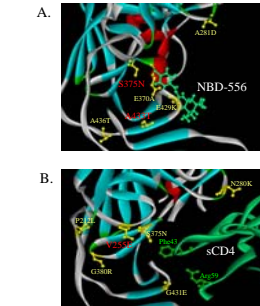


Fig.5 Map of the sites of mutations (yellow) that confer resistance to NBD-556 (A) and sCD4 (B) onto the core structure of HIV-1 IIIb gp120. The side chains of residues of mutations appeared during in vitro selection are shown. The amino acid substitutions that confer resistance in HIV-1 induced in red.

Fig.6 Sensitivities of luciferase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistant envelope mutations to NBD-556, sCD4 and MAbs

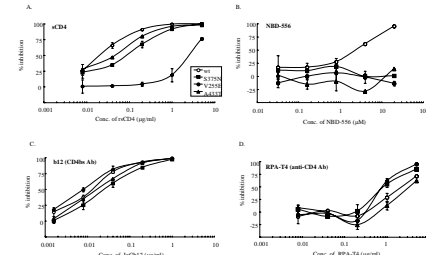


Fig.6 Sensitivities of luciferase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistant envelope mutations to sCD4(A), NBD-556(B), b12(C) and RPA-T4(D), sCD4, NBD-556, b12 and RPA-T4 at various concentrations and a pseudovirus suspension corresponding to 100 TCID<sub>50</sub> were preincubated for 15 min on ice, followed by addition of the mixtures to the target cells (TZM-bl). The inhibitory effects were determined by measuring the luciferase activities on day 2 of culture.

Fig.7 Sensitivities of luciferase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistant envelope mutations to CD4i MAb with or without NBD-556

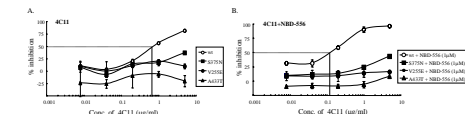


Fig.7 Sensitivities of luciferase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistant envelope mutations to CD4i Ab 4C11 with (A) or without (B) NBD-556. 4C11 at various concentrations and a pseudovirus suspension corresponding to 100 TCID<sub>50</sub> were preincubated with or without NBD-556 (1  $\mu$ M) for 15 min on ice, followed by addition of the mixtures to the target cells (TZM-bl). The inhibitory effects were determined by measuring the luciferase activities on day 2 of culture.

## Conclusions

In this study, we observed that NBD-556 could bind a CD4 binding site followed by induction of conformational changes in gp120 similar to those observed upon sCD4 binding. We also found highly synergistic interactions between NBD-556 and anti-gp120 MAbs. These data provide a rational basis for testing of combinations of the NBD compounds and therapeutic MAbs, such as KD-247 in vivo.