

A homogeneous time-resolved fluorescence-based high-throughput screening for discovery of inhibitors of Nef-sdAb19 interaction

XIAOQIN FAN^{1,3*}, JINMEI WEI^{1*}, HAITING XIONG¹, XIAOHUI LIU¹,
SERGE BENICHO², XUEJUAN GAO¹ and LANGXIA LIU¹

¹Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering, Jinan University, Guangzhou 510632, P.R. China; ²Cochin Institute, INSERM U1016, Centre National de la Recherche Scientifique UMR8104, Université Paris-Descartes, Paris, France

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Abstract. The human immunodeficiency virus (HIV) protein negative factor (Nef) is important for AIDS pathogenesis. An anti-Nef single-domain antibody (sdAb19) derived from camelids has been previously generated and shown to effectively block the physiological functions of Nef *in vitro* and *in vivo* in *nef*-transgenic mice. However, sdAb19 must be ectopically expressed within the target cell to be able to exert its neutralizing effect on Nef, while the extra-cellular administration method turned out to be ineffective. This might suggest a default of the stability or/and deliverability of sdAb19. The identification of small molecule compounds capable of inhibiting the Nef-sdAb19 interaction and mimicking the neutralizing activity of sdAb19 *in vivo* would therefore be the means of circumventing the problem encountered with sdAb19. Here we describe the development of a high-throughput screening method combining the homogeneous time-resolved fluorescence (HTRF) and the microscale thermophoresis (MST)

techniques for the identification of small-molecule compounds inhibiting the Nef-sdAb19 interaction by binding to Nef protein. Eight small-molecule compounds have been selected for their ability to significantly inhibit the Nef-sdAb19 interaction and to bind to Nef. These molecules could be further assessed for their potential of being the Nef-neutralizing agents in the future.

Introduction

Despite the considerable progress made in the field of anti-HIV drug development, the acquired immune deficiency syndrome (AIDS) is still a serious health concern across the world since its discovery more than thirty years ago. Due to its high mutation rate, HIV is often able to evade the host immune system, and also the antiviral therapies (1,2). Therefore, the development of novel drugs and therapeutic methods against the virus is important. The HIV accessory proteins represent the alternative targets for anti-HIV drug development. In particular, the negative factor (Nef), an accessory protein of HIV which plays a critical role in the physio-pathogenesis of AIDS could be a good target for the drug research and development. Nef is expressed early in the viral life cycle and targeted to the plasma membrane (3,4). It contains an N-myristoylation cytoplasmic region that is required for its association with cellular membranes and virtually critical for its complex biological activities. Nef exists as homodimers in cells, and all types of Nef are conserved with six structured core domains which forms one α -helix and five β -layer structures (5). The flexible regions of Nef, such as its large surfaces accessible for interactions and important conformational changes, are involved in increasing infectious viral particles, activating CD4 lymphocytes and preventing infected cells from apoptosis (6-8).

Recently, antibody therapy has obtained remarkable results. The single-domain antibodies (sdAb) derived from camelids is a major breakthrough in the development of antibody therapy (5,9,10). The sdAb19, consisting only of a single variable domain which recognizes the antigenic epitope of the target protein, is relatively small with a molecular weight of 13 kDa, and can penetrate in cavities located on the surface of antigens. Previous studies have demonstrated

Correspondence to: Dr Xuejuan Gao or Professor Langxia Liu, Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering, Jinan University, Guangzhou 510632, P.R. China
E-mail: tgaoxj@jnu.edu.cn
E-mail: langxialiu@gmail.com

Present address: ³Longgang Central Hospital, ENT Hospital, Shenzhen ENT Institute, Shenzhen 518116, P.R. China

*Contributed equally

Abbreviations: HTRF, homogeneous time-resolved fluorescence; MST, microscale thermophoresis; sdAb, single-domain antibodies; HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome; FRET, fluorescence resonance energy transfer technology

Key words: homogeneous time-resolved fluorescence, microscale thermophoresis, Nef, single domain antibody, small molecule compound

the sdAb fragments displayed efficient therapeutic activity in many diseases such as cancer, neurodegenerative diseases and rheumatoid arthritis (11-13). The sdAb19 generated by Bouchet *et al* targeted the HIV Nef protein with a high affinity and inhibited biologic activities of Nef both *in vitro* and *in vivo* in a *nef*-transgenic mouse model, suggesting that inhibition of Nef function is a potential method to block HIV (9). However, sdAb19 must be ectopically expressed via an expression vector within the target cell to be able to exert its neutralizing effect on Nef, while the extra-cellular administration method turned out to be ineffective. This might suggest a default of the stability or/and deliverability of sdAb19. The identification of small molecule compounds capable of mimicking the neutralizing activity of sdAb19 and displaying better stability or/and deliverability *in vivo* would therefore be the means of circumventing the problem encountered with sdAb19. In this study, we took advantage of the homogeneous time-resolved fluorescence (HTRF) technology to carry out a high-throughput screening of a library of small molecules. HTRF technology is an ideal platform used for drug discovery in high-throughput screening, which combines fluorescence resonance energy transfer technology (FRET) with time-resolved measurement (TR). The time-resolved characteristic of HTRF technology allows for the removal of nearly all environmental and compound interference effects. As a sensitive and reliable method due to its reduced inter-well variation and fluorescence interference, this technology has been developed for many antibody-based assays including GPCR signaling, kinases, cytokines and biomarkers, bioprocess, as well as the assay for biomolecular interaction (14-18). We selected the molecules capable of inhibiting the Nef-sdAb19 interaction by this method, then used the microscale thermophoresis (MST) technique to identify, among these candidate molecules, those that fix on Nef. These are the molecules which probably inhibit the Nef-sdAb19 interaction by competition of Nef binding with sdAb19, and could possibly mimic the anti-Nef activity *in vivo*.

