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A Novel High-Throughput Screening Assay to Identify Inhibitors of HIV-1 gp120 Protein Interaction with DC-SIGN

Thuong H. Tran[1], Rasha El Baz[1], Andrea Cuconati[2], James Arthos[3], Pooja Jain[1] and Zafar K. Khan[1]*

[1]*Drexel Institute for Biotechnology and Virology Research, and Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA 19129, USA*
[2]*Institute for Hepatitis and Virus Research, The Hepatitis B Foundation, Pennsylvania Biotechnology Center, Doylestown, PA 18902, USA*
[3]*Laboratory of Immunoregulation, National Institute of Allergies and Infectious Disease, Rockville Pike, Bethesda MD 20892, USA*

Abstract

The 2010 UNAIDS report states that approximately 34 million people are living with human immunodeficiency virus type 1 (HIV-1), despite highly active antiretroviral therapy (HAART). Despite being effective, ARV therapy has many disadvantages including a cost trajectory unsustainable for economically challenged countries, serious side effects, and the development of drug-resistant strains. Several measures are under way to develop alternatives for ARV therapy, particularly for the control of early HIV-1 infection, but lack of efficient drug targets and assays hinders the search of potential ARV molecules. The dendritic cells present in the mucosal tissue, together with $CD4^+$ T lymphocytes and macrophages, are among the first cells to encounter HIV-1. The dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) molecule plays a crucial role in binding HIV-1 through high affinity interaction with viral envelope glycoprotein gp120. DC-SIGN, a mannose-binding C-type lectin expressed on cells in the mucosal tissue of the rectum, uterus and cervix, facilitates early HIV-1 infection after sexual transmission. In this study we report a novel target-specific high-throughput screening (HTS) assay capable of quantifying the binding as well as the inhibition of DC-SIGN and gp120. The specificity of the assay was determined through competitive inhibition while optimization occurred for DMSO tolerance (0.5%), Z' factor (0.51), signal-to-noise ratio (3.26), and coefficient of variation (5.1%). For assay validation previously recognized antagonists of DC-SIGN/gp120 binding were tested to detect inhibition demonstrating the suitability of the assay for future HTS screen of potential inhibitors that block the binding between DC-SIGN and gp120 which may prevent early HIV-1 infection.

Keywords: DC-SIGN; gp120; High-throughput screening assay

Abbreviations: ARV: Antiretroviral; CV%: Coefficient of variation; DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; F.U.: Fluorescence units; GN: *Galanthus nivalis;* HHA: *Hippeastrum hybrid;* HIV-1: Human immunodeficiency virus type 1; HTS: High-throughput screening; NP: *Narcissus pseudonarcissus;* S/N: Signal-to-noise ratio**;** iDCs: Immature dendritic cells

Introduction

The human immunodeficiency virus (HIV-1) pandemic afflicts approximately 34 million people worldwide. Approximately 3 million people in low- and middle-income countries are receiving HIV-1 antiretroviral (ARV) therapy; however, the escalating cost of the medicine, weak or inadequate health care infrastructure, and lack of financing have prevented wide use of combination ARV treatment in many nations [1]. Nonetheless, the prolonged use of ARV therapy is invariably associated with serious side effects, and the problem is further aggravated by the presence of opportunistic life-threatening infections and comorbidity pathogens [2]. With the failure of the most recent trial(s), the availability of an HIV-1 vaccine in the near future does not look promising [3]. Therefore prevention of early infection and transmission of HIV-1 with safe, more effective and low-cost options is a better alternative to ARV therapy [4,5].

