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# Synergistic inhibition of cell-to-cell HIV-1 infection by combinations of single chain variable fragments and fusion inhibitors

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ABSTRACT

Cell-to-cell spread of HIV permits ongoing viral replication in the presence of antiretroviral therapy and is suggested to be a major contributor to sexual transmission by mucosal routes. Fusion inhibitors that prevent viral entry have been developed, but their clinical applications have been limited by weak antiviral activity, short half-life, and the low genetic barrier to development of resistance. We examined the inhibitory activities of a series of single-chain variable fragments (scFvs) targeting the V3 and CD4i epitopes against both cell-free and cell-to-cell HIV infection. We found that all anti-V3 scFvs, including two newly constructed scFvs, showed broad neutralization activity against a panel of subtype B viruses compared with the corresponding IgGs. All scFvs neutralized cell-free infection by HIV-$1_{JR\text{-}FL\ WT}$ and fusion inhibitor-resistant mutants. In addition, all anti-V3 scFvs and some CD4i scFvs significantly inhibited cell fusion, while their IgG counterparts did not. Furthermore, scFvs-fusion inhibitors combinations, such as C34 and SC34, showed synergistic inhibition of cell fusion by both HIV-$1_{JR\text{-}FL\ WT}$ and fusion inhibitor-resistant mutants. The most prominent combinational effect was observed for 916B2 CD4i scFv with SC34. The delayed fusion kinetics of fusion inhibitor-resistant mutants partly explain their synergistic inhibition by such combinations. Our data demonstrate the advantages of using scFvs over their parent IgGs for inhibiting both cell-free and cell-to-cell infection. High synergistic inhibition of cell fusion by using scFvs-fusion inhibitors combinations suggests the possibility of intensification therapy adding this combination to current anti-HIV treatment regimens.

## 1. Introduction

Antiretroviral therapy [ART] has transformed the HIV pandemic by averting deaths, improving quality of life, and preventing new HIV infections. However, ART alone will not end epidemics as one recent report suggested [1]. ART must be continued for life because persistent viral infection as replication of residual virus or reactivation of virus from latently infected cells can occur *in vivo* [2]. One of the major problems in treatment of HIV infections is antiviral resistance [1,3]. In addition, the role of cell-to-cell infection in disease pathophysiology has drawn much attention [2]. Cell-to-cell transmission is more efficient than cell-free transmission, mediates resistance to neutralizing antibodies [4,5], facilitates the spread of virus among T cells in the presence of antiviral agents [6], and has been suggested as a major contributor to sexual transmission by mucosal routes [7–9]. Moreover, recent work suggested that cell-to-cell transmission influences the establishment and maintenance of latent infection in resting $CD4^+$ cells [10].

Structural features of the HIV-1 fusion machinery in gp41 have helped in the development of fusion inhibitors [11–13] that prevent cell-to-cell infection. However, treatment with the first-generation HIV fusion inhibitor T20 [generic name, enfuvirtide; brand name, Fuzeon] resulted in frequent development of drug resistance [14,15]. A series of peptides derived from the C-terminal heptad repeat [HR] of gp41 [e.g. C43, C34, and C28] exert anti-HIV activity in the nanomolar range by binding to the N-terminal core of gp41 [15]. Compared with T20, C34

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showed a relatively high genetic barrier for development of resistance *in vitro* [16], and was found to be a more potent HIV inhibitor with activity against T20-resistant viral strains [17]. Derivatives of C34 peptides, such as SC34, with better solubility, enhanced α-helicity, enhanced activity, and a higher barrier to resistance development [18] are under pre-clinical evaluation [18,19].

Epitopes such as the V3 loop or CD4-induced [CD4i] sites of gp120 are exposed on the surface of trimeric Env only after conformational changes induced by binding to CD4. It is difficult for IgGs [115 Å] to access these epitopes due to the close physical proximity of gp120 to the cellular membrane [45–80 Å] [20]. The importance of size reduction for access to CD4i epitopes was demonstrated by improved neutralization using single chain variable fragments [scFvs] [~40 Å] constructed from their parent IgGs. The scFvs not only neutralized primary viruses resistant to the parental IgGs [20], but also showed broader inhibitory activity than the corresponding IgGs [21,22]. Post-binding neutralization played a crucial role in the improved inhibitory activity of the scFvs.

