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A mammalian two-hybrid system-based assay for small-molecular HIV fusion inhibitors targeting gp41

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ABSTRACT

gp41 is a major component of the envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) responsible for fusion of the viral envelope with the target cellular membrane. The formation of the trimer-of-hairpins core structure of gp41, via the interaction between its N-terminal heptad repeat (NHR) and its C-terminal heptad repeat (CHR) plays a key role in the membrane fusion process. Hence, inhibitors of trimer-of-hairpins formation have become a promising new class of HIV therapeutics. In the present study, based on the mammalian two-hybrid system, we developed a cell-based assay for detecting small-molecular HIV-1 fusion inhibitors targeting gp41. The optimized assay can be adapted to high-throughput screening in 96- and 384-well microplates with high signal-to-background ratios and acceptable *Z′* factors. The known small-molecular gp41 inhibitors, ADS-J1, XTT formazan and tannin acid, tested positive in this assay, with half-maximal inhibitory concentration (IC_{50}) values of 4.9 μM, 5.6 μM and 0.8 μM, respectively. These data suggested that this novel assay is robust, sensitive and specific for identifying small-molecular HIV-1 gp41 inhibitors.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) is the major protein component on the viral surface and mediates the initial stages of viral entry (Chan et al., 1997; Eckert and Kim, 2001). Env consists of two glycoproteins: gp120, which interacts with cellular receptors to promote attachment of the virus to target cells, and gp41, which mediates fusion of the viral and cellular membranes. gp41 consists of a cytoplasm (CP), transmembrane (TM), and extracellular domain. The extracellular domain (ectodomain) contains four major functional regions: fusion peptide (FP), N-terminal heptad repeat (NHR or HR1), C-terminal heptad repeat (CHR or HR2), and a tryptophan-rich (TR) region. Following gp120 binding to CD4 and a coreceptor (CXCR4, CCR5) of the target cell, gp41 changes its conformation to a prefusion (intermediate) state by inserting its fusion peptide into the target cell membrane, and three NHR helices form the central trimeric coil configuration with three highly conserved hydrophobic grooves on the surface. Three CHR helices are then inserted into the hydrophobic groove of the NHR trimeric coil via their interaction with NHR, which forms a fusion-active gp41 core structure termed the trimer-of-hairpins (also termed the six-helix bundle, 6-HB). The trimer-of-hairpins brings the viral and target cell membranes into close proximity, which results in fusion between the HIV-1 and target cell membranes (Fig. 1). Any peptides or small molecular compounds that are able to disrupt the formation of the trimer-of-hairpins would potentially block HIV-1 entry into target cells (Cooley and Lewin, 2003; Huang et al., 2003; Chantry, 2004). Thus, gp41 has become an attractive target for the development of HIV-1 fusion inhibitors (Cooley and Lewin, 2003; Huang et al., 2003; Chantry, 2004; Root and Steger, 2004; Krambovitis et al., 2005; Esté and Telenti, 2007). Over the past decade, several peptides derived from the NHR and CHR regions of gp41, designated N- and C-peptides, respectively, were demonstrated to possess viral fusion inhibition activity (Bewley et al., 2002; Steffen and Pöhlmann, 2010). Among them, Fuzeon (also known as T-20 or enfuvirtide), one of the C-peptides derived from the CHR region of gp41, became the first member of a new class of anti-

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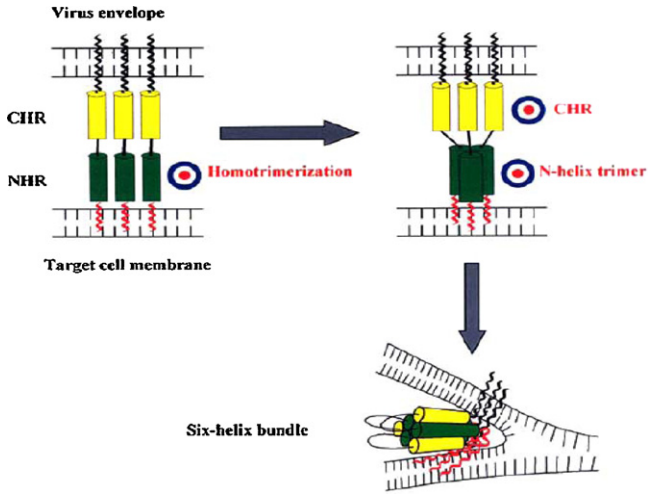

Fig. 1. The mechanism of gp41-mediated membrane fusion between HIV and target cells. HIV gp41 inserts its fusion peptide into the target cell membrane, and the gp41 N-helix trimer is formed by three molecules of NHR through homotrimerization. Three CHR domains are then packed into hydrophobic grooves with the NHR domain, through hydrophobic amino acid residues located in the NHR groove, to form the trimer-of-hairpins (also termed six-helix bundle, 6-HB) structure, which brings the viral and target cell membranes into close proximity and results in fusion between the HIV-1 and target cell membranes.
Figure adapted from Jiang et al., 1999.