CD4 antigen is an essential component of the receptor for HIV-1 infection. The infection with HIV-1 begins with the interaction of its envelope glycoprotein gp120 with a host cell receptor [6]. This binding creates a conformational change in gp120, which then opens the coreceptor binding sites for the attachment of the chemokine receptors CCR5 and CXCR4. Increasing conformational changes in gp120 activate the fusion peptide on the N-terminus of another viral envelope protein, gp41. This activation leads to the creation of a six-helix bundle complex that fuses the virus to cell membranes and eventually internalizes HIV-1 via a pH-dependent mechanism [7-10]. These factors along with other studies have indicated that the functionality of gp120 is crucial for the uptake of HIV-1 [10]. Immature dendritic cells (iDCs) present in the mucosal tissue, in conjuction with $CD4^+$ T lymphocytes and macrophages, are some of the first cells to encounter the HIV-1 virus [11-12]. Infectious HIV-1 particles, following capture by iDCs, are transported to the draining lymph nodes where the virus is efficiently transmitted to $CD4^+$ T cells. In the initial phase, HIV-1 interacts with receptors expressed on immature dendritic cells such as C-type lectin receptors [13]. The dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) molecule plays a crucial role in binding HIV-1 through the viral envelope complex [14-17] and in transmitting HIV-1 to target cells. This process is known as *trans*-infection [18] and is thought to be the pathway that HIV-1

***Corresponding author:** Zafar K Khan, PhD, Drexel Institute for Biotechnology & Virology Research, and Department of Microbiology & Immunology, Drexel University College of Medicine, 3805 Old Easton Road, Doylestown, PA 18902, USA, Tel: 215-589-6431; Fax: 215-489-4920; E-mail: zkhan@drexelmed.edu

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uses to gain access to T cells. DC-SIGN binding to gp120 is a very high affinity interaction and is thus considered to be a critical phase in the entry of HIV-1 [15,19,20].

DC-SIGN is a mannose-binding C-type lectin expressed on dendritic cells in mucosal tissue including the rectum, uterus, and cervix [21]. Antibodies to DC-SIGN inhibit the binding of the HIV-1 envelope complex to dendritic cells and prevent viral transmission [22]. Furthermore, RNA interference [23,24] and carbohydrate-binding agents [25] have been shown as potential means to block this process. RNA-based therapies, however, presented obstacles with respect to delivery, stability, and potency. Similarly, conversion of promising carbohydrates to effective inhibitors has been challenging [26]. Therefore, the identification of novel, highly selective small-molecule inhibitors of the DC-SIGN/gp120 interaction will be useful for future treatment strategies for HIV-1. The goal of this study was to design an innovative target-based cell-free assay that could be used in high-throughput screening (HTS) of DC-SIGN/gp120 antagonists. HTS assays require a specific design and optimization for speed, efficiency, low reagent use, reproducibility, and sensitivity [27,28]. Design of assays for lectins has been troublesome due to their weak binding to monovalent carbohydrate ligands [26]. Lectins typically possess shallow, solvent-exposed carbohydrate binding sites, which can be difficult to block effectively. The recently identified noncarbohydrate selectin ligands are rare examples of potent lectin inhibitors [29]. DC-SIGN too exhibits weak affinity for monosaccharide ligands and moderate affinity for oligosaccharides [30] but unusually high affinity for the HIV-1 protein, gp120 [31]. Moreover, noncarbohydrate small-molecule inhibitors (IC_{50}: 1.6-10 μM) were identified recently that demonstrated potent inhibition of DC-SIGN–carbohydrate interactions in both biochemical and cell-based assays to illuminate the mechanistic aspects of DC-SIGN functions [26]. The observations that documented the strong affinity of DC-SIGN for gp120 provided great help to our assay design. In our earlier studies we standardized a cell-based viral binding assay and screened several DC-SIGN blocking antibodies [32,33]. In the present studies once the assay was deemed suitable, it was validated with known antagonists of DC-SIGN/gp120 interactions. Other parameters tested were the specificity of gp120 binding to DC-SIGN and the effects of DMSO on the protein-protein interaction. Assay optimization was achieved through computation of three variables: signal to noise ratio (S/N), the coefficient of variation (CV%), and the Z' factor [27]. We demonstrate that this newly devised target-based HTS assay will meet the required standards as well as show significant results pertaining to the inhibition of DC-SIGN and gp120. Our overall goal is to use this HTS assay to screen a large synthetic library (over one million compounds) of small molecule inhibitors to identify potential anti-HIV-1 therapeutic agents.