In the present study, we constructed two novel scFvs from anti-V3 IgGs and demonstrated their effective and broad neutralization activity. In addition, we showed the inhibitory activity of the scFvs against both cell-free and cell-to-cell infection. Synergistic inhibition of HIV-induced cell fusion by scFvs and fusion inhibitors suggests the combination of scFvs with fusion inhibitors as a future combination to inhibit cell-to-cell persistent infection *in vivo*.

## 2. Materials and methods

### 2.1. Cells and viruses

TZM-bl [23], 293T [24], and 293A [25] cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich). Env clones from a standard panel of subtypes B and the transmitted/founder viruses were obtained from the NIH AIDS Reagent program [26–29]. Pseudoviruses were prepared by transfecting 293T cells with a plasmid expressing Env and the *env*-deficient HIV-1 backbone vector pSG3ΔEnv using Lipofectamine 2000 transfection reagent (Invitrogen, Life Technologies, Carlsbad, CA) as previously described [22].

### 2.2. Construction and purification of scFvs

Monoclonal antibodies targeting the V3 loop (0.5γ and 717G2) and CD4i epitopes (916B2, 25C4b and 4E9C) were previously described [29]. The anti-V3 antibody, 19F8, was newly isolated from the same patient, KTS376, and the recombinant IgG was constructed as previously described [29]. The scFv genes were amplified using primers shown in Supplementary Table 1, as previously described [21,22], and were inserted into pETCF [2].

The scFvs 19F8 and 717G2 were purified using His 60 Ni Superfow Resin column (Clontech), V3 peptide column and a size exclusion chromatography similarly to other scFvs [21,22].

### 2.3. Binding activity of anti-V3 IgGs and scFvs

Binding activity of anti-V3 IgGs and scFvs was assessed by ELISA using V3 peptide from HIV-1 JR-FL (NNT-20), as described previously [29]. Surface plasmon resonance (SPR) analysis was performed by a BiaCore T-200 system (GE Healthcare) using biotinylated V3 Peptide and a sensor chip SA as previously described [21]. The equilibrium dissociation constant ($k_d$) was determined.

### 2.4. Analysis of neutralization activity

The neutralization activity of IgG and scFvs was measured using TZM-bl cells and pseudoviruses as described previously [22,30]. The percent inhibition was determined by comparing relative luminescence (RLUs) in the presence and absence of the inhibitor.

### 2.5. Analysis of cell fusion inhibition

Fusion kinetics were determined using a dual split protein [DSP]-based cell fusion assay [30,31]. The 293T cells co-transfected with a plasmid expressing virus Env and $pDSP_{1-7}$ plasmid using Lipofectamine 2000 in a six-well cell culture plate. Concurrently, TZM-bl cells [$10^4$ cells/well] were transfected with the $pDSP_{8-11}$ plasmids in a 96-well white-bottomed cell culture plate (Sigma-Aldrich, Costar, Corning, Life Sciences). After 36–48 h of transfection, the TZM-bl cell media were replaced with 50 μL of fresh DMEM containing 10% FCS and 60 μM EnduRen (Promega Corporation, Madison, WI, USA). After incubating for 3–4 h, the transfected TZM-bl cells were washed with fresh medium and serially diluted antibodies were added. The transfected 293T cells were harvested using 0.05% trypsin and re-suspended at a concentration of $2 \times 10^6$ cells/mL in fresh medium then [50 μL] cell suspension was transferred into wells containing the transfected TZM-bl cells with gentle mixing several times. Renilla luciferase activity was measured with a luminometer at 0, 15, 30, 45, 60, 80, 100, and 120 min after co-culture. During co-culture, the expression level of Env in the transfected 293T cells was analyzed by staining with anti-V3 and anti-CD4i IgG/scFvs and fusion inhibitors. The expression levels of Env mutants were confirmed to be similar to that of WT Env (< 20% change in MFI). The fusion inhibition percentage was calculated using the RLU value in the presence and absence of an inhibitor at 120 min as 100% [30].

