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High-throughput assay to identify inhibitors of Vpu-mediated down-regulation of cell surface BST-2

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ABSTRACT

Bone marrow stromal cell antigen 2 (BST-2, also known as Tetherin) inhibits HIV-1 release and thereby severely impairs viral replication. HIV-1 accessory protein Vpu induces the down-regulation of cell surface BST-2, and counteracts the antiviral function of BST-2. Blocking Vpu-mediated down-regulation of cell surface BST-2 is viewed as a new opportunity for developing anti-HIV drugs. In this study, we have developed a high-throughput cell-based ELISA to identify small molecules that antagonize HIV-1 Vpu function and consequently inhibit HIV-1 replication through rescuing the antiviral activity of host BST-2. This cell-ELISA shows an excellent correlation with results obtained by flow cytometry (FACS). Under optimal conditions, a Z' factor of 0.605 was achieved in a 96-well format. Together, these results demonstrate that this assay can be used to quantify the cell surface level of BST-2 and be adapted to a high-throughput screening for novel anti-HIV compounds.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) was identified as the etiologic cause of acquired immune deficiency syndrome (AIDS) almost 30 years ago. It affects an estimated 40 million individuals worldwide (Simon et al., 2006). Although highly active antiretroviral therapy (HAART) has significantly improved the clinical outcome of HIV-1 infection and AIDS, the emergence and circulation of multi-drug resistant HIV-1 strains still drive the discovery and development of novel anti-HIV-1 agents by targeting novel targets (Clavel and Hance, 2004; Flexner, 2007).

Among a number of potential strategies, enhancing the activities of HIV-1 host restriction factors is receiving more attention (Flexner, 2007). Host restriction factors represent a group of host proteins with innate antiviral activity that function as a barrier to block virus cross-species transmission. These include the APOBEC3 family and the TRIM family (Chiu and Greene, 2008; Strebel et al., 2009; Wolf and Goff, 2008). Recently, bone marrow stromal cell antigen 2 (BST-2, also called Tetherin/HM1.24/CD317) was identified as a novel host restriction factor that inhibits the release of HIV-1 and other enveloped viruses (Jouvenet et al., 2009; Mattiuzzo et al., 2010; Neil et al., 2008; Sakuma et al., 2009). BST-2 is a 30- to 36- kDa, heterogeneously glycosylated, dimeric, type II integral membrane protein (Ishikawa et al., 1995; Kupzig et al., 2003; Tokarev et al., 2009). It tethers nascent HIV-1 virions to the surface of infected cells (Hammonds et al., 2010; Perez-Caballero et al., 2009). The tethered HIV-1 particles are subject to internalization into CD63-positive endosomal compartments for degradation (Miyakawa et al., 2009; Neil et al., 2006). Besides the inhibition of the nascent cell-free particle release, BST-2 may also restrict cell-to-cell virus transmission, the predominant mode of HIV-1 spreading in cell culture (Casartelli et al., 2010; Kuhl et al., 2010). Yet, this conclusion remains under controversy (Jolly et al., 2010).

The antiviral function of BST-2 is antagonized by HIV-1 accessory protein Vpu (Neil et al., 2007, 2008). Vpu is an 81-amino acid type I integral membrane protein (Bour and Strebel, 2003; Cohen et al., 1988; Strebel et al., 1988). Vpu can down-regulate cell surface BST-2 and thus enhances HIV-1 release (Douglas et al., 2009; Iwabu et al., 2009; Mitchell et al., 2009; Van Damme et al., 2008). Vpu appears to interact with BST-2 in the trans-Golgi network or in early endosomes, and acts as an adapter molecule to link BST-2 to the cellular ubiquitination machinery via β-TrCP for degradation at proteasomes and/or lysosomes (Douglas et al., 2009; Dubé et al., 2009; Goffinet et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009; Tokarev et al., 2011).

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The discovery of BST-2 not only reveals a novel component of innate immunity, but also offers the opportunity to develop new approaches for the treatment of HIV-1 infection. BST-2 is expressed in physiologically relevant HIV-1 target cells (Douglas et al., 2009; Miyagi et al., 2009; Sato et al., 2009; Schindler et al., 2010). Therefore, blocking Vpu-mediated BST-2 down-regulation clearly is expected to expose HIV-1 to the restriction activity of BST-2. Thus, the aim of this work is to develop a high-throughput assay to identify small molecules that antagonize HIV-1 Vpu and inhibit HIV-1 replication through enhancing the antiviral activity of host BST-2.

2. Materials and methods

2.1. Plasmid DNA, antibodies and reagents

The plasmid pVphu expresses codon-optimized Vpu (Nguyen et al., 2004). The vesicular stomatitis virus glycoprotein (VSV-G) expression vector pHIT/G, HIV-1 proviral indicator construct pNL-Luc-E$^-$ was provided by Dr. Johnny He. pNL-Luc-E$^-$-Vpu$^-$ has the first ATG of Vpu coding sequence changed to ACG. For construction of the shRNA-Vpu plasmid used in shRNA-mediated gene silencing against codon-optimized Vpu mRNA, the target sequence was chosen using Clontech RNAi Designer (sense: GGAGCATTGTGATTATTGA). The target sequence was synthesized and inserted into the pSingle-tTS-shRNA vector (Clontech).

Vpu and BST-2 anti-serum were obtained from the National Institutes of Health (NIH) AIDS Research & Reference Reagent Program. Luciferase antibody, β-actin antibody, HRP (horseradish peroxidase)-labeled donkey anti-rabbit and goat anti-rabbit IgG-FITC secondary antibodies were obtained from Santa Cruz Co. Concanamycin A, MG132 and doxycyline were purchased from Sigma (St. Louis, MO).

2.2. Cells and transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium with the addition of 10% fetal bovine serum (FBS) (Hyclone). SupT1 cells were maintained in RPMI-1640 containing 10% FBS. HeLa cells were transfected using Fugene HD transfection reagents (Roche), according to Manufacturer's instructions.