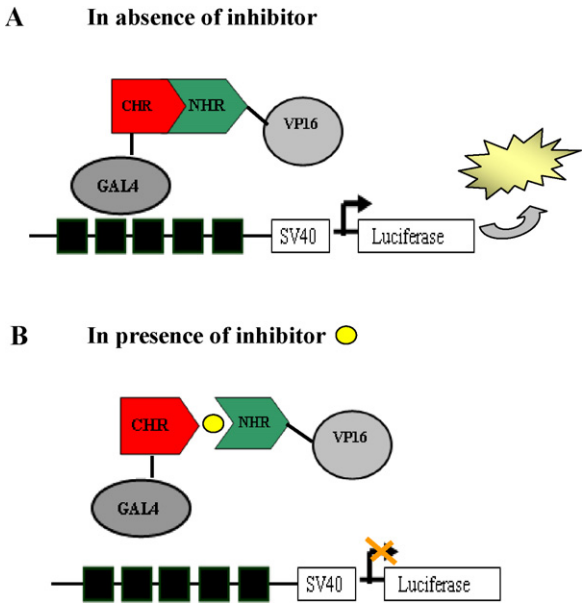

Fig. 2. The principle of the mammalian two-hybrid system-based assay for screening HIV-1 fusion inhibitors targeting gp41. (A) In the absence of inhibitors, the interaction between NHR and CHR of gp41 promotes transcription of the luciferase reporter gene. (B) In the presence of inhibitors, the interaction is interrupted, which results in the reduction of luciferase expression. The potential of a compound to inhibit the interaction between NHR and CHR can be evaluated by the reduction in luciferase activity.

HIV drugs known as HIV fusion inhibitors, and was approved by the FDA in 2003 (Fung and Guo, 2004). However, peptide-based drugs have several limitations, including low oral bioavailability, a short half-life, and a high cost of manufacturing. Given these limitations, the discovery of small molecular HIV-1 fusion inhibitors became a promising alternative therapeutic option for the treatment of HIV/acquired immune deficiency syndrome (AIDS) (Debnath, 2006; Liu et al., 2007). To date, a number of small molecular fusion inhibitors have been discovered (Krambovitis et al., 2005; Debnath, 2006; Esté and Telenti, 2007; Liu et al., 2007). One successful example is Maraviroc, a compound preventing HIV-1 from entering and infecting immune cells by blocking cell-surface receptor CCR5 (Lieberman-Blum et al., 2008). Maraviroc was approved as an anti-HIV drug by the FDA in 2006. The success of Maraviroc demonstrates that small-molecular fusion inhibitors are a promising strategy for the development of new anti-HIV therapeutic drugs.

The mammalian two-hybrid system has been widely accepted as a powerful *in vivo* method for detecting protein–protein interactions (Luo et al., 1997). Utilizing the mammalian two-hybrid system, we developed a novel cell-based high-throughput screening (HTS) assay for detecting small-molecular HIV fusion inhibitors targeting the formation of trimer-of-hairpins of gp41. The principle of the assay is shown in Fig. 2. Briefly, the NHR and CHR of HIV gp41 are fused with the yeast GAL4 DNA-binding domain and herpes simplex virus VP16 transcriptional activation domain of the mammalian two-hybrid system expression plasmids, respectively. As the two chimeric expression plasmids are cotransfected into a mammalian cell with a reporter plasmid containing GAL4 response element, the interaction between NHR and CHR of gp41 would promote the transcription of the luciferase reporter gene. In the presence of inhibitors, this interaction would be interrupted, resulting in a reduction in luciferase expression. Hence, the potential of a compound to inhibit the interaction between NHR and CHR can be validated by quantitatively detecting luciferase activity. Here, we present the design, development and validation of this novel assay.

2. Materials and methods

2.1. Materials and reagents

T4 DNA ligase, restriction enzymes, Taq polymerase, pGEM-T Easy vector, and the CheckMate™ Mammalian Two-Hybrid System were purchased from Promega (Madison, WI). RPMI Medium 1640 was purchased from GIBCO BRL (Grand Island, NY). Lipofectamine™ 2000 transfection reagent was purchased from Invitrogen Corporation (Carlsbad, CA). 96- and 384-well plates were obtained from Costar (Corning, New York). ADS-J1 was kindly provided by Professor Liu Shuwen (College of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China). XTT was purchased from Sigma–Aldrich Corporation (St. Louis, MO), and the XTT formazan was obtained as previously described (Zhao et al., 2002). Heparin, fucoidans and hyaluronan are obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Escherichia coli* polysaccharides are purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

The Chinese-hamster-ovary cell line (CHO-K1) was purchased from the American Type Culture Collection (ATCC) and cultured and passaged in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (streptomycin/penicillin) in a humidified incubator, with 5% CO_2 and a temperature of 37 °C.

