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# Sifuvirtide, a potent HIV fusion inhibitor peptide

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ABSTRACT

Enfuvirtide (ENF) is currently the only FDA approved HIV fusion inhibitor in clinical use. Searching for more drugs in this category with higher efficacy and lower toxicity seems to be a logical next step. In line with this objective, a synthetic peptide with 36 amino acid residues, called Sifuvirtide (SFT), was designed based on the crystal structure of gp41. In this study, we show that SFT is a potent anti-HIV agent with relatively low cytotoxicity. SFT was found to inhibit replication of all tested HIV strains. The effective concentrations that inhibited 50% viral replication ($EC_{50}$), as determined in all tested strains, were either comparable or lower than benchmark values derived from well-known anti-HIV drugs like ENF or AZT, while the cytotoxic concentrations causing 50% cell death ($CC_{50}$) were relatively high, rendering it an ideal anti-HIV agent.

A GST-pull down assay was performed to confirm that SFT is a fusion inhibitor. Furthermore, the activity of SFT on other targets in the HIV life cycle was also investigated, and all assays showed negative results. To further understand the mechanism of action of HIV peptide inhibitors, resistant variants of HIV-$1_{IIIB}$ were derived by serial virus passage in the presence of increasing doses of SFT or ENF. The results showed that there was cross-resistance between SFT and ENF.

In conclusion, SFT is an ideal anti-HIV agent with high potency and low cytotoxicity, but may exhibit a certain extent of cross-resistance with ENF.



## Introduction

Up to now, enfuvirtide (ENF) is the only fusion inhibitor licensed by the Food and Drug Administration [1]. It blocks the entry of HIV-1 into host cells by interfering with the process of virus-cell fusion [2]. ENF is a 36 amino acid synthetic peptide derived from the C-terminus (127–162) of the HIV-1 protein, gp41 [3,4], which is a key protein in the first step of HIV infection. The synthetic peptide binds to the triple-stranded coiled-coil formed by the three HR1 domains of gp41, thereby preventing formation of the six-helix bundle which inhibits membrane fusion [1,5]. ENF exhibited a similar level of inhibition against a panel of prototypic HIV-1 isolates with $EC_{50}$ values ranging from 0.45 to 20.22 nM (2 to 90 ng/ml) [6].

The success of ENF clearly indicates that a fusion inhibitor can be highly effective in inhibiting HIV replication. However, when ENF was discovered, the three-dimensional crystal structure of gp41 had not been completely decoded. Because of this, ENF was designed without the benefit of the full sequence of gp41. This may ultimately affect the efficiency of the drug as a fusion inhibitor. Due to the high dosage required during ENF treatment, side effects were experienced in some patients [7,8]. The development of new drugs targeting the same mechanism, but with higher efficiency is highly desirable.

In 1997, the three-dimensional crystal structure of gp41 was solved [9]. It was found that gp41 is an α-helical trimer consisting of three N-polypeptides and three C-polypeptides. The functional site of gp41 is located in the C-polypeptide. ENF covers about two third of the sequence, but is missing the deep pocket of gp41. Sifuvirtide (SFT) was designed based on the whole sequence of gp41. SFT covers the deep pocket of gp41 so that it has higher binding activity to gp41. It contains 36 amino acids with molecular weight of 4602 Da. SFT was more efficient in inhibiting HIV fusion as compared to ENF [10], and was well tolerated without serious adverse events in Phase Ia clinical trails. The plasma half-life of SFT is also much longer than that of ENF [11,12].

In the present study, the *in vitro* anti-HIV activity and cytotoxicity of SFT were evaluated. To further understand the mechanism of action of these two peptides, resistant variants of HIV-$1_{IIIB}$ were derived by serial virus passage in the presence of increasing doses of SFT or ENF.

