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Rational Design and Characterization of the Novel, Broad and Potent Bispecific HIV-1 Neutralizing Antibody iMabm36

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Abstract

While broadly neutralizing monoclonal antibodies (bNAbs) have always been considered potential therapeutic options for the prophylactic and treatment of HIV infection, their lack of breadth against all HIV variants has been one of the limiting factors. To provide sufficient neutralization breadth and potency against diverse viruses, including neutralization escape variants, strategies to combine different bNAbs have been explored recently. We rationally designed and engineered a novel bispecific HIV-1 neutralizing antibody (bibNAb), iMabm36, for high potency and breadth against HIV. iMabm36 is composed of the anti-CD4 Ab ibalizumab (iMab) linked to two copies of the single-domain Ab m36 which targets a highly conserved CD4-induced epitope. iMabm36 neutralizes a majority of a large, multi-clade panel of pseudoviruses (96%, n=118) at an IC₅₀ concentration of less than 10 µg/mL, with 83% neutralized at an IC₅₀ concentration of less than 0.1 µg/ml. In addition, iMabm36 neutralizes six replication-competent transmitted-founder viruses to 100% inhibition at a concentration of less than 0.1 µg/ml in a PBMC-based neutralizing assay. Mechanistically, improved antiviral activity of iMabm36 is dependent on both CD4 binding activity of iMab component and CD4i binding activity of the m36 component. After characterizing viral resistance to iMabm36 neutralization was due to mutations residing in the bridging sheet of gp120, an optimized m36 variant was engineered that, when fused to iMab, improved antiviral activity significantly. Together inter-dependency of this dual mechanism of action enables iMabm36 to potently inhibit HIV-1 entry. These results demonstrate that mechanistic-based design of bibNAbs could generate potential preventive and therapeutic candidates for HIV/AIDS.

Keywords

HIV; Neutralizing antibodies; Bispecific antibody; Antiretroviral; ibalizumab (iMab); m36; Entry inhibitors

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Introduction

Broad and potent antibodies represent a new generation of antiviral agents for the prophylaxis and treatment of HIV infection. Compared to small molecule antiretrovirals currently used to treat HIV, antibodies are generally considered safer and have longer half-lives, and have been shown to provide passive protection against mucosal challenge in the SHIV/macaque model. These promising features, combined with the greater breadth and potency of the newest generation of antibodies, have reignited interest in developing antibody-based drugs against HIV-1. Recently, through micro-neutralization screening of B-cell cultures and single B-cell sorting from HIV-1 infected patients, it has been successful to isolate and characterize many new monoclonal antibodies, such as PG9/16, VRC01, 3BNC117, NIH45-46, the PGT Abs and 10E8²⁻⁶. Several broadly neutralizing mAbs (bNAbs), when administered as monotherapy, can protect against HIV-1 infection in animal models^{7,8}. However, their efficacy in treating an established infection is limited⁸⁻¹¹. In particular, viral rebound occurs quickly in all patients receiving bNAbs due to the outgrowth of pre-existing or *de novo* viral escape variants¹². It has been reported that combining multiple neutralizing antibodies that each use a different mechanism of action would increase the antiviral potency and barrier to resistance *in vivo* than any one antibody alone^{8,10,11,13-15}. Therefore, novel antibodies with broader neutralizing activity and greater potency are needed in defense of HIV-1 resistance and resistance development.

HIV-1 entry is triggered by interaction of the viral envelope (Env) glycoprotein gp120 with domain 1 (D1) of the T-cell coreceptor CD4^{16,17}. Binding of CD4 by gp120 induces extensive conformational changes in gp120 leading to formation and exposure of the co-receptor (CoR) binding site, also known as the CD4-induced (CD4i) site, on gp120¹⁸⁻²⁰. The CoR binding site is typically unformed on native Env trimers prior to CD4 engagement. The CoR binding site is highly immunogenic and elicits a class of Abs known as CD4-induced (CD4i) Abs *in vivo*. The bridging sheet of gp120 is a critical component of the CoR binding site that is conserved across genetically diverse HIV-1 isolates from different clades^{21,22}. However, access of full-size Abs to the CD4i epitope (bridging sheet) is sterically restricted during viral entry into cells, most likely because the large size of a full length Ab cannot access the tight crypt within the envelope where the bridging sheet resides^{23,24}. Thus, most known full-size CD4i Abs do not have potent antiviral activity. Fragments of CD4i Abs that are smaller in size could potentially gain access to the CD4i epitope during viral entry and have been shown to inhibit HIV entry more potently than full-size Abs²⁵. m36, a single-domain Ab that is 15 kDa in size, was isolated from a naive human Ab library and targets the highly conserved, but sterically restricted, CD4i epitope on HIV Env^{26,27}. m36 has been reported to be one of the most potent and broadly cross-reactive HIV-1 engineered antibody domains (eAd) with a mean IC₅₀ in the 100nM range^{26,27}. However, similar to other antibody fragments, the m36 polypeptide is predicted to have a short half-life in circulation due to its relatively small size^{26,28}. Ibalizumab (iMab) is a mAb that has broad and potent activity against HIV-1²⁹⁻³¹. It inhibits HIV by binding mainly to domain 2 (D2) of CD4 on host target cells, inhibiting post-CD4 binding events required to infect cells³². In a large panel of diverse, clinically-relevant HIV-1