Materials and methods

Antibodies and chemicals. Eu³⁺ cryptate-conjugated anti-GST antibody (anti-GST-Eu) and XL665-conjugated anti-6xHis antibody (anti-6xHis-XL665) were purchased from Perkin-Elmer (France). Anti-his monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (USA). Tris, sodium dodecyl sulfate (SDS) and glycine were obtained from Bio-Rad (USA). Protein inhibitor was purchased from Merk (USA). Isopropyl-1-thio- β -D-galactopyranoside (IPTG), lysozyme, imidazole and reduced glutathione were purchased from Applygen (China). Glutathione-Sepharose 4B beads were purchased from GE (USA), whereas Ni²⁺ beads from Qiagen N.V. (China). Pharmacologically active compounds (LOPAC) were from Sigma (USA). Pierce BCA Protein Assay kit was purchased from Thermo (USA).

Expression and purification of recombinant proteins. The pGEX-4T-Nef construct encoding GST-Nef and pcDNA-sdAb19 expressing His-sdAb19 were transformed into *Escherichia coli* BL21. The expression of recombinant protein was induced by the addition of 0.2 mM IPTG for 6-8 h at room

temperature (RT) when transformed bacteria were grown to an OD600 of 0.6-0.8. The cells were collected and lysed by sonication in EBC buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1X complete mini EDTA-free protein inhibitors and 10 μ g/ml lysozyme. Bacterial extracts were sonicated for 20 min and centrifuged at 12,000 rpm for 30 min at 4°C to remove cell debris. GST-proteins were purified using Glutathione Sepharose 4B (GE Healthcare), and His-sdAb19 proteins were purified from supernatant using Ni-NTA Superflow (Qiagen). Glutathione and imidazole were excluded from the eluted proteins using 10 kDa-ultrafiltration (Millipore, USA). The concentration of protein was measured by BCA assay (Beyotime Biotechnology, China). The purity of recombinant proteins were determined by SDS-PAGE and coomassie blue staining (CBB).

Digestion by thrombin. Thrombin was added to digest the GST-Nef beads for 16 h at 4°C. The digestion mix was then centrifuged at 12,000 rpm for 5 min at 4°C to collect the supernatant.

GST pull-down assay. Equal amounts of GST-beads and GST-Nef-beads were incubated in EBC buffer with 500 μ g purified recombinant His-sdAb19 for 6 h at 4°C. The beads were recovered by centrifugation at 1,000 rpm for 2 min at 4°C and washed three times with EBC buffer, and proteins were eluted into 20 μ l of SDS sample buffer by heating to 95°C for 5 min. Proteins were separated using 10% SDS-PAGE gels and then electro-blotted to PVDF membrane. Membranes were blocked with 5% non-fat milk for 1 h at RT, and then incubated with anti-His mouse antibody overnight at 4°C. After washing, the blots were probed with horseradish peroxidase-conjugated anti-mouse secondary antibody (ProteinTech Group) for 1 h at RT, and then signals were visualized using ECL detection system (Beyotime Biotechnology).

Optimization of an HTRF assay for Nef-sdAb19 interaction. The following experiments were designed to determine the optimal concentration of HTRF reagents, including GST-Nef, His-sdAb19, anti-GST-Eu antibody, and anti-6xHis-XL665 antibody. i) The incubation of GST-Nef having a concentration in a range from 0 to 100 nM with 100 nM His-sdAb19, 5 nM anti-GST-Eu antibody, and 100 nM anti-6xHis-XL665 antibody was to determine the optimal concentration of GST-Nef through HTRF assay. ii) The incubation of His-sdAb19 having a concentration in a range from 0 to 100 nM with 100 nM GST-Nef, 5 nM anti-GST-Eu, and 100 nM anti-6xHis-XL665 antibody was to determine the optimal concentration of His-sdAb19 through HTRF assay. iii) Anti-GST-Eu antibody, with the changeable concentration from 0 to 5 nM, co-incubated with 100 nM anti-6xHis-XL665 antibody, 100 nM GST-Nef, and 100 nM His-sdAb19 was to determine the optimal concentration of Eu³⁺-GST antibody. iv) Anti-6xHis-XL665 antibody, with the increasing concentrations from 0 to 100 nM, co-incubated with 5 nM anti-GST-Eu antibody, 100 nM GST-Nef, and 100 nM His-sdAb19 was to determine the optimal concentration of XL665-His antibody. During these HTRF assays, GST-Nef was incubated for 4 h at 4°C, and then anti-

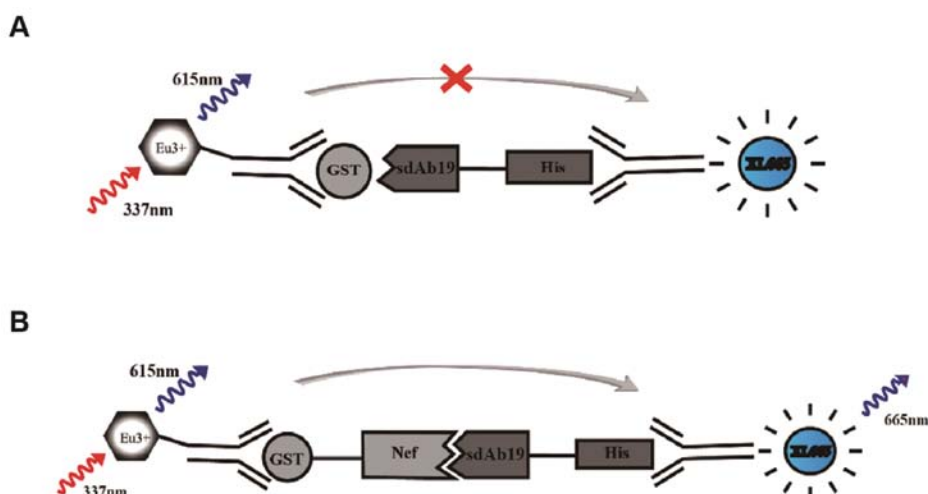


Figure 1. The schematic for homogeneous time resolved fluorescence assay. HTRF principle with Eu^{3+} and XL665 respectively as donor and acceptor. If the labeled GST-Nef binds to His-sdAb19 and each other, the two fluorophores will be in close proximity to allow FRET to occur, which result in a specific long-lived fluorescence emission of XL665 at 665 nm.