2.3. Construction of plasmids

The expression and reporter plasmids were constructed according to the scheme shown in Fig. 3. Plasmid pBIND encodes the yeast GAL4 DNA binding domain, while pACT encodes the herpes simplex virus VP16 transactivating domain. Genes of N-terminal heptad repeat (NHR), and C-terminal heptad repeat (CHR) of HIV-1 gp41 were kindly provided by Dr. Hai-qing Duan (Academy

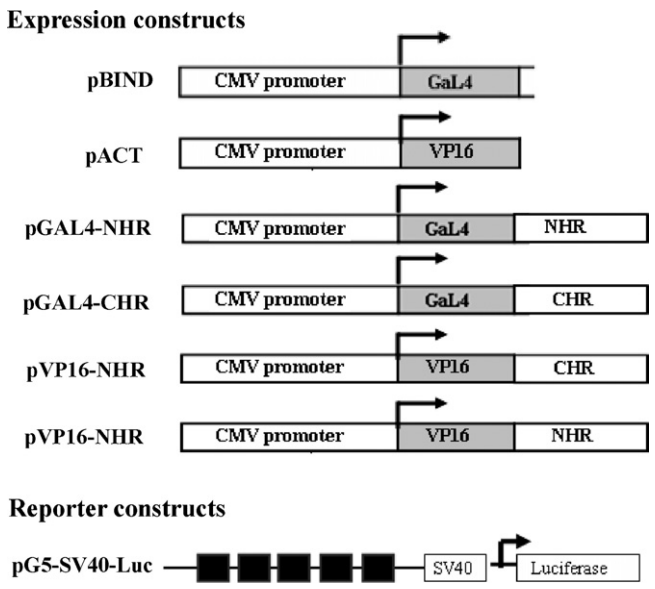

Fig. 3. The scheme of the construction of expression and reporter plasmids. Plasmid pBIND encodes the yeast GAL4 DNA binding domain, and pACT encodes the herpes simplex virus VP16 transactivating domain. Genes NHR and CHR of HIV-1 gp41 are fused to GAL4 or VP16 of the plasmid pBIND and pACT to construct chimeric fusion expression plasmids pGAL4-NHR, pGAL4-CHR, pVP16-NHR, and pVP16-CHR. The reporter plasmid pG5-SV40-Luc (firefly luciferase) was constructed by inserting five copies of the GAL4 response element (RE) (CGGAGTACTGTCCT) upstream of the SV40 promoter in the pGL3-promoter plasmid.

of Military Medical Sciences, Beijing, China) and fused to the GAL4 or VP16 domains of the expression plasmids pBIND and pACT at the *Bam*HI–*Kpn*I site to construct fusion expression vectors pGAL4-NHR, pGAL4-CHR, pVP16-NHR, and pVP16-CHR. The reporter plasmid pG5-SV40-Luc (firefly luciferase) was constructed by inserting five copies of the GAL4 response element (RE) (CGGAGTACTGTCCT) upstream of the SV40 promoter of the pGL3-promoter Vector (Promega) (Zheng et al., 2010). The integrity of all constructs was verified by DNA sequencing and restriction enzyme analysis.

2.4. Transfection and luciferase assay

CHO-K1 cells were seeded in 96- or 384-well plates one day before transfection. Reporter plasmid pG5-SV40-Luc was transiently cotransfected with both of the expression plasmids (pVP16-NHR and pGAL4-CHR, or pVP16-CHR and pGAL4-NHR), or with only one of these plasmids, into cells using Lipofectamine™ 2000 (Invitrogen), following the manufacturer's instructions. After incubation for the indicated number of hours, luciferase activity was measured using Luciferase Assay System kit (Promega Corp., Cat.# E1500, Madison, WI) on the microplate reader, Wallac 1420 Victor2 (PerkinElmer, Holland). Results were expressed as the firefly luciferase relative activity or in terms of the signal-to-background ratio (S/B). The S/B ratio was calculated using the following formula:

$$\frac{S}{B} = \frac{\text{mean max. signal}}{\text{mean min. signal}} \quad (1)$$

where the max. signal is the luciferase activity after cotransfection of the reporter plasmid pG5-SV40-Luc with both expression plasmids pVP16-NHR and pGAL4-CHR, and the min. signal is the luciferase activity after cotransfection of pG5-SV40-Luc with only one expression plasmid, either pVP16-NHR or pGAL4-CHR. Data were presented as the mean ± S.D. of triplicate measurements ($n = 3$).