Methods

Reagents

DC-SIGN, with its entire carbohydrate recognition domain, was prepared as previously described [17]. FITC-gp120 and native gp120 were obtained from ImmunoDiagnostics (Woburn, MA) and Protein Sciences Corp. (Meriden, CT), respectively. The NIH AIDS Reagent Program supplied both a DC-SIGN (Clone 120507) and gp120 antibody (2G12), whereas, another DC-SIGN antibody (SC-20) was acquired from SantaCruz Biosciences (Santa Cruz, CA). Plant lectins from *Galanthus nivalis* (GN), *Hippeastrum hybrid* (HHA), and *Narcissus pseudonarcissus* (NP) were obtained from Vector Laboratories (Burlingame, CA). The noncarbohydrate inhibitors of lectins assigned K784-1848 (SI #1) and 4112-3485 (SI #2) were provided by the Fox Chase Chemical Diversity Center, Doylestown, PA, USA.

DC-SIGN/gp120 interaction assay

Black, flat-bottom, PolySorp 96-well plates (NUNC, Rochester, NY) were coated with 200nm of DC-SIGN in a 100-μl working volume of assay buffer (30mM Tris-HCl, pH 8.3, 30mM $NaHCO_3$, and 3mM $CaCl_2$). Plates were incubated overnight at 4ºC for protein adherence and subsequently washed three times with 250μl of washing buffer (1×TBS, 1mM $CaCl_2$, and 0.1% Tween-20). After washing, 100μl of blocking buffer (1×TBS, 1mM $CaCl_2$, 0.1% Tween-20, 5.0% dried nonfat milk powder, and 0.02% thimerosal) was added to each well to prevent nonspecific binding. The plates were incubated in blocking buffer for 2 hours at 4ºC and then washed again three times. To assess gp120 binding to DC-SIGN, varying concentrations (200, 300, 400, and 500 nM) of FITC-gp120 were added to the coated well. Wells with only assay buffer served as a negative control. Binding was performed at 4˚C for 1 hour, and plates were given three final washes before reading at an excitation/emission wavelength of 485/528 in a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). Each assay was performed at least in duplicate, and fluorescence intensity values were averaged. Statistical significance was determined with the Student's *t* test by comparing assay wells to the control wells. Data were considered significant if the p value was ≤ 0.05.

Miniaturization of the assay for HTS specificity

Our center has a synthetic small molecule library of over one million derivatives, therefore we wanted to make the assay more suitable in HTS format by miniaturizing from 96- to 384- well plates in order to minimize the quantity of molecules used as well as to reduce the time frame of screening completion. Black, flat-bottom, MaxiSorp 384-well plates (NUNC, Rochester, NY) were used with a working volume of 20μl, as opposed to the 100μl volume used in 96-well plate assay. To perform competitive analysis of gp120 binding to determine assay specificity, a constant amount of FITC-gp120 (500nm) was used in conjunction with the varying concentrations of native gp120 at 500, 50, 5, and 0.5nm. Each assay in 384-well plate was performed at least in duplicate and fluorescence intensity values were averaged, and data were statistically analyzed for significance as described earlier.

DMSO optimization for HTS assay

Two fold dilutions of DMSO (Mediatech., Inc., Manassas, VA) ranging 2.0-0.02%, were analyzed to determine if DMSO had any effect on DC-SIGN/gp120 binding. FITC-gp120 (500 nm) was added directly after DMSO. Positive and negative controls were set by using FITC-gp120 or assay buffer, respectively. The assay was further optimized through derivation of the Z' factor, Signal-to-Noise (S/N), and Coefficient of Variation (CV%) from the data collected from the experiment.