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## Materials and methods

*Reagents and chemicals.* Zidovudine (AZT, 3′-azido-3′-deoxythymidine), horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, Fc specific anti-mouse IgG and Phosphonoformate (PFA) were purchased from Sigma. P5F1, monoclonal antibody (MAb) to HIV-1 p24, and rabbit polyclonal anti-HIV-1 serum were prepared by our laboratory [13].

*Peptides and proteins.* Sifuvirtide (SFT) was provided by FusoGen Pharmaceuticals, Inc. Enfuvirtide (ENF) was purchased from Roche. The recombinant plasmids, pGEX-6p-1-GST-HR212 and pGEX-6p-1-GST-HR121, were from Prof. P. Tien of the Institute of Microbiology, CAS. HR121 was constructed by linking one HR1 peptide (N34) to the C-terminus of HR1–HR2 (HR1–HR2–HR1). HR212 was constructed by linking one HR2 peptide (C34) to the N-terminus of HR1–HR2 (HR2–HR1–HR2) [14].

*Cells and viruses.* A3.01, C8166, Jurkat, MT-4, H9, U937, HL-60, K562, HeLa and CEM×174 cells were obtained from the Medical Research Council (MRC) AIDS Research Project (UK) and the NIH AIDS Research and Reference Reagent Program (USA). Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated newborn calf serum (Gibco). Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by Ficoll–Hypaque centrifugation, and incubated in complete medium containing 5 μg/ml phytohemagglutinin (PHA) (Sigma) for 72 h prior to use in antiviral assays. The laboratory-derived viruses HIV-$1_{IIIB}$, HIV-$2_{CBL\text{-}20}$, HIV-$2_{ROD}$ and the ENF-resistant HIV-1 strains were obtained from the NIH AIDS Research and Reference Reagent Program and the MRC AIDS Reagent Project. $SIV_{mac239}$ was donated by Dr. B. Gao of the Institute of Microbiology, CAS. The clinically isolated HIV-$1_{KM018}$ was obtained from a naïve HIV-1-infected individual of Yunnan Province, China, as described [15]. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was determined and calculated by the Reed and Muench method. Virus stocks were stored in small aliquots at −70 °C.

*Cytotoxicity assay.* The cellular toxicity of compounds on different cells lines was assessed by MTT colorimetric assay, as described previously [16]. Briefly, 100 μl C8166, H9, Jurkat, U937 or CEM×174 ($4 \times 10^5$ cells/ml), PBMC ($5 \times 10^6$ cells/ml), HeLa or K562 ($2 \times 10^5$ cells/ml) were respectively seeded on a microtiter plate. One hundred microliters of increasing concentrations of compounds was then added, and the cells were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ for 72 h. The 100 μl supernatant was then discarded, and MTT reagent was added to the cells, which were then incubated for 4 h. The 100 μl of 20% SDS–50% DMF was then added. After the formazan completely dissolved, the plates were read on a Bio-Tek ELx 800 enzyme-linked immunosorbent assay (ELISA) reader at 595 nm/630 nm. The $CC_{50}$ was then calculated.

*The cytopathic effect (CPE) inhibition assay.* In the presence of 100 μl serial concentrations of SFT or ENF, C8166 cells or CEM×174 ($4 \times 10^5$ cells/ml) were infected with HIV-$1_{IIIB}$, HIV-$2_{CBL\text{-}20}$, HIV-$2_{ROD}$ or $SIV_{mac239}$ at a multiplicity of infection (M.O.I.) of 0.06. They were then cultured in a final volume of 200 μl. The plates were incubated in a humidified incubator at 37 °C under 5% $CO_2$. After 3 days of culture, the cytopathic effect was measured by counting the number of syncytia in each well under an inverted microscope. $EC_{50}$ was then calculated [16].