2.3. Establishing a stable transfected cell line expressing Vpu

HeLa cells were transfected with pVphu vectors, and then were selected with 400 μg/ml G418 24 h post-transfection. Using limited dilution method, several single cell colonies were obtained and then analyzed by the cell-ELISA. One clonal cell line has significantly reduced surface BST-2, which can be restored by treatment of Concanamycin A. This cell line is named HeLa-Vpu and was used for subsequent experiments.

2.4. Single-cycle HIV-1 replication assays

2×10^5 HeLa or HeLa-Vpu cells were cotransfected with 0.6 μg of pNL-Luc-E$^-$ or pNL-Luc-E$^-$-Vpu$^-$ and 0.4 μg of pHIT/G. After 48 h, the viral supernatant was harvested by filtration through a 0.45 μm filter and the concentration of viral capsid protein was determined by p24 antigen capture ELISA (Biomerieux). To normalize transfection efficiency, firefly luciferase activities of transfected cells were determined by using a firefly Luciferase Assay System (Promega). The resultant supernatant (10 μL) was also used to infect SupT1 cells (1×10^5) in 96-well plates. The SupT1 cells were lysed at 48 h post-infection and firefly luciferase activities were determined.

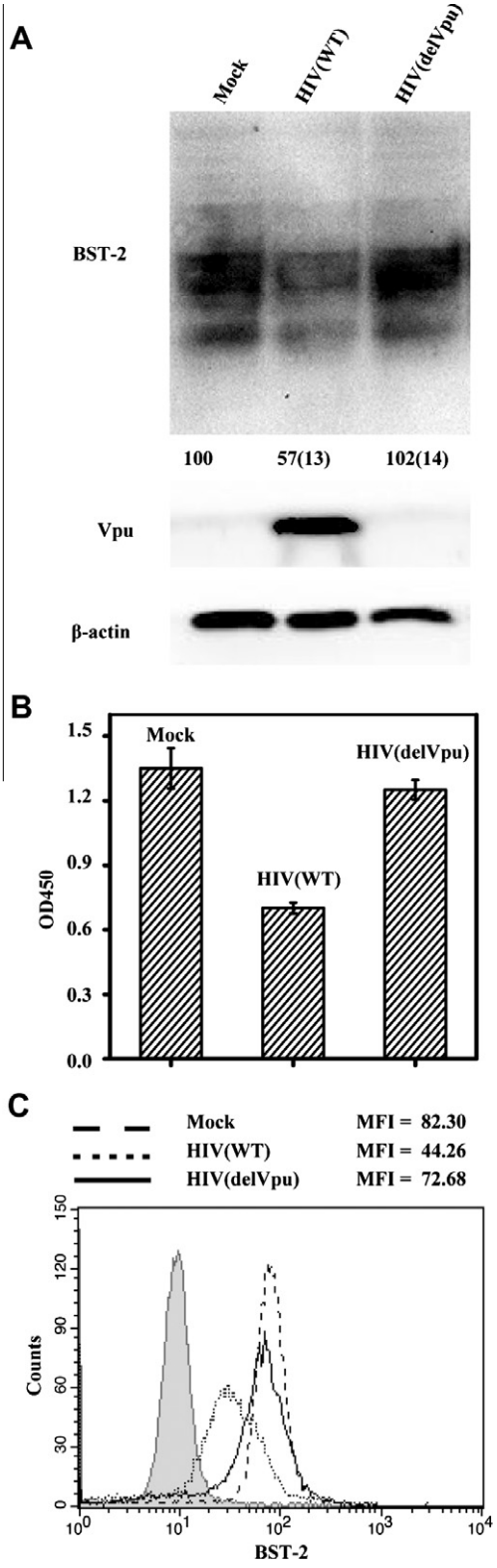

Fig. 1. Using a cell-ELISA assay to monitor Vpu-mediated down-regulation of cell surface BST-2. HeLa cells were infected with VSV-G–pseudotyped HIV-1(WT) or HIV-1(delVpu) virus at a MOI of 10, and then subjected to western blot, cell-ELISA and FACS analysis, respectively, at 48 h post-infection. (A) Western blots of cell lysates were probed with anti-BST-2 (top panel), anti-Vpu (middle panel) and anti-β-actin (bottom panel), respectively. The surface levels of BST-2 were determined using cell-EILSA (B) and FACS (C), respectively. In the panel (C), MFI values are shown beside the histogram. Dashed lines: Mock; doted lines: HIV-1(WT); full lines: HIV-1(delVpu); filled histogram: control stain with the secondary antibody only. The bar graphs represent the means of results of experiments performed at least three times, and the error bars represent standard deviations.

2.5. Cell-based ELISA

HeLa or HeLa-Vpu cells were plated into 96-well plates at 1×10^4 cells/well. After 48 h, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Then cells were washed with PBS and incubated with 50 μL BST-2 antiserum (1:5000) for 1 h at 37 °C. Cells were washed four times with PBS. Next, cells were incubated with 50 μL HRP-labeled donkey anti-rabbit second antibody (1:6000) for 0.5 h at 37 °C, and then washed four times with PBS. The cell-bound secondary antibodies were detected using 100 μL/well TMB substrate solution for 30 min at room temperature, and then the reactions were stopped by adding 50 μL/well 0.5 M H_2SO_4, followed by measuring absorption at 450 nm immediately.

2.6. Western blots

Cellular samples were suspended in loading buffer containing SDS and dithiothreitol and boiled for 10 min. Total proteins was separated on 12% polyacrylamide–SDS gels. Proteins were transferred onto nitrocellulose membranes and blotted with the antibodies to Vpu (1:1000), BST-2 (1:5000) or with antibody to β-actin (1:1000). The membranes were further incubated with either a HRP-labeled goat anti-mouse antibody or a HRP-labeled donkey anti-rabbit antibody, followed by detection with enhanced chemiluminescence.

2.7. Flow cytometry

Flow cytometry (FACS) analysis was performed as described previously (Rong et al., 2009). Briefly, Cells were trypsinized and resuspended in flow cytometry buffer ($1 \times$ PBS–3% fetal bovine serum), and then stained with hBST-2 antiserum (1:800) and a goat anti-rabbit IgG-FITC secondary antibody (1:200) on ice for 1 h, followed by fixation with 1% paraformaldehyde and analysis on a FACScalibur system.