### 2.6. Analysis of synergistic inhibition of cell fusion

The combinational inhibitory activity of antibodies and fusion inhibitors against cell fusion was analyzed using a response surface model, called Zero Interaction Potency (ZIP). This model combines the Loewe and the Bliss models, and proposed a delta score to characterize the synergy landscape over the full dose-response matrix [32,33]. Briefly, antibodies and fusion inhibitors were tested for their inhibition activity against cell fusion individually and in combination at multiple molar ratios. The percent fusion inhibitions resulting from the combination dosing of IgG/scFv and fusion inhibitors were assessed for synergistic action using R Package Synergy Finder [33] and ZIP model [32], which generates synergy scores from a dose-response matrix.

## 3. Results

### 3.1. Construction and characterization of scFvs that target the V3 loop of gp120

Three anti-V3 monoclonal IgGs (0.5γ, 19F8 and 717G2) were cloned from an HIV-1 infected patient, KTS376 [29]. SDS-PAGE analysis confirmed that the heavy and light chains of three anti-V3 monoclonal IgGs (0.5γ, 19F8 and 717G2) were approximately 50 kDa and 25 kDa, respectively (Fig. S1A). Plasmids expressing scFvs of 0.5γ, 19F8 and 717G2 were constructed by connecting the variable regions of heavy and light chains with a linker [GGSSRSSSSGGGGSGGGG]. These scFvs were expressed in *E. coli* and purified as previously reported [21]. The purified scFvs migrated as a single band around 30 kDa, demonstrating its high purity (Fig. S1A). All three anti-V3 IgGs and scFvs showed similar binding affinities by ELISA using V3 peptide as an antigen (Fig.

S1B). SPR analysis using the biotin-conjugated V3 peptide also demonstrated the high binding affinities of these IgGs and scFvs with $k_d$ values ranging from 0.14 to 0.39 nM for IgGs and from 0.27 to 1.8 nM for scFvs (Figs. S1C and S2). The $k_d$ values and binding kinetics of all anti-V3 scFvs were similar to the corresponding IgGs, with the exception that the $k_d$ value of 717G2 scFv was 10 times higher than that of the corresponding IgG.

### 3.2. Neutralizing potency and breadth of anti-V3 IgGs and scFvs

Standard TZM-bl cell-based neutralization assays were performed to compare the neutralization breadths of 0.5γ, 19F8 and 717G2 IgGs and scFvs against a panel of 27 HIV-1 subtype B viruses (Fig. S3). IgGs neutralization potencies was higher against laboratory strains than their corresponding scFvs, consistent with their higher binding affinities (Fig. S1C). In contrast, a wider breadth of neutralization was observed for scFvs than IgGs against the standard panel of subtype B viruses and transmitted/founder (T/F) viruses. The $IC_{50}$ values of 0.5γ scFv were even lower than those of 0.5γ IgG against TRO.11, AC10.0.29 and CAAN5342.A2, which required a high concentration ($IC_{50}$: > 500 nM) of IgG for neutralization. Moreover, 0.5γ scFv neutralized SC422661.8, RHPA and TRJO, but 0.5γ IgG did not. Neutralization of IgG-resistant viruses resulted in a wide neutralization breadth of the scFvs [0.5γ (74%), 19F8 (70%) and 717G2 (74%)] compared with the IgGs [0.5γ (63%), 19F8 (52%) and 717G2 (48%)], consistent with previous observations of anti-V3 and CD4i scFvs [20–22,34]. Post-attachment neutralization was suggested as a mechanism for the wider neutralization breadth of these scFvs, which can access their epitopes in the narrow space between a target cell and a virion.