*ELISA for HIV-1 p24 antigen.* HIV-1 p24 antigen in cell-free culture medium was measured using an antigen capture ELISA assay, as described previously [13,16]. Briefly, anti-p24 McAb P5F1 was added to 96-well microtiter plates coated with Fc specific anti-mouse IgG, and the Triton X-100-treated cell-free culture medium was added. The plates were then incubated with diluted rabbit polyclonal anti-HIV-1 sera, followed by incubation with HRP-labeled goat anti-rabbit IgG, and addition of OPD substrate to the wells. The optical density of the plates was read on Bio-Tek ELx800 ELISA reader at 490 nm/630 nm. The percent inhibition of p24 antigen expression was calculated. The concentration that resulted in a 50% reduction in p24 antigen expression ($EC_{50}$) was determined from the dose–response curve.

*Protection of MT-4 cells from lytic effect.* The anti-HIV-1 activities and toxicities of the peptides were assessed in HIV-$1_{IIIB}$-infected MT-4 cells. Uninfected or HIV-infected (M.O.I. = 0.1) MT-4 cells were seeded in 96-well flat-bottomed micro titer culture plates with 100 μl of varying concentrations of peptides. After 7 days of incubation at 37 °C, cell viability was determined by the MTT assay. Briefly, 20 μl of MTT stock solution (5 mg/ml in PBS) was added to each well. After 4 h of incubation at 37 °C, 100 μl of the medium was carefully removed without disturbing the cells containing the formazan crystals. Solubilization of the formazan crystals was achieved by adding 100 μl 20% SDS–50% DMF. After the formazan dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 595 nm/630 nm [16].

*Cell fusion assay.* Cell-to-cell fusion between normal C8166 cells and H9 cells chronically infected with HIV-$1_{IIIB}$ was quantified under an inverted microscope. First, $3 \times 10^4$ C8166 cells were co-cultured with $1 \times 10^4$ HIV-$1_{IIIB}$ chronically infected H9 cells in the presence or absence of SFT and ENF at varying serial concentrations. They were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$. After an 8 h incubation, the number of syncytia was counted under an inverted microscope [17].

*GST-pull down assay.* The glutathione *S*-transferase (GST) fusion proteins, GST-HR212 and GST-HR121, were purified using Glutathione–Sepharose 4B beads according to a previous report [18]. Fifty microliters of gel, containing GST-HR212 and GST-HR121, were mixed with 100 μl of 2 mg/ml SFT or ENF, respectively. The mixtures were incubated for 30 min at room temperature with rotation. One milliliter of PBS was then added to the microcentrifuge tubes, which were centrifuged at 500*g* for 5 min to pellet the Glutathione–Sepharose beads. After washing with PBS 4 times, the beads were analyzed by Tris–Tricine SDS–PAGE.

*Generation of SFT-resistant and ENF-resistant viruses.* Generation of SFT-resistant and ENF-resistant viruses was performed as described previously [4]. Cell-free HIV-$1_{IIIB}$ was allowed to adsorb to $4 \times 10^6$ cells/ml H9 cells for 1 h at 37 °C. The culture was then diluted to $2 \times 10^5$ cells/ml in SFT-containing media at a final concentration of 200 ng/ml, or in ENF-containing media at a final concentration of 1000 ng/ml. Every other day, the cell concentration was adjusted to $2 \times 10^5$ cells/ml by the addition of peptide-containing medium. Samples of cell supernatant were collected every other day and evaluated for virus production on the basis of p24 level. Blank media was used as the control. Supernatants containing the highest level of p24 were passaged again through the H9 cells at a 2-fold higher peptide concentration by the same protocol (i.e., 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 μg/ml for SFT and 1, 2, 4, 8, 16, 32 and 64 μg/ml for ENF). For the peptide-resistant virus pool, the infection was performed and maintained in the presence of peptides. The peptides were removed from the medium 72 h before the virus pool was harvested. The drug-resistant HIV-1 strains were further investigated for their susceptibility to the peptides by completing assays of HIV-1 p24 inhibition.