2.5. HTS assay and performance evaluation

The natural product (from microorganism metabolites and herbal) library and the synthetic small molecule compound library (Drug Research & Development Center of North China Pharmaceutical Group Corporation, NCPC) were used for screening the gp41 inhibitors. CHO-K1 cells were seeded in 96- or 384-well plates one day before transfection. Reporter plasmid pG5-SV40-Luc was transiently cotransfected with both of the expression plasmids pVP16-NHR and pGAL4-CHR. Two hours after transfection, 1 μl of the test compound (final concentration of 10 μg/ml) or 1 μl of DMSO (0.5%) was added to the cell culture. The luciferase activity was detected after a 24 h-incubation. All liquid handling using 96- or 384-well microtiter plates was carried out using a MICROLAB® STAR Liquid Handling Workstation (HAMILTON, Reno, NV). During routine screening, the cotransfection of plasmid pG5-SV40-Luc with pACT-NHR or pGAL4-CHR in quadruplicate we set up on each 96-well plate as the background control for calculating S/B ratio and Z' factor. Z' factor is a statistical parameter indicative of the quality of an assay. Z' factor was calculated as described in Zhang et al. (1999). The inter-plate variability was calculated with the S/B values obtained from each screened plate, and the results were presented as the percentage coefficient of variation (% CV). Inhibition by the test compound was calculated using the following formula:

$$\text{inhibition}\,\% = \frac{\text{max. signal} - \text{sample signal}}{\text{max. signal} - \text{min. signal}} \times 100\% \quad (2)$$

where the max. signal is the luciferase activity after cotransfection of the reporter plasmid pG5-SV40-Luc with both expression plasmids pVP16-NHR and pGAL4-CHR, and the min. signal is the luciferase activity after cotransfection of pG5-SV40-Luc with the expression plasmid pVP16-NHR or pGAL4-CHR. The half-maximal inhibitory concentration (IC_{50}) of the test compound was determined from a constructed inhibition curve.

The primary hits were further confirmed by re-screening and false-positive analysis.

2.6. False-positive analysis

False-positive refers to any non-specific of inhibition to the assay that can be eliminated the inhibitory activity against the luciferase activity of transfection of pG5-SV40-Luc with expression plasmid pVP16-NHR or pGAL4-CHR. Briefly, CHO-K1 cells were seeded in 96- or 384-well plates one day before transfection. Reporter plasmid pG5-SV40-Luc was transiently transfected with expression plasmid pVP16-NHR or pGAL4-CHR, respectively, using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). After incubation with the test compound or DMSO (negative control) for 24 h, the luciferase activity was measured using Luciferase Assay System Kit (Cat.# E1500) (Promega Corp., Madison, WI). Inhibition was calculated using the following formula:

$$\text{inhibition}\,\% = \frac{\text{max. signal} - \text{sample signal}}{\text{max. signal}} \times 100\% \quad (3)$$

where the max. signal is the luciferase activity of the cells incubated with DMSO. The inhibition of over 15% was considered as false positive.

2.7. Cytotoxicity assay

The cytotoxicity of compounds is the main factor that leads to false positive of the assay, and therefore can be detected to reduce the false-positive rate prior to screening. After treatment with test compounds for 24 h, the cytotoxicity was measured by assaying the lactate dehydrogenase (LDH) in the cell culture supernatant with the LDH Cytotoxicity Detection Kit (Roche, Mannheim, Germany),

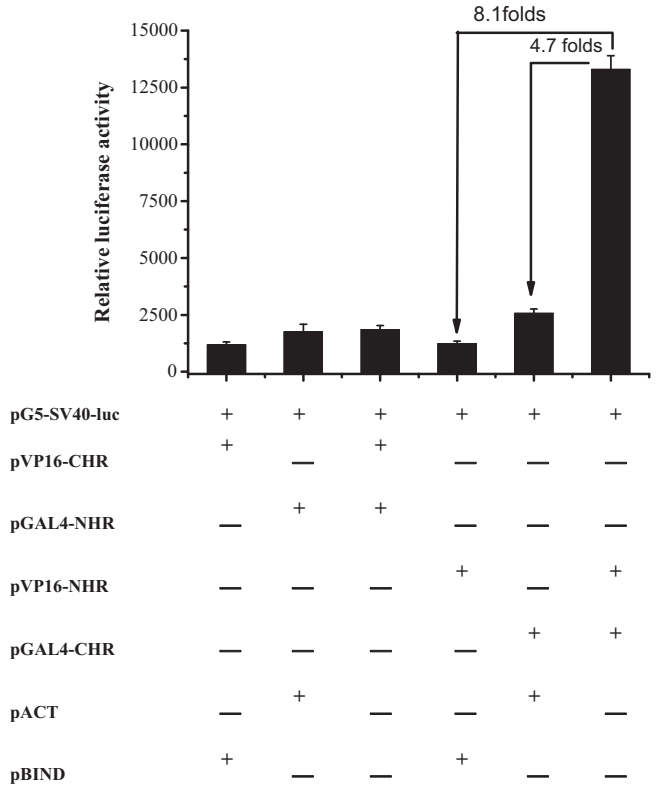

Fig. 4. Determination of the interaction between NHR and CHR in the mammalian two-hybrid-based assay. Reporter plasmid pG5-SV40-Luc was transiently cotransfected with both fusion expression plasmid pVP16-NHR and pGAL4-CHR, or with pVP16-CHR and pGAL4-NHR into CHO-K1 cells. pBIND and pACT were used as negative control plasmids. Twenty-four hours after transfection, the luciferase activity was measured. Results are expressed as the firefly luciferase relative activities (RLU) after normalization with the Renilla luciferase activity. Data are presented as the mean ± S.D., $n = 3$.