pseudoviruses (n=118), iMab neutralized 92% of viruses, as defined by 50% inhibition of infection, and 47% of viruses, as defined by 90% inhibition of infection³¹.

We have previously demonstrated that ibalizumab-based bispecific bNabs exhibit synergistic antiviral activity compared to the parental Abs, either alone or in combination, attributed in part to the enhanced local concentration of bNab activity at the site of viral entry³¹. Fine epitope mapping and crystal structure resolution of the iMab-CD4 interaction have previously shown that the iMab does not interfere with the binding of gp120 to CD4³²⁻³⁴ and preliminary data from our lab suggest ibalizumab does not impair exposure and engagement of the HIV-1 bridging sheet with the HIV-1 coreceptors. Thus, we hypothesized that fusing a CD4i Ab to ibalizumab would anchor the activity of CD4i Abs at the virological synapse prior to gp120-CD4 engagement, thereby diminishing the spatial constraints that impair native CD4i Abs from accessing their epitope and allowing the bispecific Ab to potentially inhibit HIV-1 entry at two distinct, but spatially related, entry steps. To test this, we engineered iMabm36, a novel bispecific antibody that could target both of these entry steps simultaneously. iMabm36 is comprised of the anti-CD4 Ab iMab linked to two copies of the anti-CD4i, single-domain Ab m36. We show that iMabm36 potentially inhibits viral entry of many iMab-resistant viruses without affecting the inhibition of iMab-sensitive viruses. Also, fusion of m36 to iMab is predicted to extend the short-life of m36 in vivo. Thus, the novel, rationally designed bibNab iMabm36 provides enhanced anti-HIV-1 activity compared to either parental Ab alone, or in combination, and may be a valuable new therapeutic for the prevention or treatment of HIV-1 infection.

Methods

Reagents

Recombinant soluble CD4 protein and TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Ibalizumab was provided by TaiMed Biologics USA^{31,35}.

Construction and expression of iMabm36 fusion Ab

The following primers were used:

Primer	Sequence
m36F1	5'-ctggctagccaccatggaatggagcggggtc-3'
36F2	5'-ctggtcaccgtctcctcaggtggcggaggatctggcggagggggttcaggggcggtggaagtggtcaggtccaactgcagcagt-3'
36R1	5'-tggtcaccgtctcctcaggtggcggaggatctggcggagggggttcaggggcggtggaagtggtagacatcgtgatgaccca-3'
m36R2	5'-tgaggagacgggtgaccagggttcctggcccccagta-3'
iMab 1	5'-Tactactgcgctaggcggaaggacaacgccgctaccggcgcttg-3'
iMab 2	5'-ccaagcgggtagcggcgttgctctccctagcgcagtagta-3'
m36 1F	5'-gtgcagcctctgctttcgccttcagcgcgttacgcgatgagctgggtccgcca-3'
m36 1R	5'-tggcggaccagctcatcgcgtaagcgtgaaggcgaaagcagaggctgcac-3'
m36 2F	5'-atcgagagatcaacgcaagcgcaatacatct-3'
m36 2R	5'-agatggtattgccgcttgctgtatctctccgat-3'

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m36 3      5'- gccctcgactatgaggagacgggtgaccagggttcctggccccagtagccttcaccgtatcctgatattgttgcacagtaaatagatggccgt-3'
m36-E51-1  5'- gtagcctccgtcataatctgcgtaatctccagccgctgactctccgctatgctgttgccttcacagtaaatagatggccgt-3';
m36-E51-2  5'- gccctctagactatgaggagacgggtgaccagggttcctggccccacatccatgcataatagtagcctccgtcataat-3'