Validation of the HTS assay

The validation of HTS assay was done with the assessment of DC-SIGN blocking antibodies: Clone 120507 and SC-20 at 20μg/ml. We used gp120-specific neutralizing antibody: 2G12 and some

carbohydrate small-molecule inhibitors: GN, HHA, and NP, all at 10 µg/ml; and two noncarbohydrate small-molecule inhibitors of DC-SIGN assigned: SI #1 and SI #2, at 100µM. FITC-gp120 (500nm) was added directly after blocking antibodies and inhibitors used. Positive and negative controls were set by using FITC-gp120 or assay buffer (blank).

Results

Linearity of the DC-SIGN/gp120 binding assay in two different plate settings

To observe the linearity of the newly designed binding assay, increasing concentrations of FITC-gp120 (200-500 nm) were administered to DC-SIGN-coated plates in both a 96- and a 384-well format. A linear increase in mean fluorescence intensity concurrent with increasing FITC-gp120 was observed in both plate formats (Figure 1). However, the S/N and derived p value of the 96-well format (1.3 and 0.1, respectively) fell short of that of the 384-well format (2.4 and 0.005, respectively). The best binding was observed at a 500nm concentration of FITC-gp120 in both the 96-well (505 fluorescence units, F.U.) and the 384-well plate (394 F.U.). These data indicated that a 384-well plate and 500 nm of FITC-gp120 were the best parameters to use for further experimentation.

Specificity of the DC-SIGN/gp120 interaction

To determine the specificity of DC-SIGN/gp120 binding, DC-SIGN was incubated with a standardized amount of FITC-gp120 (500nm) in the presence of varying amounts (500-0.5nm) of native gp120. Our results showed a noticeable decline in DC-SIGN/FITC-gp120 binding with increasing concentrations of native gp120 (Figure 2) and the data were found to be statistically significant ($p < 0.05$).

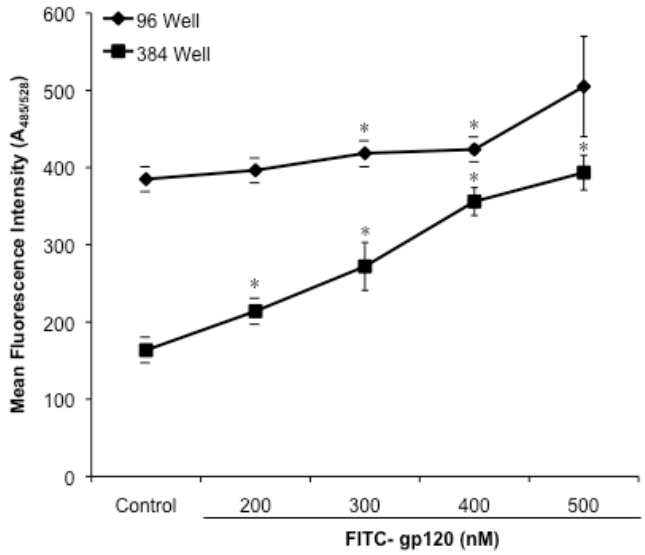

Figure 1: Reagent titration and assay miniaturization from 96 well to 384 well format. In each well, 200 nM of DC-SIGN was incubated with 200, 300, 400 or 500 nm of FITC-gp120. Wells with only assay buffer served as the control. Data represents mean fluorescence intensity ± standard error of mean (S.E.M) of three independent experiments each performed in duplicate. A Student's *t* test determined the statistical significance of a sample by having a *P*-value of ≤ 0.05 and is denoted by an asterisk (*).