## Results

### *Cytotoxicities of SFT*

The cytotoxicity of SFT against the host cells was unremarkable. The $CC_{50}$ values of SFT for all detected cell lines were higher than

**Table 1**
Inhibitory effects of SFT, ENF and AZT on different strains of HIV.

| Drugs | Cells | Virus strains | Assays | $EC_{50}$s (nM) |
|---|---|---|---|---|
| SFT | C8166 | HIV-$1_{IIIB}$ | Syncytia | 1.69 ± 0.43 |
| | | | p24 | 4.93 ± 1.28 |
| | | | Protection | 2.96 ± 0.72 |
| | | HIV-$2_{ROD}$ | Syncytia | 256.41 ± 52.15 |
| | | HIV-$2_{CBL-20}$ | Syncytia | 745.33 ± 91.26 |
| | PBMC | HIV-$1_{KM018}$ | p24 | 0.96 ± 0.07 |
| | CEM×174 | $SIV_{mac239}$ | Syncytia | 19.38 ± 5.43 |
| ENF | C8166 | HIV-$1_{IIIB}$ | Syncytia | 22.11 ± 1.26 |
| | | | p24 | 212.58 ± 49.66 |
| | | | Protection | 40.85 ± 7.21 |
| | | HIV-$2_{ROD}$ | Syncytia | 3056.18 ± 885.39 |
| | | HIV-$2_{CBL-20}$ | Syncytia | 21910.11 ± 4116.85 |
| | CEM×174 | $SIV_{mac239}$ | Syncytia | 144.72 ± 6.51 |
| AZT | C8166 | HIV-$1_{IIIB}$ | Syncytia | 9.74 ± 5.99 |
| | | | p24 | 34.45 ± 11.99 |
| | | | Protection | 6.74 ± 1.87 |
| | PBMC | HIV-$1_{KM018}$ | p24 | 253.18 ± 68.91 |

Data represent the means ± standard deviation for at least three independent experiments.

652 μM, except for that of U937 which was 406 μM. ENF also exhibited low cytotoxicity, with a $CC_{50}$ above 674 μM.

*Anti-HIV activities of SFT*

In all the infection systems, SFT had inhibitory activities against HIV-1 and HIV-2, and the $EC_{50}$ values were much lower than those of ENF (Table 1). The antiviral activities of SFT were not limited to prototypic viruses. The $EC_{50}$ values of the p24 antigen assay for the laboratory-adapted strain HIV-$1_{IIIB}$ and the primary HIV-$1_{km018}$ isolate were 4.93 nM and 0.96 nM, respectively (Table 1 and Fig. 1). SFT also exhibited inhibitory activity against HIV-2 strains (ROD and CBL-20) with $EC_{50}$ values ranging from 0.26 to 0.75 μM.

Compared with ENF, SFT exhibited lower $EC_{50}$ values in the syncytia detection assay, ranging from 1.69 nM to 0.75 μM in various HIV strains, while ENF was from 22.11 nM to 21.91 μM. The suppression of peptides in $SIV_{mac239}$ strains was also evaluated. The $EC_{50}$ values for SFT and ENF were 19.38 nM and 144.72 nM, respectively.

*Cell-to-cell fusion assay*

Cell fusion assays were performed to confirm the ability of SFT to block HIV-1 entry. Compared with ENF, SFT showed a much stronger ability to inhibit the cell-to-cell fusion between the normal C8166 cells and the HIV-$1_{IIIB}$ chronically infected H9 cells, with the extremely low $EC_{50}$ value of 0.09 nM, or 19-fold lower than that of ENF. The results showed that SFT was, in fact, more capable of inhibiting the entry of HIV than ENF.

*GST-pull down assay*

Previous studies showed that ENF can not form the α-helical complex with the N36 peptide [12]. The fusogenic core of gp41 is a trimer-of-hairpins structure (six-helix bundle), in which three HR2 helices surround a central trimeric coiled-coil formed by three HR1 helices. The GST fusion proteins, GST-HR212 and GST-HR121, were introduced to test the binding activity of SFT and ENF toward HR2 and HR1 [16]. Three molecules of the fusion proteins can form a stable six-helix bundle, while three free heptad-repeats (HR1 or HR2) are exposed to the environment and can be bound by other molecules. In our assay, both ENF and SFT could bind to GST-HR121 (lane 6 and lane 7), while neither could bind to GST-HR212 (lane 3 and lane 4) (Fig. 2). This result indicated that ENF and SFT had a comparable ability to interact with HR1 but not HR2.