### 3.3. Inhibition of cell fusion by scFvs against the V3 and CD4i epitopes

To evaluate cell-to-cell infection, the ability of anti-V3 and anti-CD4i scFvs to inhibit HIV-induced cell fusion was examined using TZM-bl cells and 293T cells expressing HIV-1 Env. We used HIV-$1_{JR\text{-}FL\ WT}$ and the fusion inhibitor-resistant mutants, C34r, I37K and SC34r [22], to compare the inhibitory activities of scFvs with those of the corresponding IgGs. Cell-free neutralization of WT and mutant viruses was observed for both IgGs and scFvs against the V3 epitope, and IgGs were more effective than scFvs (Fig. 1, **upper panel**). In the case of anti-CD4i antibodies, cell-free neutralization was only observed for scFvs but not for their parental IgGs. By contrast, HIV-$1_{JR\text{-}FL\ WT}$ was resistant to all IgGs and scFv in the cell fusion assay (Fig. 1, **lower panel).** The three fusion inhibitor-resistant mutants, however, were sensitive to inhibition of cell fusion by anti-V3 scFvs and some anti-CD4i scFvs. Among anti-CD4i scFvs, 916B2 inhibited cell fusion induced by all three fusion inhibitor-resistant mutants, while the other two anti-CD4i scFvs only inhibited cell fusion induced by SC34r.

### 3.4. Synergistic activity of scFvs and fusion inhibitors in inhibiting cell fusion

We further investigated the combinational activity of antibodies and fusion inhibitors to inhibit cell fusion, and their synergistic effect was analyzed using R Package Synergy Finder with the ZIP model. The most significant synergy was observed between C34 and the 916B2 scFv against the I37K mutant (Fig. 2). The inhibitory activities of C34 and 916B2 scFv at the highest concentrations tested were 39% and 52%, respectively (Fig. 2D), while their combination achieved more than 60% inhibition at low concentrations (Fig. 2E). This high level of synergy was observed for this combination at all concentrations tested [Fig. 2F]. By contrast, poor or very weak synergy was observed for the combination of C34 and 916B2 IgG against I37K [Fig. 2C].

The synergistic effects between antibodies and fusion inhibitors on cell fusion inhibition are summarized in Fig. 3. High synergistic inhibition of cell fusion was observed among combinations of scFvs and fusion inhibitors, but not among IgGs-fusion inhibitors combinations. The 916B2 scFv-SC34 combination showed the most prominent synergy, as shown by its ZIP model synergy score above 20 against all viruses tested. The synergy score of 916B2 scFv-C34 combination was also very high against WT and the I37K mutant, but not against C34r and SC34r. In contrast, synergy between IgGs and fusion inhibitors was sporadic and week. The synergistic effect was observed through inhibition of viruses which were not inhibited by scFv alone or viruses resistant to fusion inhibitors, resulting in the inhibition of cell fusion by these scFvs in the presence of a low concentration of fusion inhibitor (Figs. S4 and S5).

### 3.5. Fusion kinetics of cells expressing HIV-$1_{JR\text{-}FL}$ WT Env and fusion inhibitor-resistant mutants

To investigate the mechanism underlying the synergistic activities of scFvs and fusion inhibitors, the fusion kinetics of each virus was determined in the presence of serial doses of fusion inhibitors (Fig. S6). Each curve showed the progress of fusion at different time points for JR-

| Virus | Neutralization $IC_{50}$(nM) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fusion Inhibitor | | Anti-V3 antibody | | | | | | Anti-CD4i antibody | | | | | |
| | | | 0.5γ | | 19F8 | | 717G2 | | 916B2 | | 25C4b | | 4E9C | |
| | C34 | SC34 | IgG | scFv | IgG | scFv | IgG | scFv | IgG | scFv | IgG | scFv | IgG | scFv |
| JR-FL WT | 23.0 | 25.0 | 26.0 | 78.0 | 63.0 | 300 | 45.0 | 170 | >2000 | 530 | >2000 | 1190 | >2000 | 610 |
| C34r | >2000 | 65.0 | 11.0 | 59.0 | 71.0 | 365 | 34.0 | 200 | >2000 | 760 | >2000 | >2000 | >2000 | 1100 |
| I37K | 236 | 32.0 | 11.0 | 39.0 | 50.0 | 185 | 23.0 | 110 | >2000 | 330 | >2000 | 970 | >2000 | 960 |
| SC34r | >2000 | >2000 | 21.0 | 45.0 | 125 | 225 | 34.0 | 130 | >2000 | 200 | >2000 | 1240 | >2000 | 930 |
| **Envelope** | **Fusion Inhibition $IC_{50}$(nM)** | | | | | | | | | | | | | |
| JR-FL WT | 67.0 | 27.0 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| C34r | >2000 | 225 | >2000 | 465 | >2000 | 1050 | >2000 | 1720 | >2000 | 1280 | >2000 | >2000 | >2000 | >2000 |
| I37K | >2000 | 105 | >2000 | 215 | >2000 | 1540 | >2000 | 1420 | >2000 | 1120 | >2000 | >2000 | >2000 | >2000 |
| SC34r | >2000 | >2000 | >2000 | 225 | >2000 | 135 | >2000 | 185 | >2000 | 410 | >2000 | 910 | >2000 | 1550 |