GST-Eu and anti-6xHis-XL665 antibodies were added to incubate away from light for 2 h at RT. The corresponding GST protein was used as negative control for all the tests. All HTRF reagents were diluted with Tris-HCl buffer and added to the 384-well plates to a final volume of 50 μl . Plates were stored in the dark at RT before reading using Perkin-Elmer VICTORTM X5 (Perkin-Elmer, USA). The excitation fluorescence of 337 nm was used to excite Eu^{3+} and the fluorescence emission at 665 and 615 nm were detected simultaneously. Signal was expressed in terms of HTRF ratio $\Delta F\%$, as the previous description (19). $R = [\text{signal } 665 / \text{signal } 615] \times 10,000$; $\Delta R = R_{\text{Nef}} - R_{\text{GST}}$; $\Delta F\% = (\Delta R / R_{\text{GST}}) \times 100$ (Fig. 1).

Nef-sdAb19 interaction inhibition assay. The library of pharmacologically active compounds (LOPAC, Sigma-Aldrich, USA) contains 1,280 well-characterized chemical compounds. The compounds of LOPAC library are stored at a concentration of 10 mM, dissolved in DMSO in 96-well plates at -20°C . Before the experiments, we diluted the compounds with the following procedure. Each compound (5 μl) was mixed with 95 μl DMSO in other 96-well plates. Each diluted compound (10 μl) was used for high-throughput screening and added into the 384-well black plate. Then, 10 μl of 250 nM GST-Nef (a final concentration of 50 nM) was incubated with the compounds for 2 h at 4°C , following by incubated with 10 μl of 100 nM His-sdAb19 (a final concentration of 20 nM) for 4 h at 4°C . Ten microliters of the donor fluorophore Eu^{3+} cryptate-conjugated anti-GST antibody (a final concentration of 2.5 nM) and 10 μl of the acceptor fluorophore XL665-conjugated anti-6xHis antibody (a final concentration of 100 nM) were added and incubated in dark condition for 2 h at RT. The GST protein was used as comparison substance. The total volume of the reaction was 50 μl . There were 3 multiple pores in every dosage group and negative control group. Plates were read by Perkin-Elmer VICTORTM X5 by measurement of fluorescence emission at 665 and 615 nm. The percentage of interaction inhibition was analyzed according to the following formula (19). Inhibition (%) = $((F_{\text{Nef}} - F_{\text{GST}}) - (F_{\text{com}} - F_{\text{GST}})) / (F_{\text{Nef}} - F_{\text{GST}}) \times 100$; $F = [\text{signal } 665 / \text{signal } 615] \times 10,000$. F_{Nef} is the

fluorescence emission ratio of GST-Nef group treated with DMSO; F_{com} is the fluorescence emission ratio of GST-Nef group treated with the small molecular compound; F_{GST} is fluorescence emission ratio of GST negative control group treated with DMSO; F is fluorescence emission ratio of 665 nm/615 nm.

Excluding the false-positive reaction. Microscale thermophoresis (MST) was used to determine the binding affinities between Nef and the candidates of HTRF. a fixed concentration of 200 nM Nef, the candidates which were titrated with 122.07 to 25,000 nM in PBS buffer. In order to allow binding, samples were incubated at least 30 min at room temperature followed by centrifugation for 5 min at 15,000 g to eliminate potential precipitates. Experimental measurements were performed in standard or hydrophilic capillaries using a NanoTemper MonoliteTM NT.115 instrument (NanoTemper Technologies GmbH, Germany) for red fluorescence dye. Thermophoresis signals for each of the 12 capillaries were monitored, which harbor different ratios of binding partners. The normalized fluorescence at a given time point was plotted against the concentration of unlabeled candidates. The resulting sigmoidal curves were normalized, and each data point was determined. Data points were finally fitted by the Hill slope, and KD value were obtained. Kd Formula (law of mass action): $F(c) = \text{unbound} + (\text{bound-unbound}) / 2 * (\text{FluoConc} + c + Kd - \sqrt{(\text{FluoConc} + c + Kd)^2 - 4 * \text{FluoConc} * c})$ (20).