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A bi-specific fusion Ab was constructed based on m36 and a derivative IgG1 version of iMab. As shown in figure 1 schematic, m36 was linked to the C-terminus of the heavy chain of iMab via a flexible (G4S)₃ linker peptide (GGGSGGGSGGGSG). cDNA sequence of the fusion construct was generated by overlap PCR using primers m36F1, m36F2, m36R1 and m36R2. Subsequently the products were digested with *Nhe*I and *Xho*I and cloned into the pVAX expression plasmid (Life Technologies). PRO140m36, (G4S)₁ linked m36 and (G4S)₅ linked m36 were cloned into pVAX in a similar manner. To generate iMabm36, the iMabm36 gene was amplified by mutagenesis PCR primers (iMab 1 and iMab 2) using the iMabm36-encoding plasmid as a template. Similarly, the iMab m36 gene was obtained by mutagenesis PCR by primers (m36 1F, m36 1R, m36 2F, m36 2R and m36 3). m36 (CDR3 E51) fragments were amplified by overlap PCR and cloned into a pVAX expression plasmid. All constructs were confirmed by direct nucleotide sequencing. Plasmid DNA was isolated by anion exchange using endotoxin-free Maxi kits (Qiagen). For expression of different Abs, 293 A cells were transiently co-transfected by Polyethylenimine (PEI) at a final concentration of 5µg/ml and 10µg of pVAX vectors expressing the heavy chain iMabm36 fusion and the light chain of ibalizumab. After 72 hrs, cell culture supernatants were harvested and analyzed for the presence of antibody by capture ELISA. Ab-containing culture supernatants were filtered and purified by affinity chromatography using a ProteinA column (Pierce) and concentrated with a centrifugal filter (Millipore).

***In vitro* structural integrity of iMabm36**

Rabbits were immunized with purified m36 protein (300µg/dose) in CFA at week 0 and subsequently boosted in IFA twice at weeks 4 and 8. Anti-m36 Ab titers were determined in the serum sample collected 4 weeks post last boost immunization. The *in vitro* integrity of the iMabm36 fusion Ab was determined by incubation of the fusion Ab in 20% mouse serum in PBS at 37°C for up to 7 days. Aliquots of the untreated (day 0) and treated fusion Ab were taken at the indicated time points and stored at -20°C. The presence of intact iMabm36 was examined by the functional binding activity of iMabm36 to sCD4 and determined by anti-iMab Fc direct ELISA and anti-m36 sandwich ELISA, respectively. The presence of functional, intact iMabm36 was also assessed antiviral activity by the TZM-bl neutralization assay.

Purified iMabm36 was assessed for soluble CD4 (sCD4) binding in a competition ELISA assay

Soluble hCD4 was adsorbed onto 96-well (0.1µg/well) high-binding ELISA plates (Costar/Corning). The plates were then blocked with 4% dehydrated milk and 1% BSA in PBS-T (blocking buffer). The plates were washed and a fixed concentration of HRP labeled iMab (2.5µg/ml) was then mixed with increasing concentrations of iMabm36 or unlabeled iMab and measured for sCD4 binding competition. Plates were then washed, developed by means of a streptavidin-coupled peroxidase and TMB substrate (Sigma) and measured on an ELISA plate reader at an optical density (OD) of 450 nm.

Pseudovirus preparation and generation of bridging sheet mutants

The following primers were used to generate bridging sheet mutant viruses:

Primer	Sequence
Q23-1	5'-aattgtaatacctcagtcattacacagcggtgc-3'
Q23-2	5'-aatatgtggcagagagtcgaaaagcaatgtatgccct-3'
Q259-1	5'-aattgtaacacctcagccattaccagcgttgc-3'
Q259-2	5'-aatatgtggcagagagtcgaaaagcaatgtatgccctccata-3 ;
T28-1	5'- aattgtaatacctcagccattgcacagcgttgc-3'
T28-2	5'- aatatgtggcagacagtaggaaaagcaatgtatgccctccatc-3'

Assay stocks of molecularly cloned Env-pseudotyped viruses were prepared by co-transfecting 293T cells with an Env-expressing plasmid and an Env-deficient backbone plasmid (SG3 Env) at a ratio of 1:3 using PEI. All viral supernatants were harvested 2 days after transfection and the TCID₅₀ determined on TZM-bl cells by end-point dilution. Q23.17, Q259.d2.17 and T28-50 Env-expressing plasmids coding bridging sheet residues were substituted with clade B consensus amino acids by site-directed mutagenesis according to the manufacturer's instructions (Agilent). Six Env-pseudotyped mutants (Q23-β21mut, Q23-β3β21mut, Q259-β21mut, Q259-β3β21mut, T278-β21mut and T278-β3β21mut) were also prepared as described above.