**Fig. 1. Inhibition of cell-free infection and cell fusion.** Neutralization of cell-free infection was measured by standard TZM-bl cell-based neutralization assays using pseudoviruses bearing Envs of HIV-$1_{JR\text{-}FL\ WT}$ and fusion inhibitor-resistant mutants, C34r, I37K and SC34r. Inhibition of cell fusion was measured by dual split protein fusion assay using Env-expressing cells and TZM-bl cells. Representative $IC_{50}$ values (nM) of three independent experiments are shown. Color code is as follows: red, < 100 nM; green, 100–500 nM; yellow, 500–2000 nM.

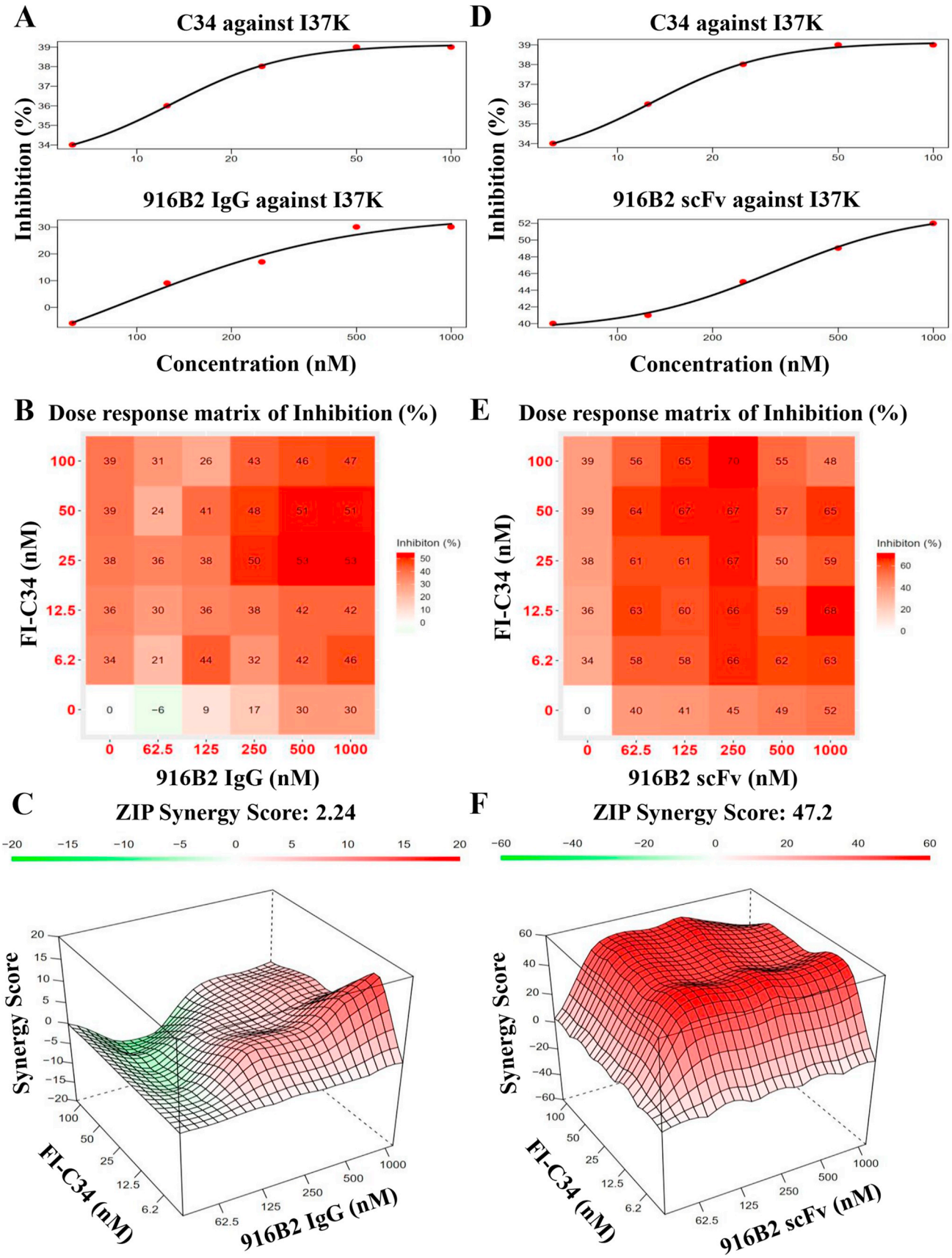


**Fig. 2. Synergistic effect of fusion inhibitors and scFvs in inhibiting cell fusion.** The combinatorial effect of the fusion inhibitor C34 and 916B2 IgG or scFv in inhibiting fusion between TZM-bl cells and cells expressing Env from the fusion inhibitor-resistant mutant, I37K, was examined. Individual dose-response curves for C34 and 916B2 IgG (A) or scFv (D) are shown. Combinatorial inhibition of C34 and 916B2 IgG (B) or scFv (E) is shown by the dose-response matrix of % inhibition. The synergistic effect of C34 and 916B2 IgG (C) or scFv (F) is shown by the 3D synergy landscape. Synergy score was calculated using synergy finder R package for drug combination data analysis.

| Virus Envelop | Fusion Inhibitor | ZIP Model Synergy Score ($\delta$-Score) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Anti-V3 antibodies | | | | | | Anti-CD4i antibodies | | | | | |