Results

Expression and purification of GST-Nef and His-sdAb19. The plasmids pGEX-4T-Nef and pcDNA-sdAb19 were transformed into *E. coli* BL21, and the recombinant bacteria was induced by IPTG in concentration of 0.2 mM for 6-8 h at RT. As shown in Fig. 2, SDS-PAGE analysis showed that GST-Nef and His-sdAb19 were expressed in inclusion bodies in the *E. coli* cells and their molecular weights were ~54 and 27 kDa (Fig. 2). The two recombinant proteins were purified with Glutathione-Sepharose 4B beads and Ni-NTA Superflow,

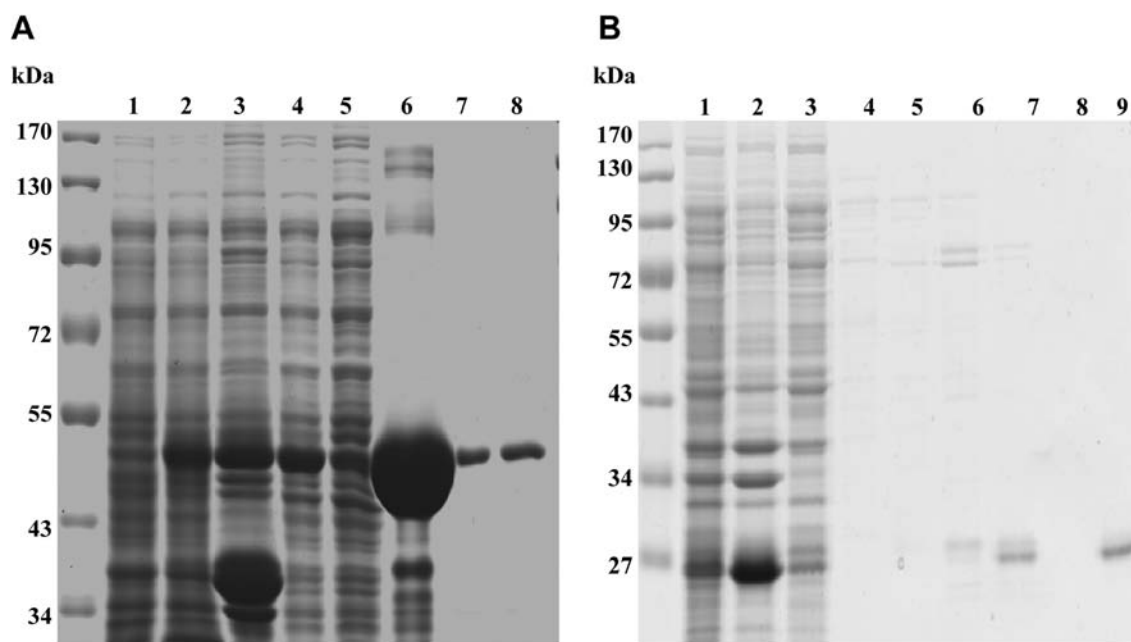


Figure 2. Expression and purification of recombinant GST-Nef and His-sdAb19. (A) The expression and purification of recombinant GST-Nef were analyzed by SDS-PAGE. Lane 1, *E. coli* BL21 transformed with pGEX-Nef without IPTG induction; lane 2, *E. coli* BL21 transformed with pGEX-Nef with IPTG induction; lane 3, the supernatant of cell lysates after IPTG induction; lane 4, the precipitation of cell lysates; lane 5, the supernatant of the flow-through; lane 6, GST-Nef recombinant protein-beads before elution; lane 7, GST-Nef recombinant protein-beads after elution with 5 mM reduced glutathione; lane 8, GST-Nef recombinant protein-beads after elution with 5 mM reduced glutathione. (B) The expression and purification of recombinant His-sdAb19 were analyzed by SDA-PAGE. Lane 1, *E. coli* BL21 transformed with pET-sdAb19 without IPTG induction; lane 2, *E. coli* BL21 transformed with pET-sdAb19 with IPTG induction; lane 3, the supernatant of cell lysates after IPTG induction; lane 4, the precipitation of cell lysates; lane 5, the supernatant of the flow-through; lane 6, His-sdAb19 recombinant protein-beads before elution; lane 7, His-sdAb19 recombinant protein-beads after elution with 10 mM imidazole; lane 8, His-sdAb19 recombinant protein-beads after elution with 50 mM imidazole; lane 9, His-sdAb19 recombinant protein-beads after elution with 100 mM imidazole.

respectively. SDS-PAGE analysis showed that there was a single band at the expected position, indicating that the proteins had been purified successfully (Fig. 2).

In vitro interaction between GST-Nef and His-sdAb19. To demonstrate the direct interaction between Nef and sdAb19 *in vitro*, we performed an *in vitro* GST pull-down experiment using GST-Nef and His-sdAb19 recombinant proteins. Fig. 3 showed that His-sdAb19 could be pulled down by the immobilized GST-Nef but not the immobilized GST, indicating the direct and specific binding of GST-Nef and His-sdAb19 exists in an acellular context.

Optimization of HTRF assay reagents. To make sure that the interaction of Nef with sdAb19 (presented as HTRF ratio $\Delta F\%$) responds sensitively to any interference coming from the small molecular inhibitors, the used concentration of four reagents must be optimized, including GST-Nef, His-sdAb19, anti-GST-Eu antibody, and anti-6xHis-XL665 antibody. As described in detail in Materials and methods, with excessive His-sdAb19 (100 nM), anti-GST-Eu antibody (5 nM), and anti-6xHis-XL665 antibody (100 nM), GST-Nef proteins having a concentration in a range from 0 to 100 nM were added for reactions. GST proteins were used as negative control. The result showed that the observed interaction (HTRF ratio $\Delta F\%$) increased linearly at the concentration of GST-Nef <50 nM, while the observed interaction (HTRF ratio $\Delta F\%$) decreased (Fig. 4A) at its concentration >50 nM, suggesting the optimal concentration of GST-Nef is ~ 50 nM.