Pseudovirion-based and PMBC-based neutralization assay

Neutralization against Env pseudoviruses were measured with a luciferase-based assay in TZM-bl cells as previously described³⁶. Briefly, five-fold serial dilutions of antibodies were performed in triplicate (96-well flat bottom plate) in TZM-bl cells (1×10^4 per well in 100μl volume) and incubated for 1 hour at 37°C. 100 TCID₅₀ of virus and 50μl 10% DMEM growth medium containing DEAE-dextran (Sigma) at a final concentration of 11μg/ml were added to each well. Assay controls included TZM-bl cells alone (cell control) and TZM-bl cells with virus (virus control). After 48-hour incubation at 37°C, assay medium was removed from each well and 40μl of lysis buffer and 60μl Galacto-Star luciferase reagents (Applied Biosystems) were added and luminescence was measured. The IC₅₀ titer was calculated as the antibody dilution that caused a 50% reduction in relative luminescence units (RLUs) compared to the virus control after subtraction of cell control RLUs.

A standard PMBC-based neutralization assay was used to assess iMabm36 antiviral activity. Briefly, neutralizing assay was performed in a 96-well plate format. PHA/IL2 activated PBMC (1.5×10^5 per well) were infected with virus in the presence or absence of antibody. PBMC were washed extensively after overnight culture. Culture supernatants were collected at day 3 and 7. Viral p24-antigen was measured by a commercial ELISA (Beckman Coulter).

Results

iMabm36 construction and characterization

To generate iMabm36, we fused m36 to the C-terminus of the heavy chain of iMab via a flexible (G4S)₃ linker peptide (Figure 1A). The iMab, m36 and iMabm36 expression cassettes correctly produced Ab molecules at the predicted molecular weights (Figure 1B). To confirm that the CD4 binding activity of iMabm36 was unaltered compared to parental ibalizumab, we evaluated its sCD4 binding activity *in vitro*. Indeed, iMabm36 was able to compete with HRP labeled iMab for sCD4 binding *in vitro* with equal potency as the parental unlabeled iMab, indicating that the fusion of m36 to iMab did not impair its CD4 binding function. As expected, m36 alone did not compete with iMab binding to sCD4 (Figure 1C).

To assess the structural integrity and stability of iMabm36, we first generated high titers of rabbit anti-m36 immune serum. The integrity of iMabm36 was examined by a secondary Ab against iMab Fc or anti-m36 rabbit immune serum upon incubation of iMabm36 at 37°C over time. No loss of CD4- or m36-binding were observed. In addition, no loss of neutralization activity was observed from samples incubated at 37°C for 7 days (data not shown). Thus, the functional activity of iMabm36 is retained for up to at least 7 days in the conditions tested.

iMabm36 fusion Ab improves antiviral breadth compared to iMab or m36 alone

To examine if the fusion of m36 to the C-terminal of iMab could result in more potent antiviral activity, we first tested iMabm36, iMab and m36 in a TZM-bl neutralization assay against a panel of 6 viruses that included iMab sensitive and resistant viruses. For all viruses tested, the potency of iMab36 was enhanced as compared to one of the parental components, m36, alone. For iMab sensitive viruses specifically (CQLDR03-A2 and SC20 8A8A), the potency of iMabm36 and iMab were similar (Figure 2A). However, iMabm36 potently neutralized all 4 iMab-resistant viruses (9077.12 B5A, TT31P 2F10, SC33 4H1 and RHPA4259.1mut)³¹ better than either iMab or m36 alone, and achieved 100% maximum percent inhibition (MPI) at low nanomolar concentrations (Figure 2B). Consistent with what was previously reported, the 50% inhibitory concentration of m36 is normally within the range of hundreds of nanomolar concentrations²⁶. Neutralizing activity of iMabm36 was also assessed in a PBMC-based 7-day neutralizing assay against 6 replication-competent transmitted-founder viruses. Both iMab and iMabm36 achieved 100% neutralization against this panel of viruses. However iMabm36 appeared to improve anti-viral potency in this assay, with 100% inhibition at a mean concentration of 0.1 µg/ml (Figure 2C).

Increased antiviral potency of iMabm36 compared to iMab and m36 mixture

The results above indicated that the combination of iMabm36 is more active than their respective individual components iMab and m36. To investigate if the greater antiviral activity of iMabm36 could also be achieved by simply mixing iMab and m36, we performed a neutralizing assay using iMab and m36 at a molar ratio of 1:2 since one iMabm36 molecule carries two m36 domains. Mixing the individual parental components, iMab and m36, indeed improved the antiviral activity as compared to either of the parental

components alone, achieving 100% neutralization of iMab-resistant viruses (RHPA4259.1mut, 9077.12 B5A and TT31P 2F10) (Figure 3A). However, iMabm36 bispecific antibody had even greater antiviral activity, being at least 10-fold more potent than the co-administration of iMab and m36 parental components.

