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A Biochemical/Biophysical Assay Dyad for HTS-Compatible Triaging of Inhibitors of the HIV-1 Nef/Hck SH3 Interaction

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Abstract: The current treatment regimens for HIV include over 20 anti-retrovirals. However, adverse drug effects and the emergence of drug resistance necessitates the continued improvement of the existing drug classes as well as the development of novel drugs that target as yet therapeutically unexploited viral and cellular pathways. Here we demonstrate a strategy for the discovery of protein-protein interaction inhibitors of the viral pathogenicity factor HIV-1 Nef and its interaction with the host factor SH3. A combination of a time-resolved fluorescence resonance energy resonance energy transfer-based assay and a label-free resonant waveguide grating-based assay was optimized for high-throughput screening formats.

Keywords: HIV, HTS, label-free technology, Nef, protein-protein interaction inhibitor, resonant waveguide grating, SH3, TR-FRET.

INTRODUCTION

The negative factor (Nef) is an accessory HIV-1 protein with an extensive cellular interactome and a broad functionality within the HIV replication cycle [1, 2]. HIV-1 long-term survivors have been found to harbor viruses with a deficient *nef* gene, indicating a critical role for Nef in the viral life cycle and in the progression towards AIDS [3, 4]. Nef orchestrates the down-regulation of important surface receptors involved in immune surveillance, such as CD4, MHCI, and MHCII, and activates host cells by triggering signaling pathways involving involving Src family kinases, such as the macrophage-specific hematopoietic cell kinase (Hck) [1, 2]. Nef's functionality is based on numerous protein-protein interactions (PPIs) [2, 5]. The SH3 interaction site on Nef has been identified as a 'hot spot' for potential therapeutic intervention due to its highly conserved character [2, 5]. SH3 interacts with a poly-proline type II helix comprising the consensus motif P_{72}xxPxR (HIV-1 NL4-3 nomenclature) and the RT-loop recognition site, a hydrophobic cleft on Nef. The compounds D14, DLC27, and the optimized compound DLC27-14 displayed inhibition of the Nef/SH3 complex formation through targeting of the hydrophobic cleft [6, 7]. However, all the compounds failed to function in antiviral cell-based assays [6, 7], thus revealing the need for alternative chemical starting points. To our knowledge, the Nef/SH3 PPI has not been targeted by a biochemical high-throughput screening approach that supports the identification of Nef-specific antivirals**.**

Here we report on the development and validation of a biochemical time-resolved fluorescence resonance energy transfer (TR-FRET)-based assay for the identification of inhibitors of the Nef/SH3 PPI. The TR-FRET-based assay was paired with an orthogonal biophysical label-free resonant waveguide grating (RWG) assay allowing not only the identification of primary hits that are TR-FRET label-dependent artifacts, but also the confirmation of specifically target-binding compounds.

MATERIAL AND METHODOLOGY

Reagents

All chemicals were purchased from commercial suppliers unless otherwise stated. The HIV-1 His-tagged Nef_{SF2}(His-Nef), His-tagged Nef_{SF2} mutant P_{72}xxPxR/AxxAxA and the

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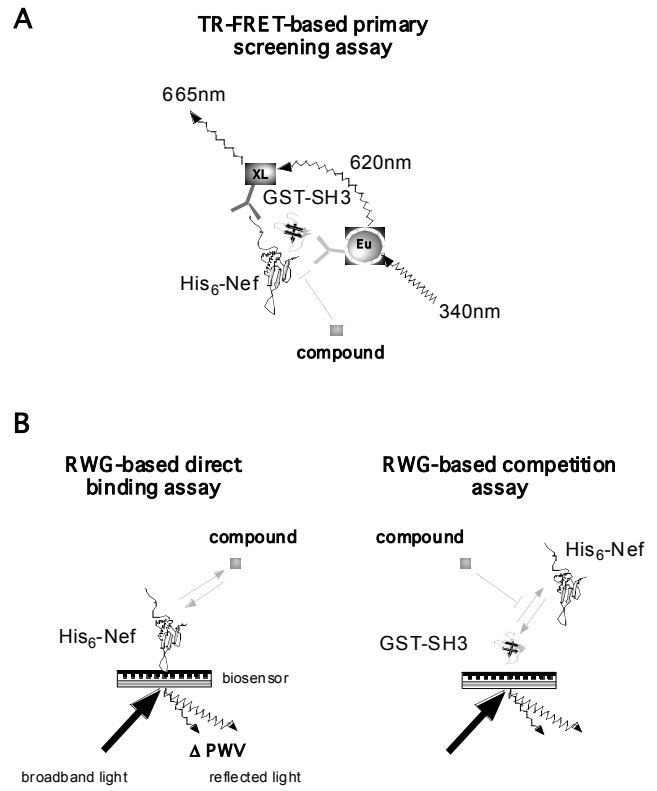