2.8. Real-time RT-PCR

Total RNA was extracted from cells using TRIZOL Reagent (Invitrogen). RNA was converted to cDNA using M-MLV Reverse Transcriptase (Promega) with random primers. The cDNAs were quantified using SsoFast EvaGreen Supermix (Bio-Rad) and Bio-Rad iCycler iQ5 Real-Time PCR systems. Primer sequences for cDNAs were as follows: BST-2 (sense: CTGCAACCACACTGTGATG, antisense: ACGCGTCCTGAAGCTTATG) (Mangeat et al., 2009), GAPDH (sense: GTCCACTGGCGTCTTCACCA, antisense: GTGGCAGTGATGGCATGGAC) (Douglas et al., 2009). The GAPDH quantification was

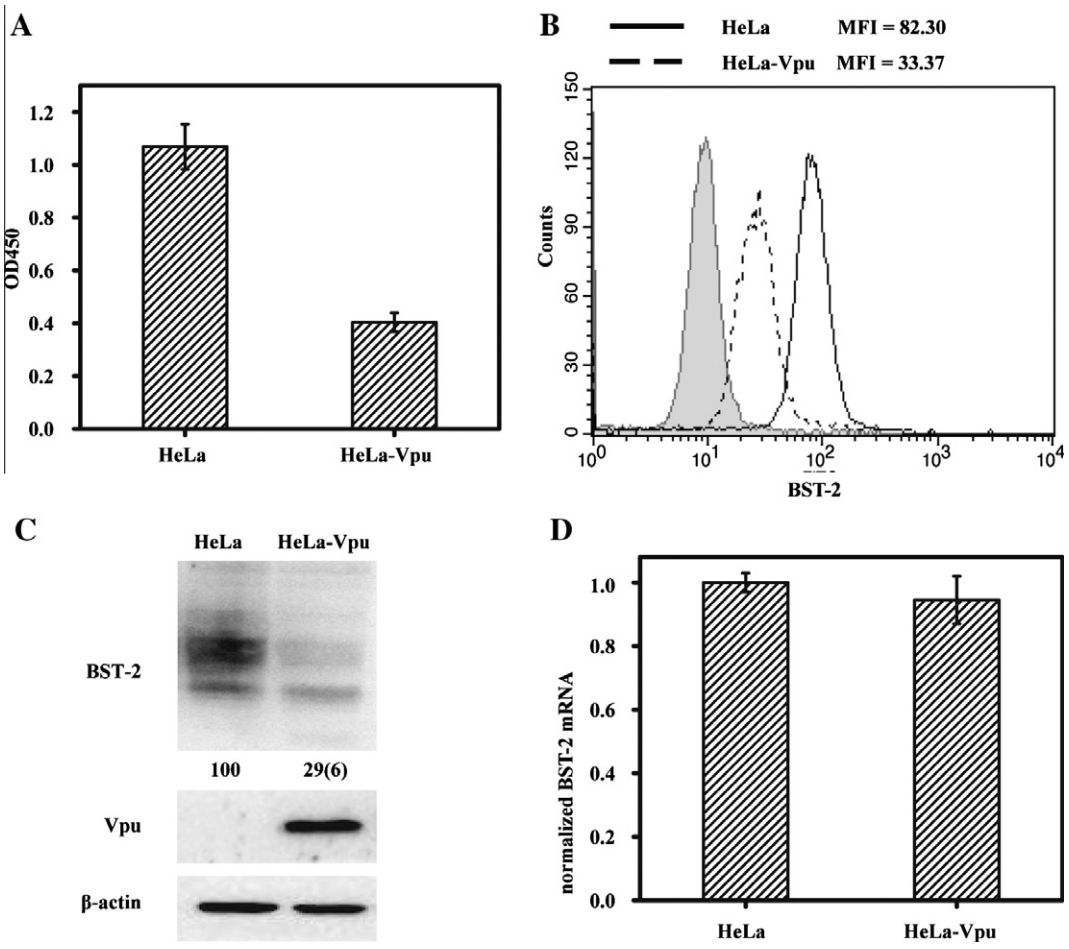

Fig. 2. Characterization of HeLa-Vpu cells, a HeLa cell line stably expressing Vpu. The surface levels of BST-2 in HeLa and HeLa-Vpu cells were determined using a cell-EILSA (A) and a FACS (B), respectively. In the panel (B), MFI values are shown beside the histogram. Dashed lines: HeLa-Vpu; full lines: HeLa; filled histogram: control stain with the secondary antibody only. (C) Western blots of cell lysates were probed with anti-BST-2 (top panel), anti-Vpu (middle panel) and anti-β-actin (bottom panel), respectively. (D) The relative abundance of BST-2 mRNA expressed in HeLa and HeLa-Vpu cells. The amount of BST-2 mRNA in HeLa and HeLa-Vpu cells were measured by real-time RT-PCR, and values presented here were the normalized BST-2 mRNA level of HeLa-Vpu relative to that of HeLa. The bar graphs represent the means of results of experiments performed at least three times, and the error bars represent standard deviations.

used to normalize the starting amount of total RNA for quantifying BST-2 mRNA.

3. Results

3.1. Measurement of BST-2 at cell surface using a cell-ELISA

Cell-ELISA is a rapid, convenient and quantitative technique for the detection of molecules expressed on the cell surface. This assay utilizes antibodies and enzyme-linked detection to perform quantitative immunocytochemistry on fixed cells grown in 96- or 384-well microplates (Liu et al., 2000; Sedgwick and Czerkinsky, 1992). In this work, we made an attempt at using this method to monitor Vpu-mediated down-regulation of cell surface BST-2. HeLa cells were first infected with VSV-G–pseudotyped HIV-1(WT) or HIV-1(delVpu) virus at a multiplicity of infection (MOI) of 10. Cell surface BST-2 was then assessed at 48 h post-infection using cell-ELISA and FACS, respectively. As expected, Vpu was only expressed in the cells infected with HIV-1(WT) but not with HIV-1(delVpu) accompanied by decreased expression of BST-2, since the latter virus misses the start codon of *vpu* gene (Fig. 1A). The results of cell-ELISA analysis showed that the infection with HIV-1(WT) reduced the surface level of BST-2 by approximate 1.8-fold, while no change was detected in HeLa cells that had been infected with HIV-1(delVpu) as compared with uninfected HeLa cells (Fig. 1B). A similar result was obtained in FACS analysis (Fig. 1C), which has been commonly used to quantify the cell surface BST-2. These data provide proof-of-concept evidence that the surface levels of BST-2 can be quantified by cell-ELISA.