To test if iMabm36 acts through increasing the local concentration of m36 at the cell surface, we compared the antiviral activity of mixing iMab with m36 at a molar ratio of 1:10 against the same small panel of viruses (Figure 3B). A mixture of iMab and m36 at a 1:10 ratio was more potent than a mixture of iMab and m36 at a 1:2 ratio. Notably, even with a 5-fold excess of m36, the antibody mixture was still less potent than the bispecific molecule. These results suggest that iMab and m36, once fused into a single antibody-like molecule contribute to the antiviral activity in a synergistic manner.

Improved Antiviral potency of iMabm36 is dependent on its CD4 binding activity and sensitivity to m36

To understand the mechanism of the improved antiviral activity of the iMab36 fusion, we ablated its ability to bind CD4 by mutating two contact residues, Glu (E) and Try (Y), in the iMab CDR H3 to alanine, yielding iMabm36 (Figure 4A, left panel)³³. An ELISA was conducted to confirm that iMabm36 lost its capability to compete with iMab for CD4 binding (Figure 4A, right panel). Correspondingly, iMabm36 also lost its neutralization activity against the viruses tested (CQLDR03-A2 and RHPA4259.1mut) (Figure 4B), indicating the CD4-binding activity of iMabm36 is critical for its activity.

Since the gp120 bridging sheet interacts directly with the HIV-1 coreceptors, we assessed whether iMabm36's activity was dependent on CD4-binding activity specifically, or if anchoring m36 to CCR5 via the anti-CCR5 mAb PRO140 could also augment the activity of m36. Thus, we replaced the iMab component of iMabm36 with PRO140³⁷ to generate PRO140m36 and assessed its activity against the prototypic CXCR4-tropic virus, NL4-3 and which is therefore resistant to PRO140. Here, PRO140m36 was indeed approximately 10-fold more potent than m36, indicating that both CD4-anchoring and CCR5-anchoring of m36 can augment m36's neutralization activity (Figure 4C).

To assess the contribution of m36 specificity in the context of iMabm36, we replaced all acidic and tyrosine residues of the m36 CDRs with alanine, since acidic and tyrosine residues are considered vital to the activity of CD4i Abs^{21,35,38}. These substitutions in iMab m36 abolished the improved neutralization activity of iMabm36 such that its neutralization activity closely resembled that of iMab (Figure 4D). These results indicate that the antiviral activity of iMabm36 is dependent on both its CD4 binding activity as well as its intrinsic m36 activity. Furthermore, in analyzing the neutralization data against nine iMab-resistant viruses, we noticed a highly significant direct correlation ($r^2 = 0.93$, $P < 0.001$) of the IC₅₀ of m36 and the IC₅₀ of iMabm36 (Figure 4E). Together, these results suggest that, in the context of iMab-resistant viruses, the potency of iMabm36 is determined by the virus sensitivity to m36.

To more comprehensively assess its breadth and potency against HIV-1, iMabm36 activity was further subjected to testing against a large panel of HIV-1 pseudoviruses (n=118)

representing all major circulating HIV-1 subtypes. While iMabm36 did not exhibit significantly improved breadth of neutralization compared to ibalizumab ($P=0.4$), neutralizing 96% and 92% of the panel, respectively, iMabm36 was significantly more effective at inhibiting HIV-1 infection, achieving on average 91.5% MPI compared to only 81.8% by ibalizumab ($P<0.001$). A majority (83%) of this panel of viruses were neutralized by iMabm36 at an IC_{50} of less than $0.1\mu\text{g/mL}$ compared to 75% for iMab (Figure 5A). iMabm36 exhibited IC_{80} values of $<7\text{nM}$ ($<1\mu\text{g/mL}$) for 45.7% of the viruses tested, compared to 40.7% for iMab (Figure 5B). These results suggest that fusion of m36 to iMab (iMabm36) improves the antiviral activity of iMab.