Fig. (1A). Principle of a TR-FRET-based Nef/SH3 protein-protein interaction assay. The quaternary complex of His-Nef, GST-SH3, α-GST Eu-labeled mAb, and α-His XL665-labeled mAb generates the proximity for a FRET pair between the donor lanthanide fluorophor europium and acceptor fluorophor XL665. The Eu chelates emit light at 620 nm after excitation at 340 nm and thereby excite neighboring XL665 FRET acceptors. Excited XL665 decays under the emission of photons with a wavelength of 665 nm. The 665 nm emission or the 665/620 nm ratio is proportional to the number of quaternary complexes formed. Excited Eu chelates have a long half-life. The time-resolved detection minimizes interference with extrinsic fluorescence. (**B**) Schematic of the resonant waveguide grating (RWG)-based assay for monitoring protein-ligand or protein-protein interactions in real time. Here, the target protein His-Nef (or off-target GST-SH3) is immobilized on the pre-activated aldehyde surface of an optical biosensor whose photonic crystal composition allows the reflection of broadband light only in a narrow range of wavelengths, the peak wavelength value (PWV). Binding of a chemical (compound) or biological (protein) ligand to His-Nef results in a change of the refractory index of the biosensor, measured as a shift of the peak wavelength value (ΔPWV) that is proportional to the change in mass on the surface.

GST-tagged Hck SH3(GST-SH3) domain were synthesized as recombinant proteins as described previously [8, 9]. The monoclonal Eu-cryptate-conjugate α-GST(α-GST mAb-Eu) and the APC (XL665)-conjugated α-His (α-His mAb-XL) antibodies were purchased from Invitrogen (Carlsbad, CA, USA) and Cisbio-US (Bedford, MA, USA), respectively. The compound D14 was kindly provided by the AIDS reagents program. DLC27 was synthesized as described previously [7].

TR-FRET-Based Assay

The TR-FRET-based assay was carried out in white solid-bottom 1536-well plates (Greiner, San Diego, CA, USA). 500 nM His-Nef, 10 nM GST-SH3, 0.5 nM α-GST mAb-Eu and 5 nM α-His mAb-XL were used in PBS in a final detection volume of 5 µl at room temperature. After 2 hrs incubation the fluorescence at 620nm and 665nm was detected using a Pherastar FS plate reader (BMG Labtech, Ortenberg, Germany). All experiments were performed in triplicates.

High Throughput Screening

For the Library of Pharmacologically Active Compounds (LOPAC) (Sigma Aldrich, St Louise, MO, USA) screen individual 50-nl compound aliquots from 1 mM DMSO stocks were transferred using an acoustic dispenser (Labcyte, Sunnyvale, CA, USA) to a well containing the quaternary complex (His-Nef, GST-SH3, α-GST mAb-Eu and α-His mAb-XL) in a 5 µl volume, which was then incubated for 2 h before the TR-FRET-based assay was measured.

RWG-Basedcompetitive Assay

The RWG experiments were carried out in 384-well GA3 aldehyde plates from SRU Biosystems (Woburn, MA) using a SRU BIND® SCREENER reader (SRU Biosystems, Woburn, MA). 5 µM GST-SH3 was coated on a pre-activated biosensor via its primary amines at room temperature. The binding kinetic of 0.15 µM Nef in presence of 50 µM compound or DMSO was measured over time in a final volume of 50 µl, PBS + 1 % (v/v) DMSO buffer.