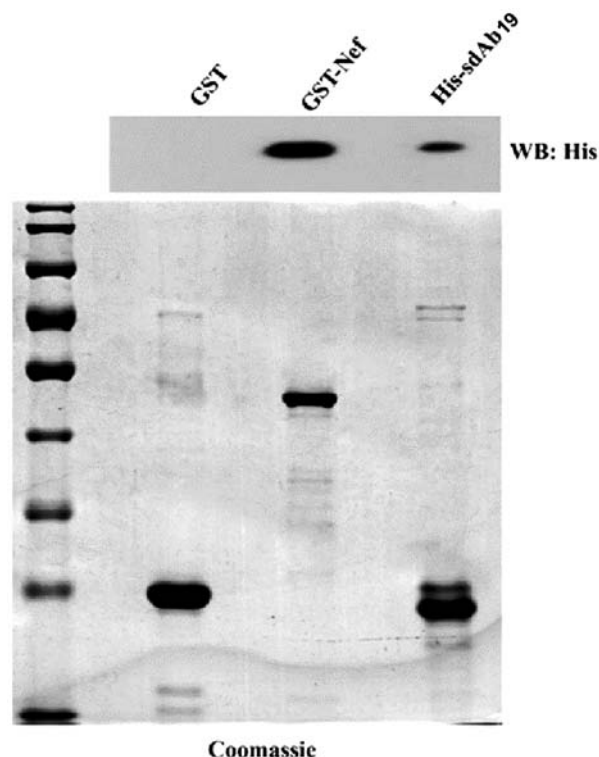


Figure 3. The direct interaction between Nef and sdAb19 determined by GST pull-down. Purified GST (lane 1) or GST-Nef recombinant protein (lane 2) were immobilized on Sepharose-Glutathione beads and incubated with purified His-sdAb19 protein. Lane 3 represents the input of His-sdAb19. The amounts of GST and GST-Nef used in the assays were checked by coomassie blue staining (lower panel).

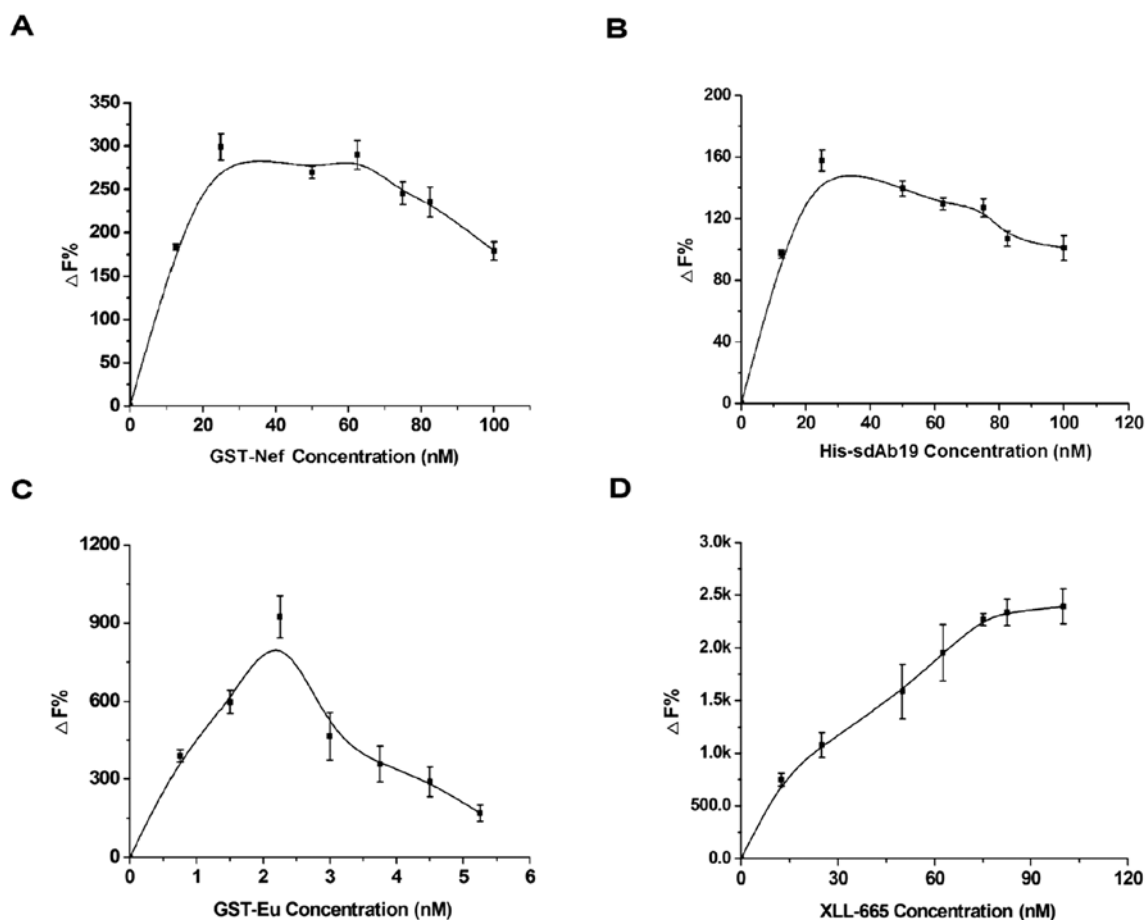


Figure 4. Optimization of HTRF assay reagents. (A) Optimization of the concentrations of GST-Nef for high-throughput screening. His-sdAb19 (100 nM), anti-GST-Eu antibody (5 nM), and anti-6xHis-XL665 antibody (100 nM) were mixed with different concentrations of GST-Nef from 0 to 100 nM in black 384-wells. GST-Nef and His-sdAb19 were incubated for 4 h at 4°C, then the anti-GST-Eu and anti-6xHis-XL665 antibodies were added for 2-h incubation at RT. Plates were read using Perkin-Elmer VICTOR X5. HTRF ratio $\Delta F\%$ is positively correlated with the Nef-sdAb19 interaction. (B) Optimization of the concentrations of His-sdAb19 for high-throughput screening. GST-Nef (100 nM), anti-GST-Eu antibody (5 nM), and anti-6xHis-XL665 antibody (100 nM) were mixed with different concentrations of His-sdAb19 from 0 to 100 nM in the black 384-wells, followed by a similar procedure of HTRF assay. (C) Determination of the concentrations of anti-GST-Eu antibody for high-throughput screening. GST-Nef (100 nM), His-sdAb19 (100 nM), and anti-6xHis-XL665 antibody (100 nM) were mixed with different concentrations of anti-GST-Eu antibody from 0 to 5 nM in the black 384-wells, followed by a similar procedure of the HTRF assay. (D) Determination of the concentrations of anti-6xHis-XL665 antibody for high-throughput screening. GST-Nef (100 nM), His-sdAb19 (100 nM), and anti-GST-Eu antibody (5 nM) were mixed with different concentrations of anti-6xHis-XL665 antibody from 0 to 100 nM in the black 384-wells, followed by a similar procedure of the HTRF assay.