| | | 0.5γ | | 19F8 | | 717G2 | | 916B2 | | 25C4b | | 4E9C | |
| | | IgG | scFv | IgG | scFv | IgG | scFv | IgG | scFv | IgG | scFv | IgG | scFv |
| JR-FL WT | C34 | -19.0 | 19.0 | -22.0 | 17.0 | -1.04 | 21.0 | -11.0 | 29.0 | 4.43 | 7.02 | -9.01 | 18.1 |
| C34r | | -20.0 | 14.0 | -5.03 | 6.01 | 1.01 | 19.0 | -5.42 | 2.11 | -1.01 | 8.11 | 20.0 | 20.0 |
| I37K | | -24.0 | 10.0 | 17.0 | 25.0 | 8.14 | 23.0 | 2.24 | 47.2 | 2.01 | 23.0 | 4.23 | 18.2 |
| SC34r | | 1.03 | 3.04 | -6.02 | 5.03 | 3.02 | 9.01 | 10.0 | 5.01 | -5.03 | 5.01 | 1.31 | 11.2 |
| JR-FL WT | SC34 | 8.01 | 12.0 | -3.04 | 12.0 | 7.01 | 9.03 | -1.41 | 30.0 | -1.03 | 12.1 | 9.04 | 7.03 |
| C34r | | -5.04 | 16.0 | 2.01 | 16.0 | -6.31 | 17.0 | -11.2 | 33.4 | 12.0 | 24.0 | -2.02 | 4.14 |
| I37K | | 7.23 | 14.0 | 7.03 | 15.0 | -7.14 | 9.23 | 1.02 | 25.4 | 2.01 | 13.1 | -1.13 | 19.1 |
| SC34r | | 19.0 | 21.0 | -5.04 | 6.01 | 11.0 | 35.0 | 8.03 | 22.3 | 6.01 | 12.2 | 2.01 | 15.3 |

**Fig. 3. Synergistic effect of fusion inhibitors and IgGs or scFvs.** Combinations of fusion inhibitors and IgGs or scFvs were examined for their activities to inhibit cell fusion of TZM-bl cells and cells expressing Envs derived from HIV-$1_{JR-FL}$ WT and the fusion inhibitor-resistant mutants, C34r, I37K and SC34r. ZIP model synergy scores were calculated using the synergy finder R package for drug combination data analysis, and representative synergy scores from three independent experiments are shown. Color code is as follows: white, < 5; yellow, 6–9; green, 10–19; red, > 20.