3.2. Establishing a HeLa-derived cell line stably expressing Vpu

Vpu alone is sufficient to reduce the surface levels of BST-2 in the absence of other HIV-1 proteins (Douglas et al., 2009; Iwabu et al., 2009; Mitchell et al., 2009; Van Damme et al., 2008). It is therefore conceivable that with the cell-ELISA method, Vpu-expressing HeLa cells can be used to develop a high-throughput screening assay to identify small molecules that inhibit Vpu-mediated down-regulation of BST-2. To establish such an assay, we first transfected HeLa cells with a plasmid expressing codon-optimized Vpu, then selected for stably transfected cell lines with G418. The cell surface BST-2 of G418-resistant cell colonies was quantified by cell-ELISA. A single cell clone showed the greatest degree of BST-2 reduction (Fig. 2A), and the expression of Vpu in the cells was confirmed by western blot (Fig. 2C, middle panel). This cell line was named HeLa-Vpu. The control HeLa and the HeLa-Vpu cells showed a similar proliferation rate, suggesting that the stable expression of Vpu has no significant effect on the proliferation and viability of HeLa cells (result not shown). The cell surface level of BST-2 in HeLa-Vpu cells decreased approximate 3-fold more than control

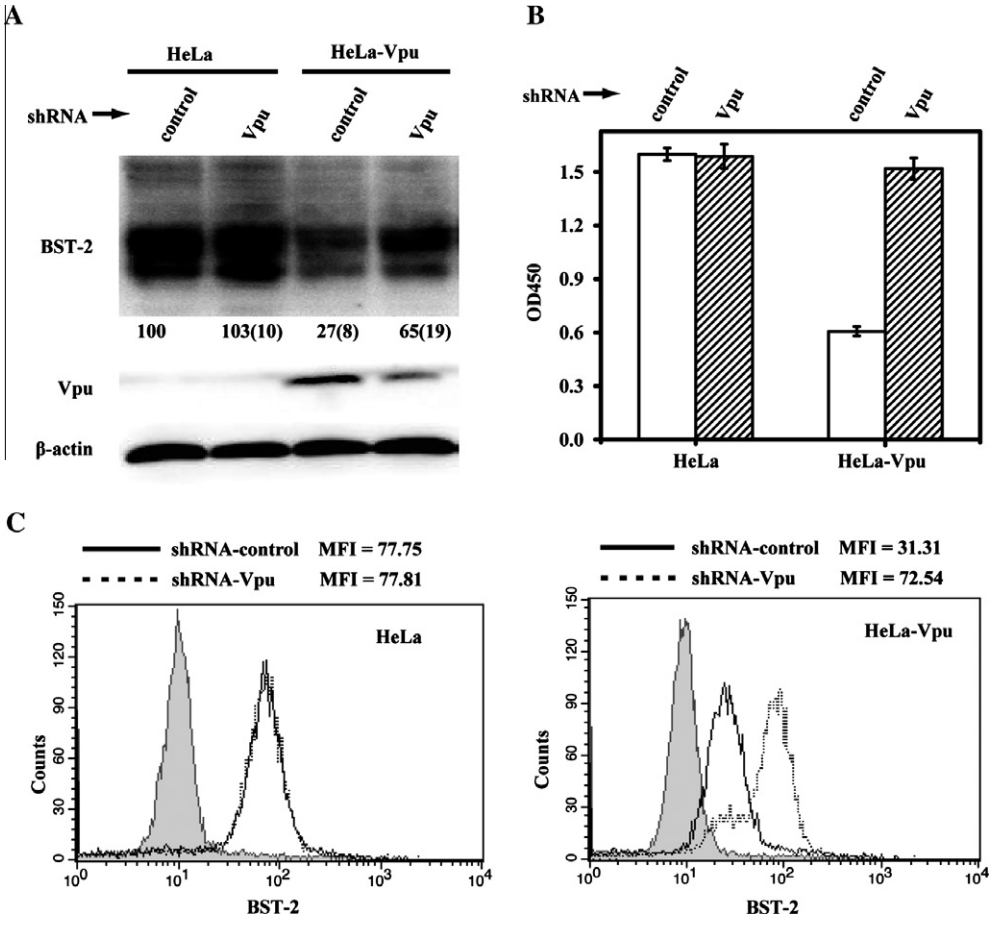

Fig. 3. Effect of silencing Vpu expression on the surface level of BST-2 in HeLa-Vpu cells. HeLa and HeLa-Vpu cells were transfected with either plasmid expressing shRNA-Vpu or control vector as indicated, and then were cultured in the presence of 1 μg/ml doxycyline for 48 h. (A) Western blots of cell lysates were probed with anti-BST-2 (top panel), anti-Vpu (middle panel) and anti-β-actin (bottom panel), respectively. The surface levels of BST-2 were determined using a cell-ELISA (B) and a FACS (C), respectively. In the panel (C), MFI values are shown beside the histogram. Full lines: shRNA-control; dotted lines: shRNA-Vpu; filled histogram: control stain with the secondary antibody only. The bar graphs represent the means of results of experiments performed at least three times, and the error bars represent standard deviations. The experimental procedure was described in Section 2.

HeLa cells measured by cell-ELISA (Fig. 2A) and FACS analysis (Fig. 2B). In addition, total cellular BST-2 was also significantly reduced, as shown by the results of western blot of cell lysates (Fig. 2C, top panel). The decreased amounts of total cellular and cell surface BST-2 in HeLa-Vpu cells did not result from a lower BST-2 expression independent of the presence of Vpu, since the result of qPCR analysis showed a similar amount of BST-2 mRNA in HeLa-Vpu and control HeLa cells (Fig. 2D).