according to the manufacturer's instructions. Briefly, 20 μl medium from each well was transferred to a fresh plate and mixed with 100 μl the reagent and incubated at room temperature for 10 min. The absorbance was measured at 490 nm on the microplate reader, Wallac 1420 Victor2 (PerkinElmer, Holland). The medium from the cells incubated with 2% of Triton X100 (v/v) for 1 h was used as positive control.

3. Results and discussion

3.1. Determination of the interaction between NHR and CHR in the mammalian two-hybrid system

According to the scheme shown in Fig. 3A, the genes NHR and CHR of HIV-1 gp41 were inserted into pACT or pBIND to produce four chimeric fusion expression vectors: pVP16-NHR, pVP16-CHR, pGAL4-NHR, and pGAL4-CHR. The interaction between NHR and CHR was determined by cotransfection of the reporter gene vector pG5-SV40-GAL4 with pVP16-NHR and pGAL4-CHR, as well as with pGAL4-NHR and pVP16-CHR. The mass ratio of the three plasmids DNA was maintained at 0.12 μg/0.12 μg/0.12 μg per well. pBIND and pACT were used as negative control plasmids. CHO-K1 cells were seeded in 96-well plates at 1×10^4 cells/well one day before transfection. Twenty-four hours after transfection, the luciferase activities were measured. As shown in Fig. 4, the cotransfection of reporter plasmid pG5-SV40-luc with pVP16-NHR and pGAL4-CHR had about an 8.1- and 4.7-fold signal response over that of cotransfections with pVP16-NHR and pBIND, and with pGAL4-CHR and pACT, respectively. In contrast, no significant increase was observed in the cotransfection of pGAL4-NHR and pVP16-CHR. These results indicated that there was a strong interaction between fusion proteins GAL4-CHR and VP16-NHR, but no interaction between GAL4-NHR and VP16-CHR in this mammalian two-hybrid system. We hypothesized that the C-terminal domain of NHR might interrupt the binding of the GAL4 DNA binding domain to its response elements, or some conformational change of CHR induced by the fused VP16 protein might affect the interaction between the NHR and CHR domain of gp41. Hence, the following assay development experiments were performed with pGAL4-CHR and pVP16-NHR.

3.2. Optimization of the assay

To achieve a robust and sensitive signal, we further optimized the transfection conditions. First, we optimized the cell density by plating CHO-K1 cells in 96-well plates at various cell densities of 1×10^3, 2.5×10^3, 6.4×10^3, 1.6×10^4, 4×10^4, and 1×10^5 per well. As shown in Fig. 5A and B, 4×10^4 cells per well produced the highest luciferase activity and an S/B value of over 9.2.

To maximize the signal response, the DNA mass ratio of expression plasmid to reporter plasmid was further optimized. We maintained the ratio of pVP16-NHR to pGAL4-CHR at 1:1 and varied the amounts of reporter vector and expression vector to get different mass ratios of pVP16-NHR/pGAL4-CHR/pG5-SV40-luc: 1:1:20, 1:1:10, 1:1:3, 1:1:1, 3:3:1, and 10:10:1. The total amount of DNA was maintained at 0.36 μg per well. When the mass ratio of pVP16-NHR/pGAL4-CHR/pG5-SV40-luc was at 1:1:10 (0.03 μg/0.03 μg/0.3 μg), the cotransfection yielded the highest signal response (Fig. 5C and D) and an S/B value of 11.1.

Next, we varied the detection time point after transfection within the range of 8–32 h to determine the optimal incubation time. Fig. 5E and F shows that 24 h-incubation produced the highest luciferase activity and signal response. In addition, because all of the screened compounds were dissolved in DMSO, the highest tolerance of DMSO in this assay was also determined. Our results showed that when the concentration of DMSO was lower than 1%, it had no significant impact on the assay (Fig. 5I and J).

3.3. Validation of the assay

ADS-J1, a synthetic small molecular compound, was discovered as a gp41 inhibitor by a computer-aided molecular docking technique, and further was proven to be able to disrupt the formation of the N and C-peptide six-helix bundle and block HIV-1 Env-mediated membrane fusion *in vitro* at micromolar concentrations (IC_{50} values ranging from 4 to 30 μM) (Debnath et al., 1999; Naicker et al., 2004; Wang et al., 2009). XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] is a dye that is widely used for evaluating cell viability. Its reduced formazan product (Fig. 6B) has been proven to block the trimer-of-hairpins formation between NHR and CHR by binding to the conserved hydrophobic pocket on the surface of the gp41 NHR-trimer (Zhao et al., 2002).