RWG-Based Direct Binding Assay

A 384-well GA3 aldehyde plate was coated with 5 µM His-Nef (or GST-SH3) and the binding kinetic of 50 µM compound was detected in a final volume of 50 µl in PBS + 1 % (v/v) DMSO buffer using SRU BIND® SCREENER reader (SRU Biosystems, Woburn, MA).

RESULTS

Fig. (**1**) shows a schematic of the assay cascade comprising a TR-FRET-based primary screening assay and an orthogonal label-free RWG-based assay. The GST-tagged Hck SH3 domain (GST-SH3) forms a stable complex with the His-tagged Nef protein (His-Nef) in solution (Fig. **1A**). The affinity of this interaction was quantified previously and found to have a K_d of 0.25 - 1.5 µM [9, 10]. The interaction can be detected homogeneously and therefore automation friendly by TR-FRET between a europium(Eu(III))-conjugated anti-GST monoclonal antibody (α-GST mAb-Eu) and an anti-His XL665-labeled mAb (α-His mAb-XL) in a quaternary complex (Fig. **1A**). Upon the Eu (III)-cryptate donor excitation at 340 nm, part of the emission at 620 nm activates the acceptor, allophycocyanin XL665, whose emission is detectable at 665 nm. The longevity of the 620 nm emission allows the time-resolved assessment of the 665/620 nm intensity ratio (as a measure of the binding of SH3 to Nef)100 µs after UV excitation of the quaternary complex. Both the large Stokes shift of the Eu(III)-fluorescence and the time resolution of the measurement eliminate most sources of fluorescence interference, making Eu(III) especially well-suited for high-throughput screening of large and diverse compound libraries [11, 12]. In some cases compounds may have inherent fluorescence that confounds the use of screening using a TR-FRET-based assay. The use of