3.3. Vpu-dependent down-regulation of BST-2 in HeLa-Vpu cells

Although the above result showed a significant reduction in cell surface BST-2 in HeLa-Vpu cells, it is still unclear whether the reduction resulted from the presence of Vpu, and whether the surface level and anti-HIV activity of BST-2 could be restored when the activity of Vpu is inhibited. The answers to these two questions are important for determining the potential use of the HeLa-Vpu

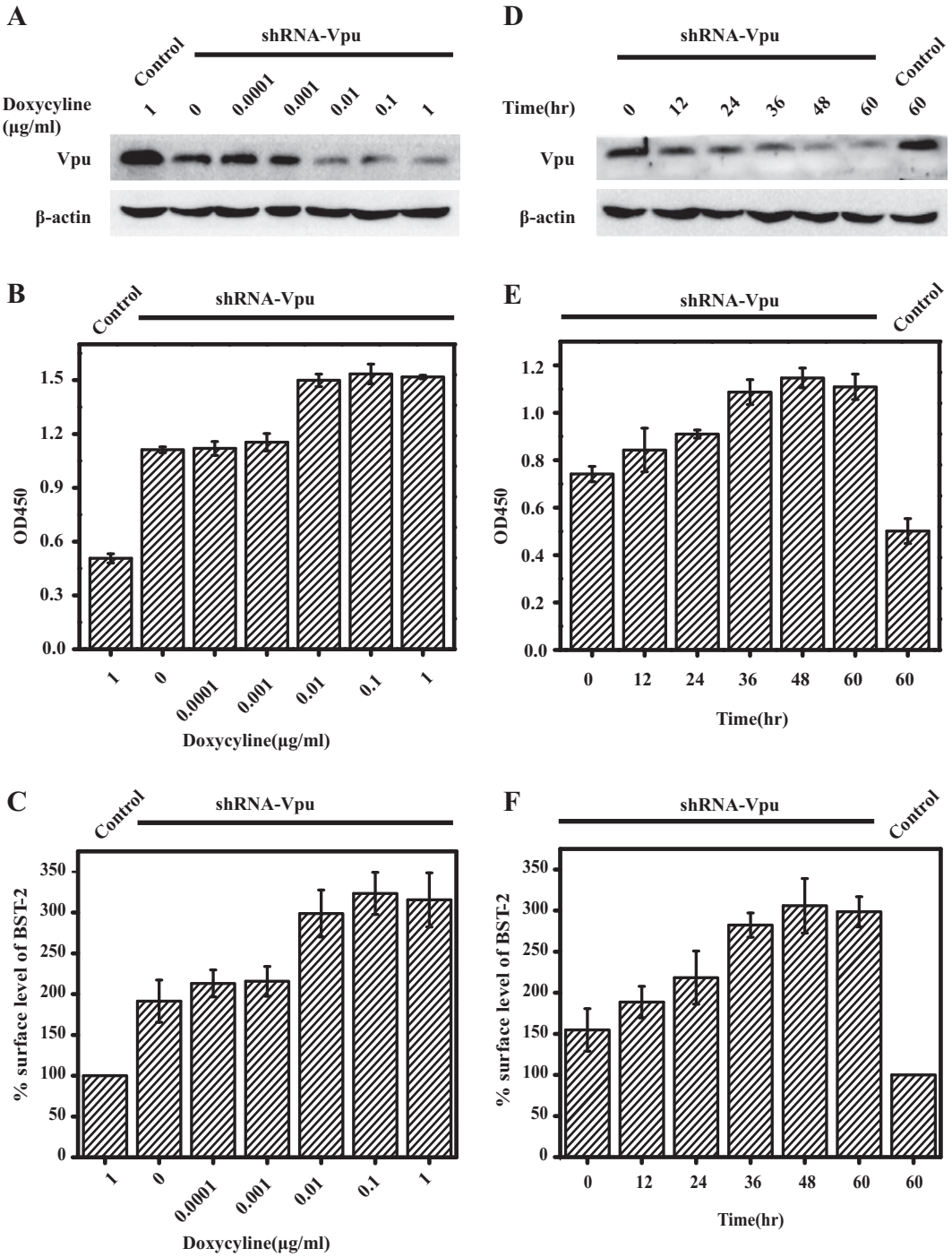

Fig. 4. The restoration of surface BST-2 in HeLa-Vpu cells quantitatively correlates with the depletion of Vpu. HeLa-Vpu cells expressing shRNA-Vpu were cultured either in the presence of doxycyline at various concentrations as indicated for 48 h (A–C) or in the presence of doxycyline (1 μg/ml) for various time periods as indicated (D–F). (A and D) Western blots of cell lysates were probed with anti-Vpu (upper panel) and anti-β-actin (lower panel), respectively. The surface levels of BST-2 were determined using cell-ELISA (B and E) and FACS (C and F), respectively. The bar graphs represent the means of results of experiments performed at least three times, and the error bars represent standard deviations. The experimental procedure was described in Section 2.

cells for the development a cell-ELISA assay to monitor the Vpu-mediated down-regulation of cell surface BST-2. To answer these questions, we first investigated whether the BST-2 level could be restored when Vpu is depleted by a Vpu-specific shRNA. The plasmid shRNA-Vpu was constructed to produce the Vpu-specific shRNA in the presence of doxycyline. This shRNA was designed to inhibit codon-optimized but not viral Vpu expression. HeLa and HeLa-Vpu cells were transfected with either the plasmid expressing shRNA-Vpu or a control vector, respectively, followed by cell-ELISA, FACS and western blot analysis. As shown in Fig. 3, when the expression of Vpu was reduced by shRNA-Vpu (Fig. 3A), cell-surface BST-2 in HeLa-cells was restored to a level comparable to that in control HeLa cells (Fig. 3B and C). In contrast, the expression of Vpu-specific shRNA had no effect upon cell-surface BST-2 of HeLa cells in the absence of Vpu (Fig. 3B and C).