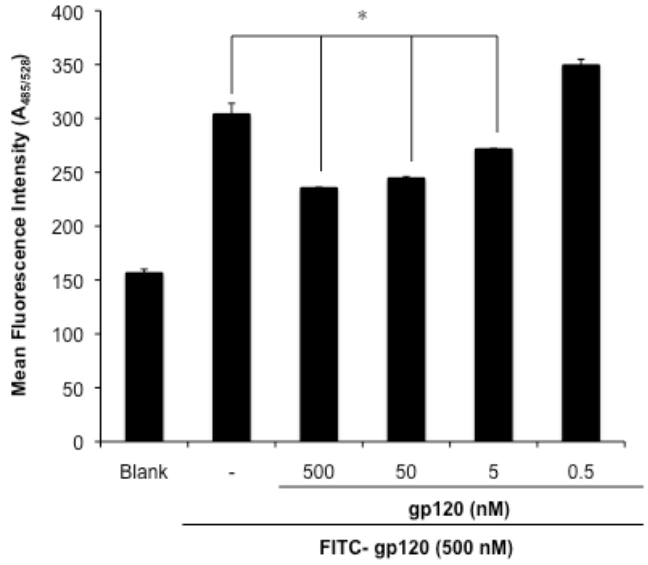

Figure 2: Competitive inhibition of gp120 binding to determine assay specificity. DC-SIGN was exposed to decreasing concentrations (500 - 0.5 nm) of native gp120, followed by the immediate addition of FITC-gp120. Positive and negative controls were demonstrated by the exposure only to FITC-gp120 or to assay buffer, respectively. Gradual increase of FITC-gp120 binding occurred as the concentration of native gp120 was lowered, showing the specificity of the assay. Data represent mean fluorescence intensity ± S.E.M from an experiment performed in duplicate. Student's *t* test was used to determine the statistical significance of a sample for p value ≤ 0.05 and is denoted by an asterisk (*).

Optimization of the HTS parameters

The binding of FITC-gp120 to DC-SIGN was analyzed in the presence of DMSO to determine the effect on protein binding. As shown in (Figure 3), the presence of DMSO, even as high as 2%, had no significant effect on the binding of FITC-gp120 to DC-SIGN. Assay optimization was done by calculation of Z', signal background as opposed to signal noise (S/N), and CV%. Z' was derived by following the procedure noted in past studies and was equated to 0.51, indicating an excellent assay [27]. We also obtained a reasonable S/N Ratio of 3.26 by dividing the average values of the positive control with those of the negative control. CV% resulted in an exceptional value of 5.1%. All together, these parameters confirmed the suitability of the assay in 384-well for the HTS and allowed us to move forward with the validation process of the assay.

Validation of the assay with known inhibitors

To test the validity of the assay, we screened a variety of inhibitors of DC-SIGN and/or gp120 by adding the test compounds before exposure to FITC-gp120 (Figure 4). Two DC-SIGN-specific antibodies (Clone 120507 and SC-20), previously shown to possess blocking potential [32], once again demonstrated significant reduction in binding (F.U. 284 and 289, respectively, compared to positive control F.U. 911). Plant lectins HHA and NP exhibited a 66% and 67% decrease in fluorescence, respectively. Although GN had shown promising inhibition in a previous study, it was only seen here with a 33% decrease ($p = 0.06$), deeming it insignificant [25]. The gp120 neutralizing antibody 2G12 also demonstrated significant reduction in binding with a reading of 268 versus 911 F.U. (72% decrease). Previously reported small-

molecule inhibitors of lectin/monosaccaharide (SI #1 and SI #2) were also observed to be strong antagonists of DC-SIGN/gp120 binding, with F.U. of 244 (74% decrease) and 269 (71% decrease), respectively [26]. Overall, these significant results provided confidence for the use of the DC-SIGN/gp120 interaction assay as a HTS tool of novel anti-HIV-1 molecules.