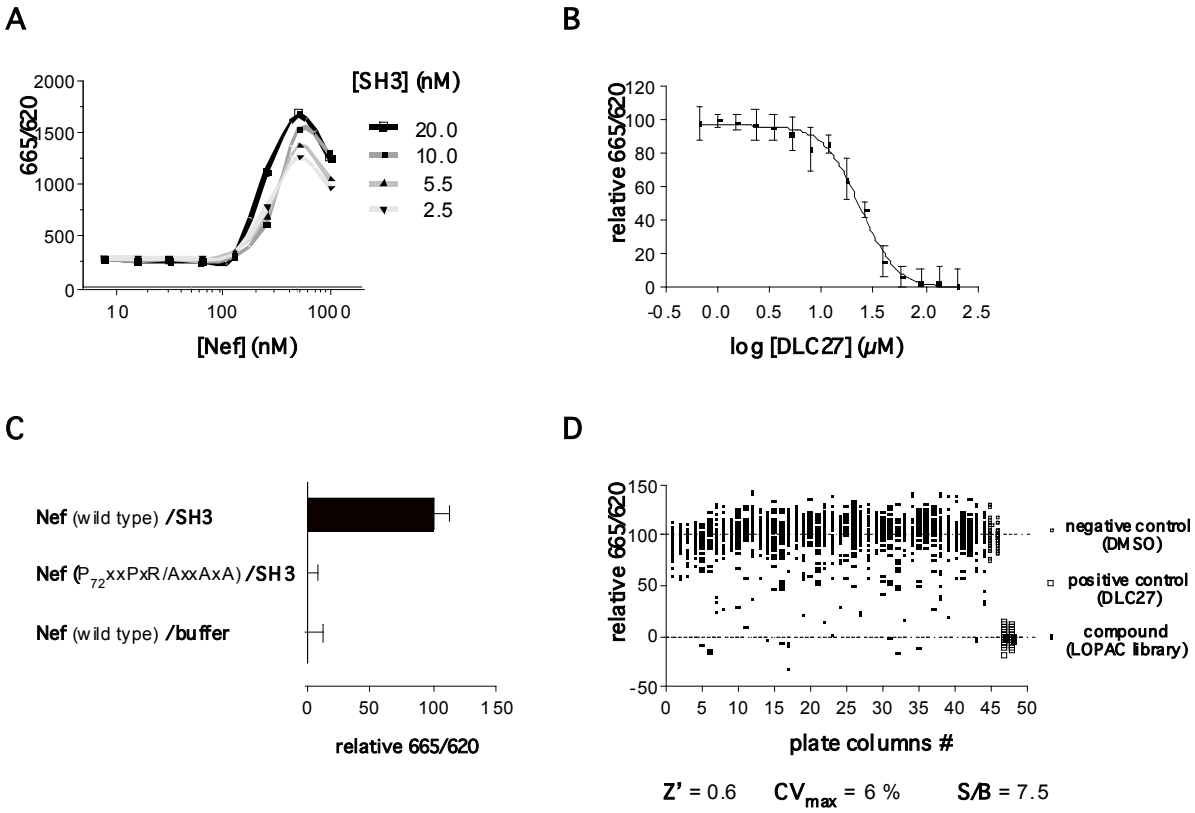


Fig. (2A). Optimization of the TR-FRET signal of the quaternary complex. Here, a concentration matrix of His-Nef vs. GST-SH3 was incubated with 0.5 nM Eu-conjugated anti-GST antibody and 5 nM XL-conjugated anti-His_6 antibody in a total volume of 5 µl for 2 h at room temperature. (**B**) TR-FRET-based IC_{50} analysis of the PPII DLC27. At 500 nM His-Nef and 10 nM GST-SH3 an IC_{50} of 23.51 ± 1.31 (mean ± s.d.) µM was calculated using a four parameter logistic modelfrom GraphPad Prism 5.0. (**C**) TR-FRET-based PPI analysis of the Nef mutant P_{72}xxPxR/AxxAxA.10 nM GST-SH3 was incubated with 500 nM wild type Nef, buffer (control) or Nef P_{72}xxPxR/AxxAxA and binding detected using the antibody-based TR-FRET. (**D**) Scatterplot of a 1536-well LOPAC (Library of Pharmacologically Active Compounds) assay plate. The controls, DMSO (open circles) and DLC27 (open squares), were used to determine the Z'-factor of 0.6 The TR-FRET signal was normalized to the median of the controls.

the label-free resonant waveguide grating (RWG) technology for evaluation of fluorescent compounds negates this concern (Fig. **1B**). The RWG technology utilizes a photonic crystal biosensor surface that reflects broadband light in a narrow range of wavelength (peak wavelength value, or PWV). Any alteration of the mass of an immobilized biomolecule on the biosensor surface, such as through the binding of a compound, alters the dielectric permittivity of the biosensor material, which causes a shift of the reflected wavelength (**Δ**PWV) proportional to the change in mass [13]. Thus, immobilization of the biomolecule on the biosensor surface, followed by the addition of putative chemical or biological binding partners can be monitored kinetically so that one can discriminate binding events on the basis of multiple criteria such as stoichiometry and association kinetics [14].

The optimal TR-FRET-based assay conditions were defined in a multi-dimensional approach by varying the protein concentrations of both His-Nef and GST-SH3 and varying the ratios of α-GST mAb-Eu to α-His mAb-XL. Fig. (**2A**) shows an example of signal development in response to increasing His-Nef and GST-SH3 concentrations at fixed mAb concentrations. As can be seen, the TR-FRET-based assay signal increased in a His-Nef and GST-SH3 concentration-dependent manner, until at high concentrations free His-Nef competed for the binding of detection antibody and blocked the formation of the quaternary complex, resulting in a decrease of the 665/620 nm ratio. Concentrations of 500 nM His-Nef, 10 nM GST-SH3, 0.5 nM α-GST mAb-Eu and 5 nM α-His mAb-XL in a reaction volume of 5 µl in PBS displayed the best signal to background (S/B = 7.5) values with an adequate signal robustness (coefficient of variations < 10%). These assay parameters tolerated dimethyl sulfoxide (DMSO) concentrations of up to 2 % (v/v), and the signal was stable in a time frame of t = 60 – 180 min after antibody addition (**data not shown**).