The quantitative correlation between the surface level of BST-2 and the inactivation of Vpu was further investigated using shRNA-Vpu in either a dose response or a time course experiment. HeLa-Vpu cells expressing shRNA-Vpu were grown in the presence of doxycyline at various concentrations (0–1 μg/ml) for 48 h, which would induce the expression of the shRNA in a dose-dependent manner. The cell surface of BST-2 was then determined using cell-ELISA and FACS. As shown in Fig. 4, with the increasing addition of doxycyline, an increasing reduction in Vpu expression was detected (Fig. 4A), concomitant with increasing surface levels of BST-2 (Fig. 4B and C). The partial Vpu depletion was also observed without the addition of doxycyline, which probably resulted from a leaky expression of shRNA. We further determined the correlation between the restoration of cell surface BST-2 and the depletion of Vpu in the presence of doxycyline (1 μg/ml) for various time periods. As expected, the increase in surface level of BST-2 (Fig. 4E and F) paralleled the depletion of Vpu (Fig. 4D), and reached a maximum after 48 h treatment of doxycyline. Taken together, these data show a correlative response between the surface level of BST-2 and the expression of Vpu in HeLa-Vpu cells, and demonstrate the potential usefulness of the HeLa-Vpu cells for the development of cell-ELISA to monitor the Vpu-mediated down-regulation of cell surface BST-2. It is worthy of note that, in all these experiments, results of the cell-ELISA analysis perfectly match those of the FACS analysis, suggesting that the cell-ELISA analysis measures the cell surface level of BST-2 as well as FACS does.

The release of HIV-1 particles was inhibited in the absence of Vpu in HeLa cells. Reducing the cell surface level of BST-2 would increase the viral release and the viral infectivity (Douglas et al., 2009; Mitchell et al., 2009; Van Damme et al., 2008; Zhang and Liang, 2010). To determine the biological relevance of BST-2 down-regulation in HeLa-Vpu cells regarding the release of HIV-1, we assessed whether Vpu-defective HIV-1 particles were efficiently released in this cell line. HeLa and HeLa-Vpu cells were cotransfected with VSV-G expression vector pHIT/G and either pNL-Luc-E$^-$ or pNL-Luc-E$^-$-Vpu$^-$. Both pNL-Luc-E$^-$ and pNL-Luc-E$^-$-Vpu$^-$ produce viral membrane protein (Env) deficient HIV-1 containing a firefly luciferase gene as a reporter, and the latter one contains an addition mutation that abolishes Vpu expression. pHIT/G provides the VSV-G membrane protein for Env-deficient HIV-1 *in trans* to rescue a one-round infection of recombinant viruses. At 48 h post-transfection, the culture supernatant containing virus particles was harvested, and amounts of p24 in the supernatant were quantified to determine particle release efficiency using p24 ELISA as described in the Section 2. Furthermore, an equal amount of the supernatants was used to infect SupT1 cells. The activity of luciferase in infected cells was determined to monitor the viral infectivity. As shown in Fig. 5A and B, the particle release and the infectivity of Vpu-deficient virus produced from HeLa were reduced by approximately 3- and 9-fold, respectively, compared to that of wild type virus that expresses Vpu. In contrast, only a less than 30% difference was observed when both of the above viruses were produced from HeLa-Vpu. These data suggest that the decreasing surface level of BST-2 in HeLa-Vpu is capable to rescue the release and the infectivity of Vpu-deficient HIV-1. Moreover, expression of the shRNA-Vpu, which inhibited the

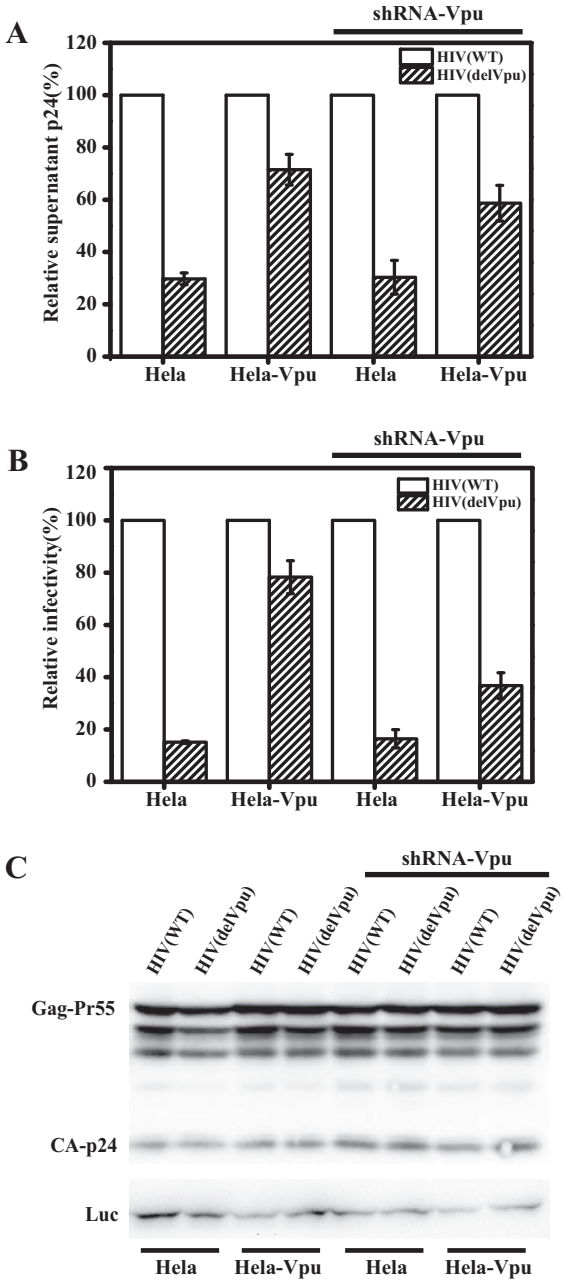

Fig. 5. The release of HIV-1 produced from HeLa and HeLa-Vpu in the presence or absence of shRNA-Vpu. HeLa and HeLa-Vpu cells, both of which were either left untreated or introduced with plasmid expressing shRNA-Vpu, were cotransfected with pNL-Luc-E$^-$ or pNL-Luc-E$^-$-Vpu$^-$ and pHIT/G, and the VSV-G–pseudotyped viruses were harvested by filtration of culture supernatant at 48 h post-transfection. (A) Quantification of virus release. Amounts of p24 in the supernatant were quantified to determine particle release efficiency using p24 ELISA as described in Section 2. (B) Relative infectivity of the resultant viruses. Values presented here (A and B) were the normalized amounts of p24 and viral infectivity of Vpu-deficient HIV-1 relative to that of HIV-1(WT) viruses produced from the same treated cells, respectively. The bar graphs represent the means of results of experiments performed at least three times, and the error bars represent standard deviations. (C) Western blots of lysates of transfected cells probed with anti-p24 (upper panel) and anti-luciferase (lower panel), respectively.