Thus, we tested the two compounds by our assay. To obtain maximum inhibition, these two compounds were added at different time points from 2 h to 12 h after transfection. As shown in Fig. 6A and B, ADS-J1 and XTT formazan inhibited the luciferase activity in cotransfection of the reporter plasmid pG5-SV40-Luc with both expression plasmids pVP16-NHR and pGAL4-CHR in a dose-dependent manner. In comparison, ADS-J1 and XTT formazan added 2 h after transfection exhibited higher inhibitory activities with IC_{50} values of 4.9 μM and 5.6 μM, respectively. When the addition times were longer than 2 h, however, the inhibitory activities of both of these compounds decreased, which may be due to the increase in the interaction between HNR and CHR with time. Fig. 6C showed that ADS-J1 at the concentration of up to 70 μM had not

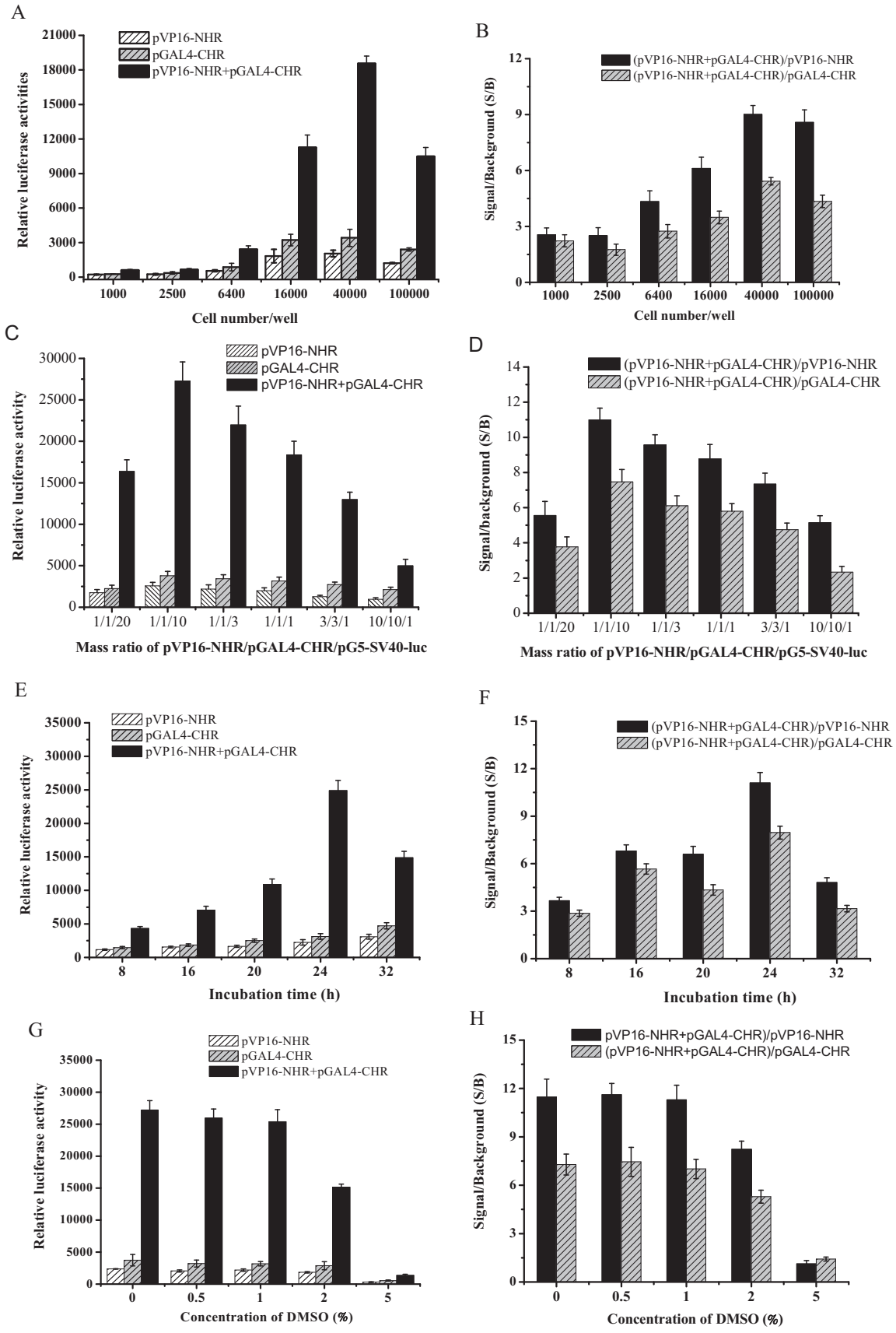

Fig. 5. Optimization of the assay. (A and B) Optimization of the per-well cell density plated in a 96-well plate; (C and D) optimization of the mass ratio of pVP16-NHR/pGAL4-CHR/pG5-SV40-GAL4 per well; (E and F) optimization of the incubation time after transfection; (G and H) determination of the tolerance of DMSO of the assay. Cotransfection of pG5-SV40-GAL4 with pVP16-NHR or pGAL4-CHR was used as a background control. Results are expressed as the firefly luciferase relative activities or in terms of the signal/background ratio (S/B) after normalization with Renilla luciferase activity. Data are presented as the mean ± S.D., $n = 3$.