Understanding iMabm36 resistance

In analyzing the neutralization data from the large panel of HIV-1 pseudoviruses ($n=118$), m36 fusion to iMab improved the neutralization activity of iMab against the viruses of all clades except clade A and G (Figure 6A). To assess whether clade A viruses were resistant to iMabm36 because they lack the m36 epitope, we aligned and compared the sequences of their gp120 bridging sheets ($\beta 3$, $\beta 2$, $\beta 21$ and $\beta 20$ strands) that contains the putative epitope of m36 to the consensus B sequence²⁷. Based on the comparison, we substituted specific residues in the $\beta 2$ and $\beta 21$ sheets of Q23.17 (clade A), Q259.d2.17 (clade A) and T28-50 (clade AG) Env with clade B consensus amino acids (Table 1), since m36 was initially isolated by phage display using a clade B gp120 as bait^{26,27}. As shown in Figure 6B, the resistant viruses with $\beta 21$ site-directed mutations to clade B consensus residues were more sensitive to iMabm36. Furthermore, the combination of the $\beta 3\beta 21$ mutations rendered the viruses highly sensitive to iMabm36, indicating that bridging sheet sequence divergence of clade A/AG viruses confers iMabm36 resistance.

Modifications to improve iMabm36 antiviral activity

To investigate the impact of the linker length on the antiviral activity of iMabm36, we compared the neutralization activity of the original iMabm36 (G4S)3 to iMabm36 with a short linker (G4S)1 and iMabm36 with a long linker (G4S)5. iMabm36 (G4S)3 had greater antiviral activity than iMabm36 with a short linker (G4S)1. iMabm36 with a long linker (G4S)5 was more active against iMabm36 resistant viruses 1006_11_C3_1601 and du172.17 (Figure 7A). Thus, a longer linker between iMab and m36 improves the antiviral activity of the fusion Ab.

To improve iMabm36 activity against clade A viruses, we modified the m36 CDRs. In particular, we substituted m36 CDR H3 with that of the CDR H3 from the CD4i Ab E51, since this CDR has more favorable electrostatic interactions and a greater binding interface with gp120 than m36^{39,40}. When tested against iMabm36 resistant pseudoviruses (Q23.17 and Q259.d2.17), the resultant modified iMabm36 (CDR3 E51) exhibited more potent neutralizing activity against these two viruses tested (Figure 7B).

Finally, we combined the CDR H3 modification [m36(CDR3 E51)] with iMabm36L5 [the iMabm36 variant with the longer linker (G4S)5] to produce an optimized iMabm36, termed iMabm36opt. When tested against iMabm36-sensitive and resistant pseudoviruses, the iMabm36opt retained its antiviral activity against iMabm36 sensitive viruses, and

neutralized resistant viruses more effectively than iMabm36, iMabm36L5 and iMabm36(CDR3 E51), achieving an MPI of 86%-98% compared to only 40%-87% for iMabm36, 65%-94% for iMabm36L5, and 75-91% for iMabm36(CDR3 E51) (data not shown). In analyzing MPIs from nine iMabm36-resistant viruses tested, a highly significant improvement ($P=0.0029$) of the MPI for iMabm36opt was observed (Figure 7C). Thus, these data suggest that the optimized variants of iMabm36 can exhibit greater potency antiviral activity than the original iMabm36.

Discussion

In this study, we engineered iMab linked to two copies of m36, characterized iMabm36 antiviral activity, and developed optimized variants of this novel bispecific Ab to further enhance its antiviral activity. iMabm36 has increased antiviral activity over iMab and m36 alone. We also sought to understand its dual mechanism of action since we found that the improved activity of iMabm36 was dependent on both CD4 binding and sensitivity to m36. The inter-dependency of this dual mechanism of action enables the high potency and breadth of iMabm36. Moreover, we investigated the influence of linker length and m36 composition and specificity on its antiviral activity for further optimizing antiviral potency and breadth.

We attribute the improved antiviral activity of iMabm36 to its inter-dependent, dual mechanism of action. iMabm36 binds with high affinity to CD4 via its ibalizumab component, pre-concentrating m36 on the target cell surface in the vicinity of viral entry. Engagement of HIV-1 Env with CD4^{33,34,41} and subsequent formation and exposure of the bridging sheet of gp120 (unpublished data), both of which are unimpaired by ibalizumab, leads to formation of the m36 epitope, which can then be efficiently targeted by the m36 domains fused to the C-terminus of the ibalizumab heavy chain due to the reduced steric constraints imposed by the virological synapse and thus interfere with CoR engagement. Since almost all HIV-1 isolates use CD4 as a primary entry receptor in vivo and the m36 targeting site is relatively conserved across all HIV-1 isolates, simultaneously targeting of these two sites provides potent antiviral activity and likely a high barrier against viral resistance. Interestingly, no antiviral activity enhancement was observed when m36 was fused to the N-terminal of the heavy or light chain of iMab (data not shown). On the other hand, fusion of m36 to the C-terminal of iMab may block the adjacent “entry complex” of gp120 and CoR interaction. As such, the unique location and enriched local concentration of m36 when fused to the C-terminal of iMab may overcome temporal and steric restrictions during viral entry, thus further enhancing antiviral potency and breadth as compared to either m36 or iMab alone.