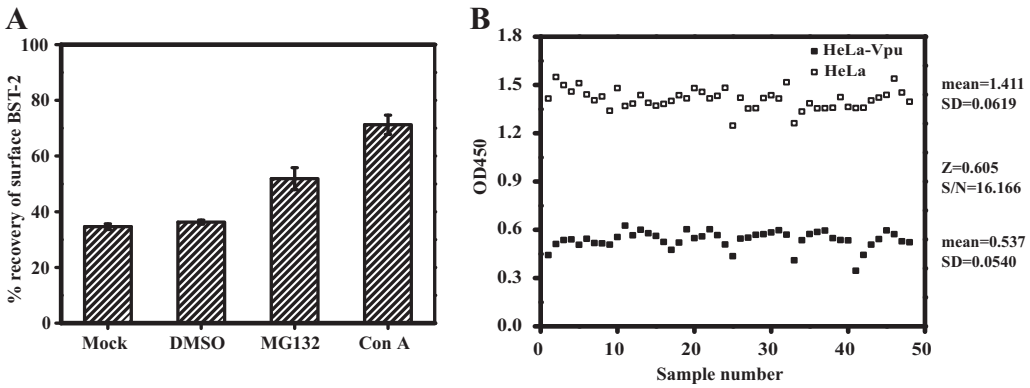

Fig. 6. Validation of the cell-ELISA assay. (A)The effect of lysosomal inhibitors on the surface levels of BST-2 in HeLa-Vpu cells. HeLa and HeLa-Vpu cells were seeded into 96-well mciroplates, and then treated with indicated compounds for 16 h, followed by measurement of the surface levels of BST-2 using cell-ELISA. The final concentrations of MG132 and Concanamycin A (ConA) were 20 μM and 50 nM. The surface levels of BST-2 in HeLa-Vpu cells were calculated as a percentage of that of HeLa cells under the treatment of same compound, and values were presented in the bar graph. The bar graphs represent the means of results of experiments performed at least three times, and the error bars represent standard deviations. (B) Determination of Z' factor and S/N for the cell-ELISA assay. The same number of HeLa or Vpu-HeLa cells were seeded into 96-well tissue culture plate and cultured for 24 h. Then the cells were treated by 0.5% DMSO for 24 h, followed by a cell-ELISA analysis. The Z-factor value and S/N were calculated in the equations: $Z = 1 - (3 \times \text{SD of HeLa-Vpu} + 3 \times \text{SD of HeLa})/(\text{mean of HeLa} - \text{mean of HeLa-Vpu})$; S/N = (mean of HeLa − mean of HeLa-Vpu)/SD of Hela-Vpu.

expression of codon-optimized Vpu (Figs. 3 and 4) but not viral Vpu, significantly improved the restriction of Vpu-deficient HIV-1 in HeLa-Vpu cells, providing further evidence for the biological relevance of BST-2 down-regulation in HeLa-Vpu cells. In contrast to shRNA-Vpu, transfection of HeLa and HeLa-Vpu with an empty shRNA vector had no effect on viral release and infectivity (results not shown), which is consistent with the results shown in Fig. 3. Fig. 5C showed similar expression of viral protein Gag-Pr55 and CA-p24, demonstrating that the changes in viral infectivity and particle release did not result from different expression levels of viral proteins.

3.4. Validation of the screening assay

The above results suggest the potential use of the HeLa-Vpu cells for the development of a cell-ELISA to monitor the Vpu-mediated down-regulation of cell surface BST-2. To realize this goal, we adapted the HeLa-Vpu cell line to a high-throughput screening assay as described in Section 2. It has been reported that BST-2 is degraded by Vpu through a lysosomal degradation pathway in HeLa cells, and that the inhibitors of the endosome–lysosome pathway, e.g. Concanamycin A and bafilomycin A1, are able to restore cell surface level of BST-2 (Douglas et al., 2009; Mitchell et al., 2009). Only prolonged treatment with proteasomal inhibitor MG-132 can moderately up-regulate the surface level of BST-2 in the presence of Vpu, which may result from depletion of the cellular pool of ubiquitin (Mitchell et al., 2009). Based on these previous studies, we first tested whether these known lysosomal inhibitors or proteasomal inhibitor could restore the surface levels of BST-2 in HeLa-Vpu cells and act as positive hits so as to validate the screening assay. 1×10^4 of HeLa or HeLa-Vpu cells were seeded into 96-well plates. The lysosomal inhibitors or proteasomal inhibitor were added into the culture medium and the cells were treated for 16 h, following which the cell surface level of BST-2 was analyzed by cell-ELISA. Compared with the treatment of DMSO, Concanamycin A resulted in a twofold increase in the cell surface BST-2 in HeLa-Vpu cells, i.e., approximately from 35% to 70%, and the proteasomal inhibitor MG132 also showed a moderate effect (Fig. 6A). This observation was consistent with previous results obtained from FACS analysis and immunofluorescence (Mitchell et al., 2009).

The Z-factor is widely utilized to evaluate the quality of high-throughput screening assays. It reflects both the assay signal dynamic range and the data variation associated with the signal measurements (Zhang et al., 1999). To ensure the quality of the assay, it is also recommended that one of traditional assay performance measures (APMs), e.g. signal-to-noise ratio(S/N), should be used along with Z-factor (Iversen et al., 2006). Here we use these two parameters to evaluate the quality of the high-throughput screening assay we established here. As shown in Fig. 6B, the Z-factor is 0.605 and S/N is 16.166 for the assay, both of which meet acceptance criteria for a high throughput assay.