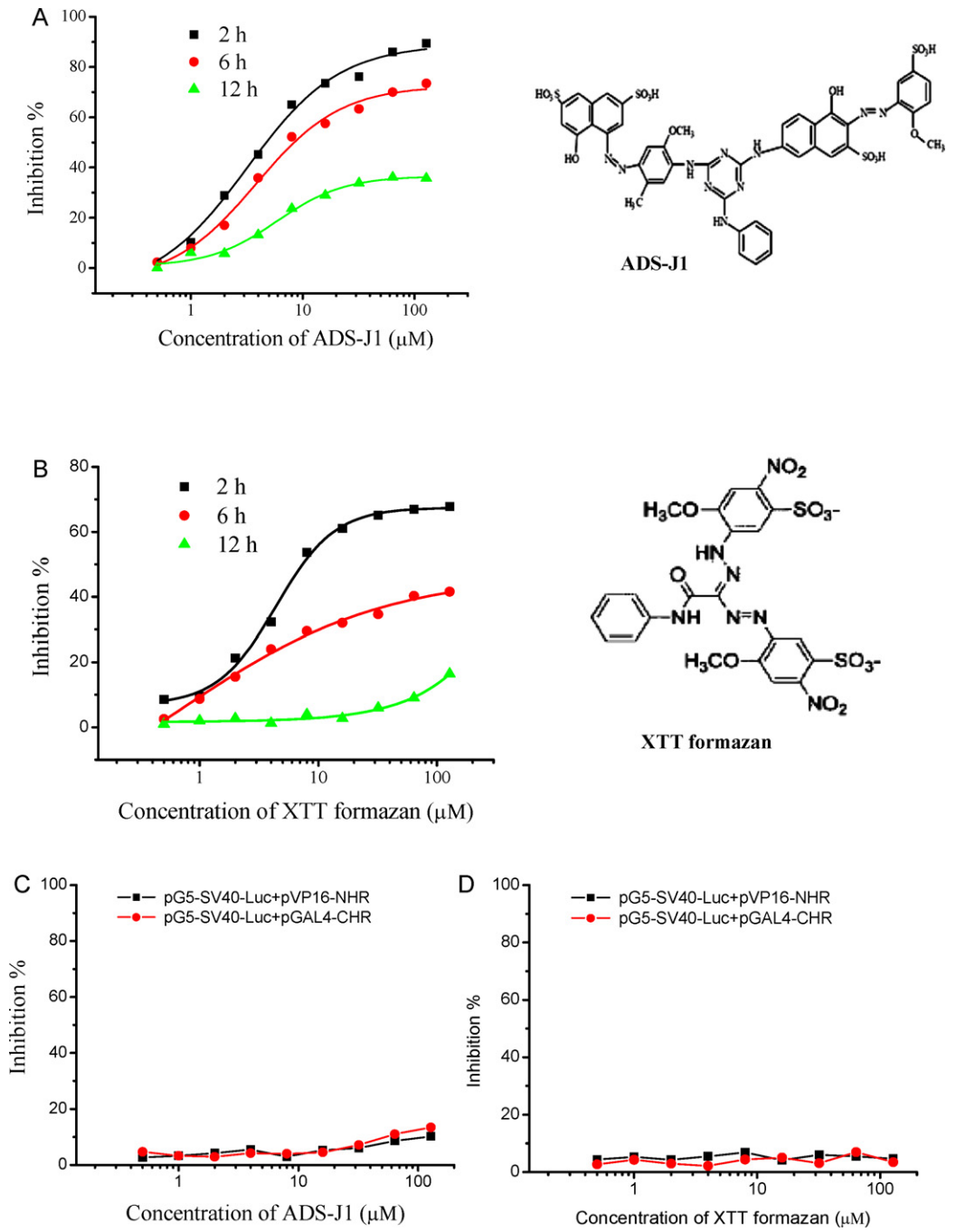

Fig. 6. Assay validation with known small molecular gp41 inhibitors. (A and B) The inhibitory activitiy of ADS-J1 and XTT formazan to the interaction between VP16-NHR and GAL4-CHR. CHO-K1 cells were seeded in 96-well plates at 1×10^4 cells/well one day before transfection. Then, pVP16-NHR, pGAL4-CHR, and pG5-SV40-luc were cotransfected into cells at a mass ratio of 0.03 μg:0.03 μg:0.3 μg per well. Serial dilutions of ADS-J1 and XTT formazan were added at different time points: 2 h (■), 6 h (●), and 12 h (▲) after transfection. Twenty-four hours after transfection, the luciferase activities were measured. (C and D) False-positive analysis of ADS-J1 and XTT formazan. The inhibitory activities of ADS-J1 and XTT formazan to the background of cotransfections of pG5-SV40-luc with only pVP16-NHR (■) or pGAL4-CHR (●), were evaluated as described above. All experiments were undertaken in triplicate and repeated at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant inhibition against the luciferase activity of cotransfection of pG5-SV40-Luc with the expression plasmid pVP16-NHR or pGAL4-CHR, and showed over 15% inhibitory activity only at higher concentration. Fig. 6D showed that no non-specific inhibitory activity was observed in XTT formazan. These data indicate that ADS-J1 and XTT could interrupt the interaction between gp41 NHR and CHR in the mammalian two-hybrid system.

However, several studies have been performed to present evidence that ADS-J1 inhibits HIV-1 entry by interacting with gp120 (Armand-Ugón et al., 2005; Manetti et al., 2006; González-Ortega et al., 2010). The mechanism of ADS-J1 action is controversial. At present, it is difficult to explain the discrepancy of these results. Maybe this problem is just like that of AR177, one of the most potent inhibitors of the HIV integrase. AR177-resistant strains mutated in HIV-1 gp120, and no mutations were found in the integrase enzyme (Esté et al., 1998). We thought that extensive studies on the mechanism of action of ADS-J1 are still needed, and the further insight into the mechanism of action of ADS-J1 will aid our understanding

Table 1
Inhibitory activity of sulfated, non-sulfated and glucuronic acid polysaccharides at a concentration of 10 μg/ml against the luciferase activity of various cotransfections.

Polysaccharide	Charged group	Cotransfection of pG5-SV40-Luc with expression plasmid		