Based on the optimized assay conditions the potency of a known inhibitor of the Nef/SH3 interaction, DLC27, was evaluated (Fig. **2B**) [6]. A DLC27 concentration series was evaluated and the IC_{50} calculated with a four-parameter logistic model from GraphPad Prism 5.0. The IC_{50} of 23.51 ± 1.31 (mean ± s.d.) µM correlated with previously published data [6, 7]. As a second proof of concept, we tested a His-Nef mutant lacking the canonical PxxPxR motif: it did not show a TR-FRET-based assay signal (Fig. **2C**). The feasibility of miniaturization and automation of the TR-FRET-based assay for HTS purposes was examined by testing a 1536-well compound plate from the Library of Pharmacologically Active Compounds (LOPAC, Sigma-Aldrich, St. Louis, MO, USA) (Fig. **2D**). Individual 50-nl compound aliquots from 1

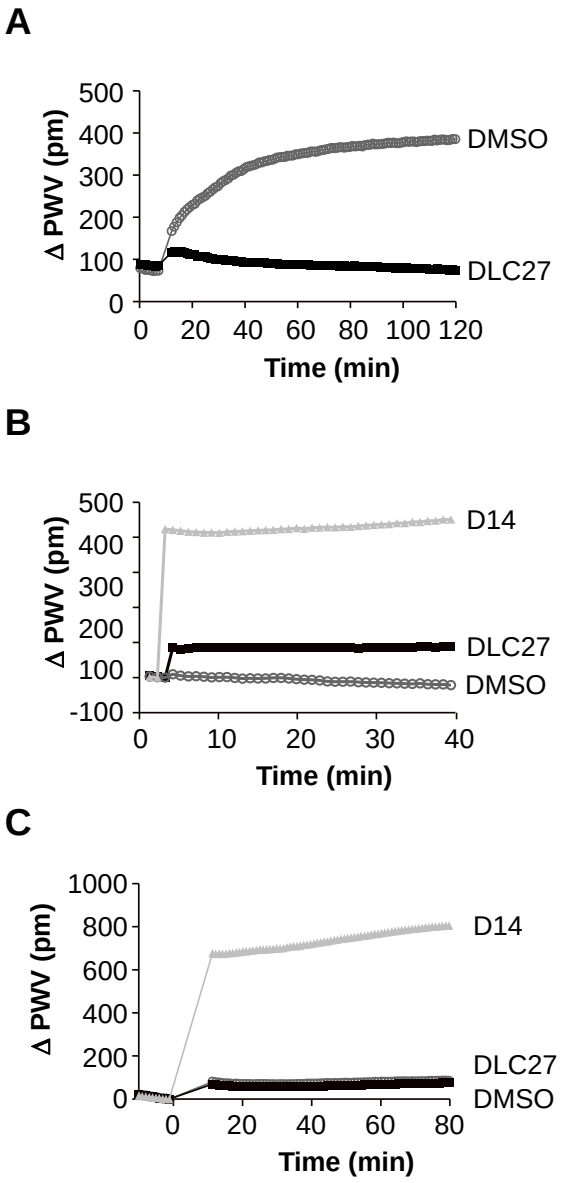


Fig. (3A). RWG-based competition assay: Inhibition of His-Nef/GST-SH3 complex formation by DLC27. The binding of 150 nM His-Nef to 5 µM GST-SH3 immobilized to the biosensor surface was monitored by RWG in the presence of 1 % (v/v) DMSO or 50 µM DLC27 in PBS in real-time. (**B**) RWG-based direct binding assay: Binding kinetics of D14 and DLC27 to His-Nef. The peak wavelength shift (ΔPWV) was assessed after the addition of 50µM DLC27 or D14 in 1 % (v/v) DMSO in PBS to 5 µM immobilized His-Nef in real-time. (**C**) Binding kinetics of D14 and DLC27 to GST-SH3. The ΔPWV was assessed after the addition of 50 µM DLC27 or D14 in 1% (v/v) DMSO in PBS to 5 µM immobilized GST-SH3 in real-time.

mM DMSO stocks were transferred using an acoustic dispenser (Labcyte, Sunnyvale, CA, USA) to a well containing the quaternary complex in a 5 µl volume, which was then incubated for 2 h before the TR-FRET-based assay was measured. The Z' factor [15] of this representative test compound plate was calculated to be 0.6. Among the LOPAC compounds, we also placed our positive control DLC27 and D14 as test samples. DLC27 and D14 had been identified as Nef-binding small molecules and inhibitors of the Nef/SH3 protein-protein interaction [6]. Research on DLC27 has continued in a hit-to-lead study, while D14 was not followed up for further antiviral testing [7]. Both compounds could be identified as hits and be confirmed in IC50 experiments (Table **1**). The robustness and dynamic range of the TR-FRET-based assay allows for both an automated ultra-HTS campaign and ranking of primary hits according to their IC_{50} values.