*Inhibitory activity of peptides in drug-resistant viruses*

Inhibitory activities of SFT and ENF against drug-resistant viruses were investigated in two virus systems: resistant virus strains and *in vitro*-derived drug-resistant HIV-1. One ENF-sensitive and three ENF-resistant HIV-1 strains, with well defined genetic mutations known to confer the drug resistance, were used in the study. Both SFT and ENF are active against these strains, while SFT was much more potent than ENF, with an $EC_{50}$ about 50-fold higher than that of SFT (Table 2). The $EC_{50}$ of SFT against ENF-resistant virus ranged from 0.37 to 1.28 μM, and the susceptibility decreased by 7- to 25-fold, compared to the sensitive strain. The trend of these results is consistent with our observations reported above using both laboratory-adapted and primary HIV-1 viruses.

*In vitro*-derived drug-resistant variants of HIV-$1_{IIIB}$ were obtained by repeated passage of the uncloned HIV-$1_{IIIB}$ through the H9 cell line, in the presence of increasing concentrations of the peptides, after six virus passages over about 1 year. The drug-resistant HIV-1 strains were named $HIV_{rT-20}$ and $HIV_{rSi}$, which were resistant to ENF and SFT, respectively. $EC_{50}$ values of ENF and SFT against $HIV_{rT-20}$ were 147.59 μM and 12.76 μM—both about 7000-fold greater than that of the parental HIV-$1_{IIIB}$ (Fig. 3A). The $EC_{50}$ values of ENF and SFT against $HIV_{rSi}$ were 169.13 μM and

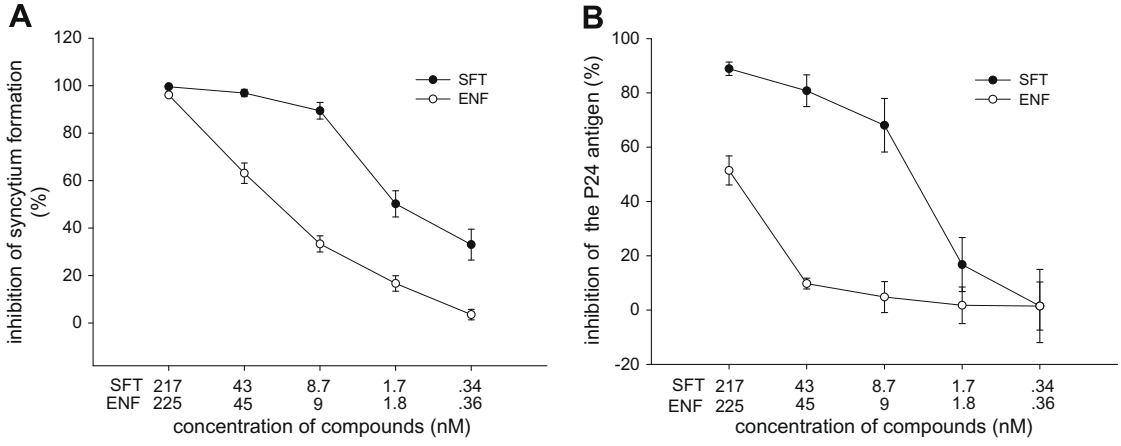


**Fig. 1.** The antiviral effects of SFT and ENF on HIV-$1_{IIIB}$ in C8166 cells. Inhibition of HIV-$1_{IIIB}$ replication was assessed by syncytium formation (A) and p24 antigen (B). Results are expressed as mean ± SEM.