Although the CD4i Abs contact a relatively conserved gp120 element, changes in the major gp120 variable loops can influence the activity of these CD4i Abs^{27,42}. Our studies showed that viruses with clade A and clade G Envs were resistant to iMabm36 neutralization. However, mutating the bridging sheet of some Clade A viruses (Q23.17, Q259.d2.17 and T28-50) to resemble that of clade B viruses can render them sensitive to iMabm36. Such neutralization differences between these viruses and their mutants provide a natural explanation for the limited sensitivity of clade A viruses to neutralization by iMabm36. m36 was selected based on clade B Envs (JRFL, Bal, and R2)^{26,43}, and our results indicate that

the activity of iMabm36 is partially determined by the virus sensitivity to m36. Therefore, further modification of m36, i.e. selection based on divergent Envs, might be a potential way to further enhance the antiviral activity of the iMabm36 bibNAb.

Previous studies showed that the limited patches of conserved sequence on the co-receptor-binding surface are available to be accessed by CD4i antibodies²⁵. The linker length between m36 and iMab may restrict the flexibility of m36 to access its target. Indeed, the results obtained using constructs with different linker lengths indicate that lengthening the linker between iMab and m36 could improve the MPI against the resistant viruses. One possibility could be that the longer linker provides m36 with greater flexibility to adequately cover and recognize the binding site more efficiently than that of a short linker.

It is reported that CD4i antibodies include two groups based on antibody CDR length^{21,35}. In the CDR short group (CDR H3 10~14 AA) the CDR H3s were not very acidic, whereas the CDR H2s displayed an unusual concentration of three or four acidic residues at the loop tip. In the CDR long group (CDR H3 19~25 AA), the CDR H3s were acidic and comprised more tyrosines in contrast to the short group. Because the conserved CD4i epitope component is located at the interface of the gp120 outer domain and bridging sheet, electrostatic interactions provided by net charge could influence its interaction with gp120 and drive conformational changes related to virus entry. Thus, through the CDR H3 (E51) substitution^{38,44,45}, an improved version of iMabm36(CDR3 E51) was generated that achieves greater MPI. These data suggest CDRH3 modifications similar to the tyrosine rich and acidic N-terminal region of CCR5 may provide better recognition and binding to gp120 than unmodified iMabm36. This approach indicates such iMabm36 CDRH optimization, even though slightly, might provide tyrosine posttranslational mimicry of the CCR5 N-terminus and enhanced electrostatic interaction to reduce the binding energy between the modified m36 CDR and gp120, especially when the high local concentration of m36 was achieved by anchoring it near the co-receptor binding site by iMab or PRO 140. Strategies to combine bNAbs against HIV-1 have been explored in order to confront the emergence of resistant mutants; and bispecific antibodies continue to be an area of great interest in the pursuit of next-generation monoclonal antibodies against disease. Rationally designed, dual-targeting fusion antibodies such as our previously described PG9-iMab, PG16-iMab and now iMabm36 and PRO140m36 may improve the antiviral activity of bNAbs by anchoring bNAb activity at the site of viral entry, enhancing both their local concentration and accessibility to their cognate epitope. Such bibNAbs may improve the overall activity of bNAbs and antagonize HIV-1 escape better than antibody combinations and could provide a high barrier against HIV-1 escape.

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Figure 1. Generation of iMabm36 fusion antibody

1A. Schematic representations of iMabm36 fusion construct (iMab-G4S $\times$ 3-m36). m36 was linked by a flexible glycine-serine linker (G4S $\times$ 3) to C-terminal of the iMab heavy chain.

1B. iMab36 fusion and iMab were produced in HEK293A cells, m36 was produced in E.coli.

1C. iMabm36 binds sCD4. X-axis indicates increasing concentrations of the unlabeled iMab36, iMab, or m36. Y-axis indicates the competitive level of binding of HRP-labeled ibalizumab to sCD4.

Figure 2. iMabm36 antiviral activity against iMab-sensitive and resistant viruses

2A. CQLDR03-A2 and SC20 8A8A: iMab-sensitive viruses.

2B. 9077.12 B5A, TT31P 2F10, SC33 4H1 and RHPA4259.1mut: iMab-resistant viruses.

2C. Antiviral activity of iMab and iMabm36 against 6 replication-competent transmitted-founder viruses in a PBMC-based neutralizing assay. Y-axis indicates 100% inhibition concentration.