Subsequently, the proper concentration of His-sdAb19 was determined by HTRF assay by the addition of enough GST-Nef (100 nM), anti-GST-Eu antibody (5 nM), and anti-6xHis-XL665 antibody (100 nM) with His-sdAb19 (the concentrations from 0 to 100 nM). The result showed that the strongest interaction between GST-Nef and His-sdAb19 was observed when the concentrations of His-sdAb19 reached 40 nM (Fig. 4B). Due to the fact that $\Delta F\%$ of 20 nM is almost half of 40 nM, and that 100 nM GST-Nef was used in this reaction twice, than the chosen 50 nM for screening, we decided on the concentration of 20 nM for the following high-throughput screening. Whereas, GST at concentration of 100 nM was the negative control.

Similarly, the suitable concentration of anti-GST-Eu antibody was detected with enough GST-Nef (100 nM), His-sdAb19 (100 nM), and anti-6xHis-XL665 antibody (100 nM) mixed together with anti-GST-Eu antibody with the concentrations from 0 to 5 nM for HTRF detection. The

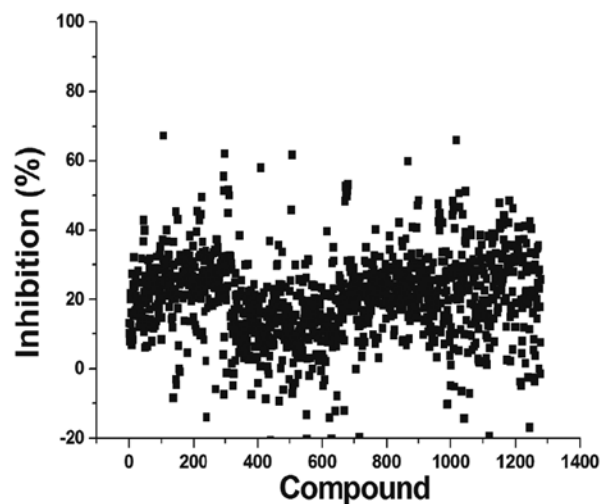


Figure 5. High-throughput screening of the small molecular inhibitors against Nef-sdAb19 interaction. Small box shows the inhibition (%) of the screened compounds.

Table I. The candidates compounds screened with HTRF and MST assay.

No.	Name	Class	HTRF	MST
1	Trovaflaxin mesylate	Immunomodulators and antibodies	+	+
2	CL316243	Adrenoceptor	+	+
3	Felodipine	Ca ²⁺ channel	+	+
4	R(+)-IAA-94	Cl ⁻ channel	+	+
5	Loratadine	Histamine	+	+
6	Isoliquiritigenin	Cyclic nucleotides	+	+
7	IC261	Phosphorylation	+	+
8	A3 hydrochloride	Phosphorylation	+	+
9	Cystamine	Glutamate	+	-
10	Artemether	Immunomodulators and antibiotics	+	-
11	Colchicine	Cytoskeleton and ECM	+	-
12	CNS-1102	Glutamate	+	-
13	Enoximone	Cyclic nucleotides	+	-
14	Ketorolac tris salt	Prostaglandin	+	-
15	Niclosamide	Antibiotic	+	-
16	Auranofin	Phosphorylation	+	-

HTRF, homogeneous time-resolved fluorescence; MST, microscale thermophoresis.

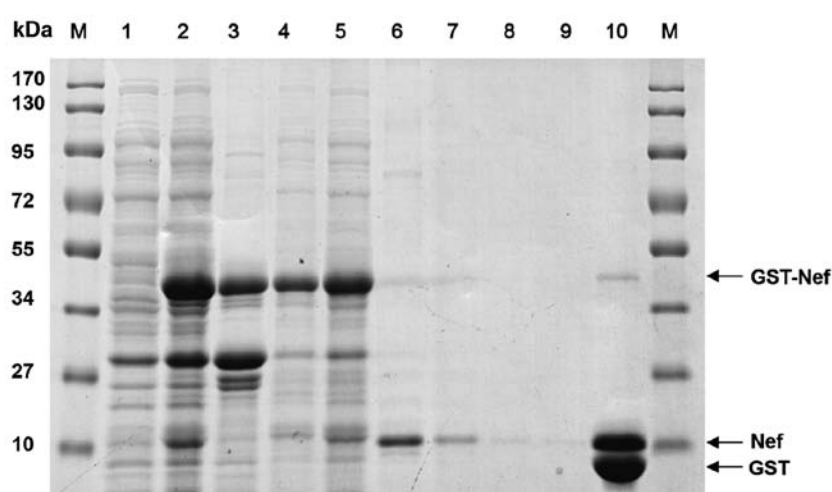


Figure 6. Affinity purification of Nef. Coomassie blue staining of the purification of Nef. Lane 1, *E. coli* BL21 transformed with pGEX-Nef without IPTG induction; lane 2, *E. coli* BL21 transformed with pGEX-Nef with IPTG induction; lane 3, the precipitation of ultrasound; lane 4, the supernatant of ultrasound after IPTG induction; lane 5, the supernatant of ultrasound flowed through the Glutathione Sepharose 4B; lane 6, the filtrate of GST-Nef fusion protein beads digested by thrombin; lane 7, the digested beads eluted with 3 ml PBS first time; lane 8, the digested beads eluted with 3 ml PBS second time; lane 9, the digested beads eluted with 3 ml PBS third time; lane 10, the beads after digested by thrombin and eluted by PBS.