FL WT and fusion inhibitor-resistant mutants. We observed that C34 induced delayed fusion of JR-FL WT at concentrations as low as 15.63 nM (Fig. S6A). The delay of cell fusion was also observed for the I37K mutant, though a high concentration of C34 was required (Fig. S6C). In contrast, no delay in fusion was observed for the C34r and SC34r mutants. JR-FL WT and the I37K mutant, which showed the delayed cell fusion, also showed a higher synergy for the C34-scFvs combination than other two mutants which showed no delayed fusion kinetics [Fig. 3]. This was clearly the case for 916B2 scFv-C34 combination, suggesting that fusion delay may be responsible for synergistic inhibition.

In the case of SC34, delay of cell fusion was observed at very low concentrations for JR-FL WT, C34r, I37K (Fig. S6E, F and G). However, no delayed fusion kinetics was observed against SC34r mutant, which the SC34 and scFv combinations showed strong synergistic inhibition against (Fig. 3). These results suggested that, along with delayed fusion kinetics, other mechanisms contribute to the synergistic inhibition of cell fusion by scFvs-fusion inhibitors combinations.

## 4. Discussion

In this study, we demonstrated a combinational inhibitory effect of scFvs with fusion inhibitors in preventing cell-to-cell infection of HIV-1. We found that anti-V3 and anti-CD4i scFvs were effective inhibitors of cell-free infection and cell-to-cell infection in the presence of fusion inhibitors. Individual or combinational effects of neutralizing antibodies against different epitopes has been reported against both cell-free and cell-to-cell infections [35]. In addition, combinations of various fusion inhibitors [36] and combinations of fusion inhibitor and antibodies [37,38] were reported to prevent cell-free infection. However, no studies have addressed scFvs in combination with fusion inhibitors for preventing both cell-free and cell-to-cell infections.

Ability to inhibit cell-to-cell infection, as evaluated by cell fusion, was different among scFvs. Difference in V3 loop exposure during the entry process impacts the efficacy of anti-V3 antibodies against cell-free and cell-to-cell infections, which may account for differences in inhibition potency [4,39]. Similarly, differences in the binding epitope of 916B2 (hairpin 1 region) and 25C4b or 4E9C (β20, β21 and V3 stem region) may account for the differences in their neutralization profiles [22].

Combinations of fusion inhibitors and scFvs showed highly synergistic inhibition of cell fusion. The slower fusion kinetics in the presence of fusion inhibitors may partly account for the strong synergy of the combination, but other mechanisms also contributed to the synergy. It is possible that conformational changes of envelope trimers [39] induced by binding to scFvs may allow fusion inhibitors to interact with gp41 [30]. Moreover, mutations within the cytoplasmic tail of gp41 may play a crucial role in exposure of key neutralizing epitopes during cell-to-cell infection [40,41].

Our study brings a fresh perspective to small antibody-based treatment and prevention of cell-to-cell infection for HIV-1. Application of scFvs instead of IgGs [20,34] as antibody-based therapies may provide broad neutralization coverage against both cell-free [21,22] and cell-to-cell infection. The current data suggest application of combinations of scFvs and fusion inhibitors as a next generation treatment that can inhibit cell-to-cell HIV infection, which is not suppressed by ART, even for fusion inhibitor-resistant mutants. The scFvs-fusion inhibitors combinations also a candidate for prevention of viral transmission, in which cell-to-cell infection plays an important role [9]. The ability of such combinations to suppress cell-to-cell infections in animal model should be examined, with an eye toward clinical applications. Exploration of efficient combinations of next-generation fusion inhibitors and broadly-neutralizing antibodies is also an important goal.

## Conflicts of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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## Appendix A. Supplementary data

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