Discussion

The year 2011 marks three decades of AIDS pandemic and during that period AIDS has claimed more than 25 million lives, and the epidemic continues in many developing countries with a growing population of HIV-1 infected patients. In the latest United Nations General Assembly, sixty-fifth session report the agenda: Implementation of the declaration of commitment on HIV/AIDS articulated a new vision for uniting for universal access towards zero new HIV infections, zero discrimination and zero AIDS-related deaths. The UNAIDS believes that a substantial number of countries will fail to meet Millennium Goal 6: halting and reversing the spread of HIV-1. Global statistics have shown that, since the introduction of the global AIDS pandemic in 1990s, only 35% of victims actually have access to ARV therapy. ARV is available to patients located in developed nations, but availability of ARV therapy has progressed slowly in countries with underdeveloped economies because of its extremely high cost [1]. In addition to the expense, ARV therapy causes serious adverse health effects. These include, but are not limited to, hepatotoxicity, osteoporosis, peripheral neuropathy, lactic acidosis, hyperglycemia, pancreatitis, cardiomyopathy, and depression [2]. As such, these drugs are not a viable treatment option in some patients with common co-morbid conditions. Many researchers agree that a more suitable approach to HIV-1 therapy is to prevent early infection and transmission [4,5]. One of the more promising targets proposed for new treatment is prevention of the DC-SIGN/gp120 interaction [15]. However, to date, neither a specific inhibitor to block DC-SIGN/gp120 binding nor a specific HTS assay that can identify such inhibitors has been reported. Thus, we began to construct, optimize, and validate a target-based assay that would be suitable for the HTS of potential DC-SIGN/gp120 inhibitors.

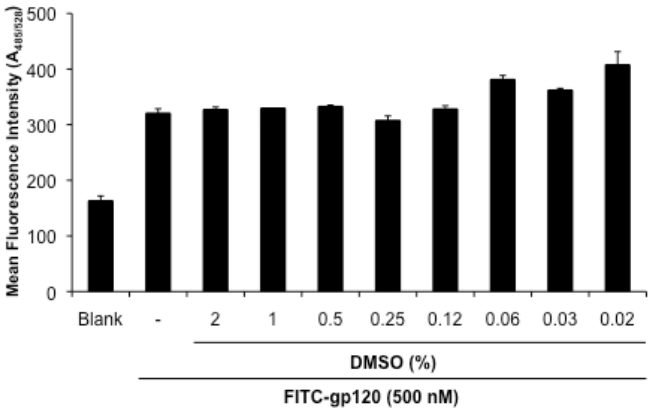

Figure 3: Optimization for DMSO tolerance. DC-SIGN was introduced to decreasing concentrations (2.0% - 0.02%) of DMSO to determine if DMSO had any effect on gp120 binding to DC-SIGN. FITC-gp120 was added directly after DMSO. Positive and negative controls were demonstrated by exposure only to FITC-gp120 or to assay buffer, respectively. The results showed that DMSO had no effect on gp120 binding because none of the DMSO had statistically different readings from the positive control. Data represent mean fluorescence intensity ± S.E.M. from an experiment performed in duplicate.

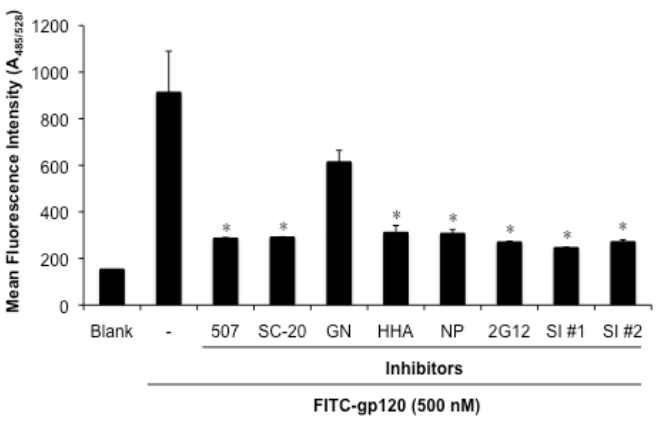

Figure 4: Assay validation through screening known DC-SIGN and gp120 inhibitors 507 (20μg/mL), SC-20 (20μg/mL), GN (10μg/mL), HHA (10μg/mL), NP (10μg/mL), 2G12 (10μg/mL), SI #1 (100μM), and SI #2 (100μM). All inhibitors were combined with DC-SIGN and then followed by FITC-gp120. Incubation occurred at 4°C for 1 hour. Positive and negative controls were demonstrated by the exposure to only FITC-gp120 or to assay buffer, respectively. All of the samples except GN showed at least a 66% decrease in fluorescence, indicating that inhibition of DC-SIGN/gp120 binding had taken place. GN had only a 33% decrease, which was calculated to be insignificant. Data represent mean fluorescence intensity ± S.E.M from an experiment performed in triplicate. Student's *t* test was used to determine the statistical significance of a sample for p value ≤ 0.05 and is denoted by an asterisk (*).