result showed that the maxima of $\Delta F\%$ appeared at 2.5 nM anti-GST-Eu antibody, and the strongest interaction occurred at the anti-6xHis-XL665 antibody concentration >40 nM (Fig. 4C and D). To guarantee the effective detection of HTRF signal, the antibodies against GST-Eu and 6xHis-XL665 need to be excessive. In consideration of these factors, we chose respectively 2.5 and 100 nM as the proper concentrations of anti-GST-Eu and anti-6xHis-XL665 antibodies for the following high-throughput screening.

High-throughput screening of the small molecules as inhibitors of Nef-sdAb19 interaction. After determination of the

proper concentrations of four reagents for HTRF detection, 50 nM GST-Nef, 20 nM His-sdAb19, 2.5 nM anti-GST-Eu and 100 nM anti-6xHis-XL665 were used for screening the small molecular inhibitors against Nef-sdAb19 interaction. As described in Materials and methods, the high-throughput screening by HTRF tests were performed in 384-well plates in a total 50 μ l reaction volume containing the above reagents with their proper concentration and each small molecular compound (100 μ M) or control DMSO. After incubation, the 384-well plates were analyzed by Perkin-Elmer VICTOR™ X5. The percentage of interaction inhibition was calculated with the formula: Inhibition (%) = $((F_{\text{Nef}} - F_{\text{GST}}) -$

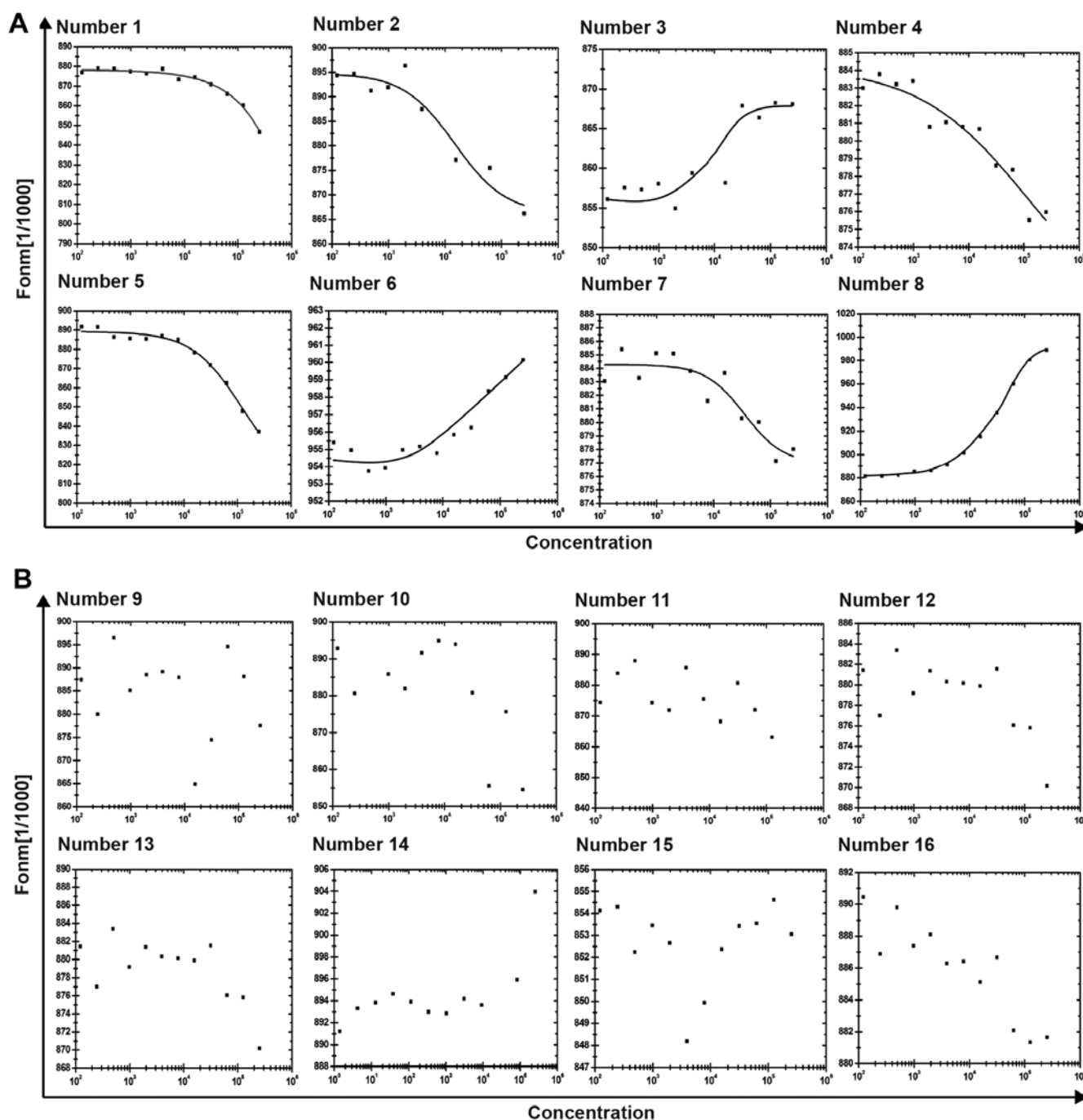


Figure 7. Excluding the false-positive of HTRF using MST. The red fluorescent dye-labeled Nef was kept constant at 200 nM, and the sixteen candidate compounds were titrated from 100 to 25,000 nM, then plotted for analysis of thermophoresis and T-Jump. The K_D is in a good agreement with literature values. (A) The positive MST; (B) The negative MST.