To exclude compound artifacts due to fluorescence interference or inhibition of the Nef/SH3 interaction by the virtue of colloidal compound aggregate formation [16], we paired the primary TR-FRET-based assay, in an orthogonal approach, with a label-free RWG-based assay format using a 384-well plate setup (Fig. **1B**). Using the tool compounds DLC27 and D14 for follow up studies, we employed RWG-based competition assays as well as RWG-based direct binding assays to evaluate hits regarding their protein-protein interaction inhibition and direct target binding properties [17]. Fig. (**3A**) shows the binding kinetics of His-Nef to GST-SH3 immobilized on an aldehyde-coated biosensor plate in the presence of 50 µM DLC27 or a control (DMSO). Only the control sample shows the expected shift of the reflected wavelength (ΔPWV), confirming that DLC27 disrupts the interaction of Nef and SH3. To further investigate the binding specificity of compounds to the protein target, we immobilized the viral Nef protein or the human SH3 domain on the biosensor surface and then treated each with DLC27 or D14 (Fig. **3B** and **C**). The real-time binding kinetics of DLC27 or D14 to immobilized His-Nef or GST-SH3 demonstrated that DLC27 selectively bound to Nef, whereas D14 produced a positive PWV shift with both His-Nef and GST-SH3 (Fig. **3B, C** and Table **1**). These findings could indicate that D14 shares a binding site between His-Nef and GST-SH3. However, given that the magnitude of the PWV shifts caused by D14 exceeded what can be expected from either the molecular mass or the stoichiometry of compounds, it appears that D14 is a promiscuous compound that disrupts Nef/SH3 interaction.

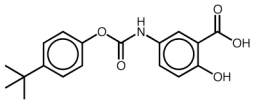
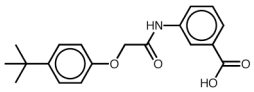
DISCUSSION

In summary, we have developed a robust and HTS-compatible assay comprising a primary TR-FRET-based assay for identifying inhibitors of Nef/SH3 complex formation. Taking advantage of the emergence of HTS-amenable biophysical methodologies, we combined the TR-FRET-based assay with an orthogonal biophysical label-free RWG-based assay, the HTS resolving power is extended, by allowing screening of fluorescent compounds, as well as the identification of Nef/SH3 promiscuous binders. In combining these two distinct methodologies into a high-throughput assay cascade, novel Nef/SH3 inhibitors can be rapidly selected and ranked from large chemical libraries by their inhibitory activity and specific binding early in a drug discovery campaign (Table **1**).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

Table 1. Summary of Biochemical and Biophysical Screening Data

Name	Structure	TR-FRET Assay (Nef-SH3) IC50 (μM)	RWG-Based Competition Assay (Nef-SH3)	RWG-Based Direct Binding Assay (Nef)	RWG-Based Direct Binding Assay (SH3, Off-Target)
D14		108.82 ± 21.77	+	+ (super stoichiometry)	+ (super stoichiometry)
DLC27		23.51 ± 1.31	+	+	-

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ABBREVIATIONS

TR-FRET	=	time-resolved fluorescence resonance energy transfer
RWG	=	resonant waveguide grating
PPI	=	protein-protein interaction
PPII	=	protein-protein interaction inhibitor
HTS	=	high-throughput screening
Nef	=	negative factor
His-Nef	=	His-tagged Nef
SH3	=	Src-homology domain 3
GST-SH3	=	GST-tagged SH3
GST	=	glutathione S-transferase
Eu	=	europium
mAb	=	monoclonal antibody
DMSO	=	dimethyl sulfoxide
UV	=	ultraviolet
RT	=	room temperature

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