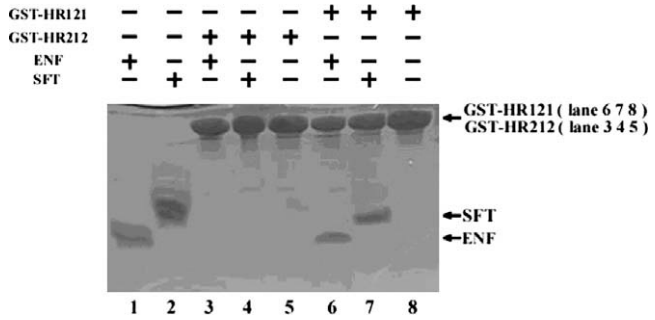


**Fig. 2.** GST-pull down assay of SFT and ENF. Results showed that both SFT and ENF can bind to GST-HR121 (lanes 6 and 7) but not to GST-HR212 (lanes 3 and 4).

25.52 μM—about 7600-fold and 15,000-fold greater than that of the parental HIV-$1_{IIIB}$, respectively (Fig. 3B). Our results show that there is cross-resistance between SFT and ENF. Cross-resistance is evidenced by the improved $EC_{50}$ values for SFT against ENF-resistant viruses. This result was consistent with the recent report referenced in He et al. [12].

## Discussion

Membrane fusion is the first step of the HIV-1 life cycle. It is an attractive target for disrupting HIV-1 replication. Recently, several antiviral drugs have been developed to impede the HIV-1 entry process [19]. A good example is ENF, which is approved by the FDA for salvage therapy [1]. It was discovered by random screen in 1992, but the 3-D structure of gp41 was not yet completely elucidated at that time. Thus, the structure–activity relationship of the drug to the target was not fully understood, and so optimization of ENF was not possible. For example, ENF cannot form stable 6-HB with N-peptides derived from the NHR region, while the 6-HB is the critical fusogenic core in the process of HIV-1 entry [20]. After the full configuration of gp41 was unveiled, it is now possible to design fusion inhibitors with higher stability and efficacy compared with the native ones. SFT was constructed with a series of alterations to increase the stability of the α-helix, and to cover the deepest pocket of gp41. These design improvements will allow tighter binding between SFT and gp41.

In the present study, drug cytotoxicities on A3.01, C8166, Jurkat, MT-4, H9, U937, HL-60, K562, HeLa and CEM×174 cells within the effective dosage range were unremarkable. Antiviral activities of SFT against a wide range of strains, from HIV-1 to HIV-2, were performed due to the possibility that the compound could be active in one HIV strain but not the other [5]. A potential anti-HIV agent should be able to demonstrate anti-HIV activities against at least three major strains of HIV: laboratory strains, drugs-resistant strains and clinically isolated strains. SFT demonstrated inhibitory activity on all these strains (Table 1).

**Table 2**
Inhibitory effects of SFT on ENF-resistant strains.

| Virus | Phenotype* | $EC_{50}$ (mean ± SD, μM) | |
|---|---|---|---|
| | | ENF | SFT |
| pNL4-3 gp41 (N42S) | S | 1.44 ± 0.18 | 0.05 ± 0.01 |
| pNL4-3 gp41 (V38A/N42D) | R | 48.59 ± 8.11 | 1.28 ± 0.34 |
| pNL4-3gp41 (V38A/N42T) | R | 51.61 ± 9.56 | 0.62 ± 0.09 |
| pNL4-3 gp41 (V38E/N42S) | R | 52.88 ± 4.39 | 0.37 ± 0.05 |

* Sensitive (S) or resistant (R) to ENF.