$(F_{com} - F_{GST}) / (F_{Nef} - F_{GST}) * 100$. The results in Fig. 5 showed that inhibition of most compounds was <50%, the generally acknowledged standard of effective inhibition in relation to high-throughput screening experiments. Notably, there were sixteen candidate compounds showing inhibition (%) >50% (Fig. 5). Their names and classes are shown in Table I).

Test of Nef binding by MST for HTRF-positive small molecules. In order to select among the candidate molecules that bind to Nef, we used microscale thermophoresis to test the interaction of each of the sixteen candidate molecules

obtained and listed above. Fig. 6 shows the SDS-PAGE of the recombinant Nef that we produced and purified for the MST assays. The red fluorescent dye-labeled Nef was kept constant at 200 nM, and the sixteen candidate compounds were titrated from 100 to 25,000 nM. The binding of Nef with the candidate compounds were monitored by the change in the thermophoretic property of the fluorescently labeled protein upon complex formation. The K_D was calculated as previously reported (19). As shown by the MST T-Jump response in Fig. 7, there were eight candidate compounds that bind to Nef.

Discussion

The protein Nef is critical for the pathogenesis of HIV, and the inhibition of its biological activity represents a potential way to eradicate HIV. The anti-Nef antibody sdAb19 derived from camelids significantly blocked the growth of HIV (23). Considering sdAb19 represent an efficient tool to elucidate the molecular functions of Nef, it is a meaningful strategy to search for new small molecular inhibitors against Nef-sdAb19 interaction for further inhibiting the function of Nef. In this study, we utilized HTRF technology to high-throughput screen the small molecular inhibitors against the interaction between Nef and sdAb19 *in vitro*. We obtained sixteen candidate compounds significantly inhibiting the interaction between Nef and sdAb19 through screening. Since the HTRF method that we used in our lab was inappropriate to monitor the interaction between Nef and small molecules, we used MST to select the Nef binding molecules. The results showed that only eight candidate compounds were positive for the binding with Nef in an extracellular context and represented therefore the candidate compounds for mimicking the anti-Nef activity of sdAb19 *in vivo*. The eight double-positive candidate molecules belong to 4 categories of compounds, including ion channel, phosphorylation, immunomodulators and antibiotics (Table I).

Ion channels are membrane proteins that are found in a number of viruses (21). In this study, felodipine and R(+)-IAA-94 displaying potency in inhibition of ion channel activity (22) were selected for their ability of inhibiting Nef-sdAb19 interaction and binding to Nef. Protein phosphorylation is a reversible post-translational modification essential for the regulation of many cellular processes. Phosphorylation can modulate protein properties including enzymatic activity, stability, subcellular localization and interaction with binding partners. The importance of phosphorylation of the replication proteins of negative-strand RNA viruses has previously been documented (23). 3-[(2,4,6-trimethoxyphenyl)methylidene]-indolin-2-one (IC261), a novel casein kinase-1 delta/epsilon (δ/ϵ) specific inhibitor, triggers the mitotic checkpoint and induces p53-dependent postmitotic effects. The effects of IC261 on CK1 δ/ϵ might be the interference of specific phosphorylation of spindle component proteins (24). Notably, several recent studies showed that the CK1 δ/ϵ is involved in the regulation of cell survival and cancer progression (25). IC261, as CK1 δ/ϵ inhibitor, may inhibit cancer development through inhibition of microtubule polymerization (26). Immunomodulators and antibiotics are bringing great prospects for clinical trials, since there have been significant advances in immunotherapeutic field in HIV over the past decade (27). Retinoids, derivatives of vitamin A, have multiple cellular functions including induction of differentiation, regulation of apoptosis and inhibition of proliferation or inflammation (28). Retinoic acid p-hydroxyanilide is a vitamin A acid analog with antiproliferative activity in cultured human breast cancer cells (29,30). Some studies found that all-trans retinoic acid (at RA) might inhibit HIV-1-induced podocyte proliferation and dedifferentiation with the cAMP/PKA pathway through Nef-induced MAPK1,2 activation by retinoic acid receptor α (31,32).

The sdAb19 that derives from camelids, binds with high affinity ($K_d=2 \times 10^{-9}$ M) and shows specificity to the conserved

epitope of Nef. The interaction between Nef and sdAb19 was confirmed by GST pull-down in our study. Small organic molecules could be revealed to be advantageous over antibody- or protein-based drugs due to their higher stability, bioavailability, deliverability and lower cost. We developed an HTRF-based screening assay for the identification of small molecule inhibitors against Nef-sdAb19 interaction that can be used also for larger scale screening. We performed HTRF assay with very low protein concentrations (Nef 50 nM; sdAb19 25 nM) to increase the chances of finding small-molecule inhibitors. This is a novel trial for screening the antiviral small molecule drugs using the HTS assays. Sixteen candidate compounds were screened by HTRF assay, and eight compounds showed positive against Nef-sdAb19 interaction using MST.

In conclusion, the research presented in this study represents a proof of concept of a novel method for the screening of small molecule compounds, and permitted the selection of 8 candidate molecules susceptible to inhibit the biological functions of HIV Nef protein and the physiopathogenesis of AIDS.

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