Figure 3. Antiviral activity of iMabm36 vs coadministration of iMab with m36

3A. RHPA4259.1mut, 9077.12 B5A and TT31P 2F10 (iMab-resistant viruses) are tested against m36, iMab, iMabm36 and coadministration of iMab and m36 at a ratio of 1:2.

3B. RHPA4259.1mut, 9077.12 B5A and TT31P 2F10 (iMab-resistant viruses) are tested against iMabm36 and coadministration of iMab and m36 at ratios of 1:2 and 1:10.

A

B

C

Figure 4. Mechanism of action of iMabm36

4A. Glu (E) and Try (Y), two contact amino acid residues in the iMab heavy chain variable CDR3 region were substituted with Ala (A) residues to ablate the CD4 binding ability of iMab (left). iMabm36 does not bind sCD4. X-axis indicates increasing levels of the unlabeled constructs for iMab36, iMab, and iMabm36; Y-axis indicates the competitive level of binding of HRP-labeled ibalizumab to sCD4 (right).

4B. CQLDR03-A2 (iMab-sensitive) and RHPA4259.1mut (iMab-resistant) viruses were tested for their viral activity in the presence of iMab36, iMab, and iMabm36.

4C. PRO140m36 antiviral activity against X4 virus NL4-3.

4D. SC33 4H1 and TT31P 2F10 (iMab-resistant viruses) were tested for their viral activity in the presence of iMab36, iMab, and iMab m36. m36 contains a combination of acidic and tyrosine mutations in the m36 CDRs that abolish m36 anti-viral activity.

4E. The correlation of iMab-resistant virus sensitivity to m36 versus iMabm36. X-axis indicates the m36 IC₅₀ (ng/mL), whereas Y-axis indicates iMabm36 IC₅₀ (ng/mL).

Figure 5. iMabm36 antiviral activity against a panel of 118 HIV-1 Env pseudoviruses
 5A. Neutralization of a panel of 118 HIV-1 Env pseudoviruses by iMab (top panel³¹) and iMabm36 (bottom panel) as measured in a TZM-bl assay. For each virus, black bars indicate the MPI when tested at antibody concentrations up to 10 μg/mL (left y-axis), and the corresponding IC₅₀ (μg/ml) (right y-axis). Viruses are ordered by descending MPI for iMab.
 5B. IC₈₀ analysis of iMab and iMabm36 against 118 HIV-1 Env pseudoviruses.

Figure 6. Mechanism of iMabm36 resistance

6A. Comparison of MPI of iMabm36 and iMab against 118 HIV-1 Env pseudoviruses grouped based on clades.

6B. Q23.17 (clade A), Q259.d2.17 (clade A), T28-50 (clade AG) and their respective bridging sheet mutants were examined for their susceptibility to iMabm36.

Figure 7. iMabm36 Optimizations

7A. 1006_11_c3_1601 and Du172.17 (iMab-resistant viruses) were tested for their linker length impact against viral activity in the presence of iMab36 (G4S×1), iMabm36 (G4S×3) and iMab36 (G4S×5).

7B. Q23.17 and Q239.d2.17 (iMabm36-resistant viruses) were tested for their viral activity in the presence of the iMabm36 and CDRH3 modification construct iMabm36(CDR E51).

7C. MPI analysis of iMab, iMabm36 and iMabm36opt on a small panel of eight iMabm36-resistant viruses.

Table 1
gp120 bridging sheet sequence comparison and mutagenesis

Virus	$\beta 2$	$\beta 3$	$\beta 20$	$\beta 21$
Consensus B	CVKLT	SVI	QIINM	VGKAMY
Q23.17 (A)	-----	SAI	-----	AGQAMY
Q23- $\beta 21$ mut	-----	SAI	-----	<u>V</u> GKAMY
Q23- $\beta 3\beta 21$ mut	-----	<u>S</u> V <u>I</u>	-----	<u>V</u> GKAMY
Q259.d2.17 (A)	-----	SAI	-----	AGQAIY
Q259- $\beta 21$ mut	-----	SAI	-----	<u>V</u> GKAMY
Q259- $\beta 3\beta 21$ mut	-----	<u>S</u> V <u>I</u>	-----	<u>V</u> GKAMY
T278-50 (AG)	-----	SAI	-----	VGQAMY
T278- $\beta 21$ mut	-----	SAI	-----	VG <u>K</u> AMY
T278- $\beta 3\beta 21$ mut	-----	<u>S</u> V <u>I</u>	-----	VG <u>K</u> AMY

Based on the comparison, specific residues in the $\beta 2$ and $\beta 21$ sheets of Q23.17 (clade A), Q259.d2.17 (clade A) and T28-50 (clade AG) Envs were substituted with clade B consensus amino acids.