The initial step in creating this assay was titration of our reagents and conversion from a 96-well plate format to a 384-well plate format to minimize the cost. A miniscule S/N in the 96-well plate was determined to be insignificant because of a p value > 0.05. In contrast, an adequate S/N was observed in the 384-well plate. All data were seen to be significant ($p<0.05$), but optimal binding to DC-SIGN was observed with 500nm of FITC-gp120. Other researchers have also had problems with high background readings while using a 96-well format for HTS [34,35]. Plausible explanations include contamination, plastic consumables, and the high sensitivity of fluorescence readers for 96-well plates. Because the 384-well plate was found to be superior to the 96-well plate in our hands, further experimentation was done in this format with an optimized 500 nm concentration of FITC-gp120. Conversion to a 384-well format also allowed our HTS to be more time-efficient as one 384-well plate could screen a number of compounds equivalent to that of four 96-well plates. Competitive inhibition analysis was done to determine the specificity of this assay. We pre-incubated DC-SIGN with native gp120 before adding FITC-gp120 and observed that with decreasing concentrations of native gp120 there was a corresponding increase in mean fluorescence. Thus, we concluded that our assay is indeed specific for the binding of FITC-gp120 and DC-SIGN. Like many other pharmaceutical libraries, our in-house library contains compounds dissolved in DMSO. Therefore, optimization of our assay for HTS began by determining its DMSO tolerability at different concentrations. The results indicated incredibly stable protein binding, especially at 2%, 1%, and 0.5% DMSO. Although some fluctuations were seen at lower concentrations, the data were found statistically insignificant, suggesting sufficient DMSO tolerability of the assay. Optimization continued as we calculated the Z' factor, S/N, and CV%. The Z' factor, a measurement that takes into account both the S/N and CV%, is an indicator of how reliable and reproducible an

assay is. Here, the Z' factor was determined to be 0.51, which classified it as an "excellent" assay and indicated that the separation band was large between the samples and the control [27]. The S/N measures the strength of the signals given by the assay. Our S/N was 3.26, which showed a threefold difference between the background and sample values. Finally, the CV%, a method that measures the variability between readings of the wells, was 5.1%, indicating a high consistency between sample readings. Analysis of these values strongly suggested that our assay was indeed optimized and ready for HTS.

In order for the assay to be ready to screen potential inhibitors, it had to be validated by identifying previously known inhibitors of DC-SIGN, gp120, or carbohydrate binding agents. These compounds included DC-SIGN blocking antibodies (Clone 120507 and SC-20), plant lectins (GN, HHA, and NP), and a gp120 neutralizing antibody (2G12). Finally, two noncarbohydrate small-molecule inhibitors, SI #1 and SI #2, were also used for assay validation [25,26,32]. All of the tested inhibitors (except for GN) showed a substantial decrease in FITC-gp120 binding to DC-SIGN, confirming that our novel assay was capable of verifying the disruption of this reaction.

Overall, this innovative assay is very specific, optimized, and capable of quantifying DC-SIGN/gp120 interactions with or without inhibitors. In the near future, we plan to screen a large pharmaceutical library to identify potential molecules that could be derivatized into novel, cheap, and safe anti-HIV-1 agents. Another promising aspect of this experiment is that it can be adapted for nano-well plates in conjunction with automated liquid handlers. Also, it can be easily adopted to include other envelope-bearing viruses as well as bacteria, fungi, or parasites that interact with DC-SIGN [36]. In conclusion, although this assay was designed for our purposes, its high degree of flexibility and reproducibility allows it to be used by other investigators who seek to take advantage of the HTS process.

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