To assess the potential of SFT as a therapeutic agent, its efficacy should be compared with other anti-HIV agents working by a similar mechanism, as well as those with different mechanisms of action. ENF, C34, T649 and T-1249 are all derived from HIV-1 gp41. The inhibitory activities ($EC_{50}$) of ENF, C34 and T649 (C36) on HIV-1-induced cell–cell fusion were 19.29 ± 0.22 nM, 8.20 ± 0.13 nM and 4.67 ± 0.07 nM, respectively [21]. T-1249 showed 1.2–3.2 times greater inhibition of HIV-1 replication than ENF [22]. In the present study, the potency of SFT is 13–42 times higher than that of ENF. These data demonstrate that SFT is comparable to other fusion blockers or even better in efficacy. When compared to anti-HIV agents working on different mechanisms, the $EC_{50}$ is similar in magnitude to AZT (Table 1).

Although SFT was designed to interact with HIV gp41, the possibility of other actions, besides membrane fusion inhibition, was tested. Neither SFT nor ENF inhibited HIV-1 replication in chronically infected H9 cells (data not shown). In contrast, SFT is able to inhibit the fusion between chronically infected H9 cells and normal C8166 cells. Neither SFT nor ENF showed an inhibitory effect on recombinant HIV reverse transcriptase or protease, or promoted the zinc ejection from HIV-1 nucleocapsid protein, Ncp7 (data not shown). Furthermore, GST-pull down assays showed that SFT can interact with HR121, which is similar to HR1 N34. However, ENF can also bind to HR121. This result is not consistent with previously published research on this topic [12]. Taken together, these results have demonstrated that SFT is a pure fusion inhibitor.

While an ENF-resistant HIV-1 strain has been derived before in Rimsky et al. [4], this study reports the derivation of the first SFT-resistant variants of HIV-$1_{IIIB}$ by similar methods. The $EC_{50}$ of SFT against ENF-resistant strains is 12.76 μM, about 7000 times higher than that of parental strains. A similar result is also observed in three ENF-resistant viruses with 7- to 25-fold improved $EC_{50}$ values. It is demonstrated from our preliminary results that there is

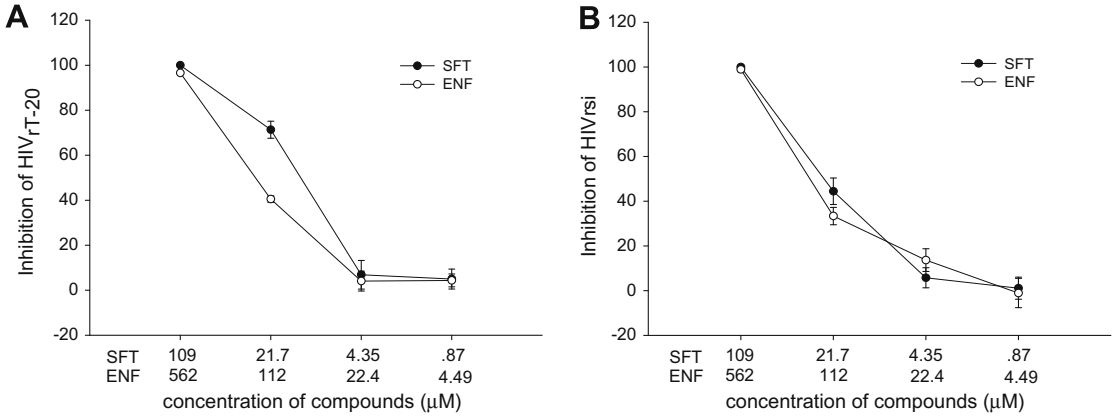


**Fig. 3.** Inhibitory effects of SFT and ENF on derived drug-resistant strains.

partial cross-resistance between SFT and ENF (Fig. 3). This cross-resistance may be due to target similarity between SFT and ENF. The mutation sites and the frequency of mutation in HIV-1 gp41 should be studied further.

In conclusion, SFT can effectively inhibit HIV replication *in vitro* and maintains high activity against ENF-resistant HIV-1 strains. Although SFT is partially cross-resistant with ENF, it has a much lower dosage and longer injection interval [12]. Thus, SFT has the potential to become a better fusion inhibitor for the treatment of HIV/AIDS patients, especially for HARRT-experienced patients.

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