4. Discussion

In this work, we reported for the first time a high-throughput assay to identify molecules that inhibit HIV-1 Vpu-mediated down-regulation of BST-2 from the cell surface. It has been shown that Vpu-mediated reduction of BST-2 at cell surface, not its total cellular content, represents the major mechanism of Vpu antagonism (Dubé et al., 2010; Goffinet et al., 2010; Mangeat et al., 2009). Thus monitoring the up-regulation of cell surface rather than total BST-2 in the presence of Vpu was more relevant for the identification of Vpu antagonist. Although FACS is a sensitive and quantitative method commonly used to quantify the cell surface BST-2 (Tokarev et al., 2009), several disadvantages of the assay, e.g. a large amount of sample (cells) needed, limit its application in high throughput drug screening. We herein quantitatively monitored the surface levels of BST-2 by an immunoenzymatic technique cell-ELISA, which proved to be simple, rapid, robust and inexpensive method. Moreover, our data showed that the results of the cell-ELISA analysis perfectly correlate with that of the FACS analysis, suggesting that the cell-ELISA analysis can quantify the cell surface level of BST-2 as well as FACS does.

In the HeLa-Vpu cells, the cell surface level of BST-2 was reduced by approximately 3-fold compared with the control HeLa cells, whereas several reports showed a more significant decrease (10-fold or greater) of BST-2 in Vpu over-expressing HeLa cells (Mitchell et al., 2009; Van Damme et al., 2008). The different magnitude of reduction may be due to the difference in either methodologies used or expression level of Vpu, i.e. transient transfection of Vpu-expression vector generally produces a high cellular level of Vpu compared with a Vpu stably expressing cell line. Nevertheless, characterization of the cell-ELISA assay revealed a Z' factor of 0.605 and S/N of 16.166, indicating that this assay is suitable for a high-throughput screening. Furthermore, we performed a series of

experiments to validate the cell-ELISA assay. Our data showed that the BST-2 reduction in HeLa-Vpu cells was the result of Vpu expression, and that either the depletion of Vpu by interfering RNA or the inhibition of lysosomal degradation by Concanamycin A was able to restore the surface level of BST-2 in HeLa-Vpu cells. We also demonstrated that HeLa-Vpu cells lost the ability to inhibit the release of HIV-Vpu$^-$ virus, and silencing the expression of Vpu restored restriction of HIV-Vpu$^-$ virus. These results collectively demonstrate that the cell-ELISA assay based on HeLa-Vpu cells can be used to identify molecules that inhibit HIV-1 Vpu-mediated down-regulation of BST-2 from the cell surface. To demonstrate proof-of-principle of a similar assay in physiologically relevant HIV-1 target cells, we also performed a modified assay using BST-2 inducible SupT1 cell lines (Rong et al., 2009), as in HeLa cells, we found approximately 4-fold reduction in surface BST-2 (data not shown). However, the complicated experiment procedure (e.g. multi-step centrifugation) limits the use of suspension T-cell line in the ELISA-based high-throughput screening.

Recent findings reveal that that enhancement of virus release by Vpu does not require cell surface down-modulation or intracellular depletion of BST-2 in CEMx174 and H9 cells (Miyagi et al., 2009). Although there is still a debate over the antiviral mechanism of BST-2 in various cell types, it is generally accepted that BST-2-mediated restriction is related to its localization at cell surface in HeLa cells (Dubé et al., 2010; Mitchell et al., 2009; Van Damme et al., 2008). More important, several line of evidence indicate that restoration of cell surface BST-2 in HeLa serves as an indicator of inactivation of Vpu's function (Dubé et al., 2010; Mitchell et al., 2009; Van Damme et al., 2008), which is also the principle of the cell-ELISA assay and has been further validated here. It is conceivable that hits able to inhibit action of Vpu would restore antiviral activity of BST-2 in CEMx174 and H9 cells, while cell surface BST-2 may not be affected.

The advantage of a cell-based ELISA described in this study is that both of Vpu and BST-2, two transmembrane proteins, are embedded in their natural surroundings and HeLa cells constitutively express high levels of endogenous BST-2, which is modified different from exogenously expressed BST-2 (Andrew et al., 2009). This prevents altered conformation and difficulties dealing with membrane-associated protein in an in vitro assay, and also limits possibility of false or biologically irrelevant positive results obtained from an artificial system. It is worthy of note that several important host machineries are also involved in the Vpu-mediated BST-2 down-regulation, such as the trans-Golgi network, the β-TrCP mediated ubiquitination and lysosomal degradation (Douglas et al., 2009; Dubé et al., 2009; Goffinet et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009). Considering the crucial functions of these pathways in regulating protein trafficking and removing mis-folded proteins, general disruption of their normal function may lead to restore the surface level and antiviral activity of BST-2, but may also cause numerous diseases, including neurodegenerative disorders and tumor. To eliminate compounds that might generally inhibit these crucial machineries, a counter-screen should be used to examine the effect of compounds on the physiological functions described above, e.g., a "real hit" should not inhibit the internalization and β-TrCP-dependent lysosomal degradation of IFNAR1 (interferon-alpha receptor1) induced by Interferon (Kumar et al., 2003), so as to identify compounds that selectively inhibit Vpu-mediated BST-2 down-regulation without generally interrupting important cellular functions.

In addition to HIV-1, BST-2 also inhibits retroviruses and filoviruses, e.g. Ebola, Marburg and Lassa (Jouvenet et al., 2009; Sakuma et al., 2009). These viruses have evolved to escape from this innate immunity through species-specific measures other than Vpu (Gupta et al., 2009; Jia et al., 2009; Kaletsky et al., 2009; Le Tortorec and Neil, 2009; Lopez et al., 2010; Mansouri et al., 2009; Pardieu et al., 2010; Zhang et al., 2009). The discovery of compounds that inhibit Vpu-mediated down-regulation of BST-2 using the assay established herein may not only lead to the discovery of novel therapeutics for HIV-1 infection, but also provide a proof-of-concept of implementing the same strategy to develop new drugs against other viruses that are sensitive to BST-2.

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