		pGAL4-CHR and pACT-NHR	pGAL4-CHR	pACT-NHR,
Heparin	Sulfate (40%) + glycuronic acid (22%)	99.4%	86.3%	89.5%
Fucoidan-1	Sulfate (41%)	61.2%	49.5%	46.8%
Fucoidan-2	Sulfate (23%)	59.3%	42.4%	38.3%
Fucoidan-3	Sulfate (34%)	85.5%	67.8%	59.4%
Hyaluronan	Glycuronic acid (44%)	56.7%	45.6%	53.1%
Polysaccharide from *E. coli*	–	4.6%	3.4%	1.1%

of the roles of gp41 and gp120 and the molecular determinants of virus binding and virus fusion.

Since both of ADS-J1 and XTT are sulfonated compounds, to investigate whether sulfated polyanions that have been reported to inhibit HIV-1 entry (Baba et al., 1988, 1990; Batinić and Robey, 1992; Esté et al., 1997; Schols et al., 1990) have an inhibitory effect on gp41 NHR–CHR interaction in the assay and whether the sulfate group or other charged group could interfere with the assay, we next tested heparin (with sulfate groups and glucuronic acid residues), three fucoidans (with different sulfate contents), hyaluronan (with glucuronic acid residues) and *E. coli* polysaccharides (without sulfate group) by the assay at concentration of 10 μg/ml. As shown in Table 1, except for the *E. coli* polysaccharides, heparin, three fucoidans and hyaluronan showed significant inhibitory activity not only against the luciferase of cotransfection of pG5-SV40-Luc with both two expression plasmids pVP16-NHR and pGAL4-CHR, but also against that of cotransfection with only one the expression plasmid pVP16-NHR or pGAL4-CHR, indicating the inhibition by these polyanions is not due to the interruption of gp41 NHR–CHR interaction, but the sulfate group and glucuronic acid interfering the assay.

It has been reported that sulfated polysaccharides can interact with various proteins, such as HIV-1 gp120 glycoprotein (Baba et al., 1988, 1990; Batinić and Robey, 1992; Esté et al., 1997; Schols et al., 1990), HIV-1 tat protein (Hui et al., 2006; Urbinati et al., 2004; Watson et al., 1999), CD4 molecules of cell surface (Lederman et al., 1989; Meiyu et al., 2003), stromal cell-derived factor-1 (Fermas et al., 2008), leucocyte function associated antigen-1 (Vermot-Desroches et al., 1991), through a sulfate (or sulfonate)-mediated interaction. In present study, three fucoidans with different sulfate contents showed different extent of non-specific inhibition, suggesting that the degree of sulfation as well as specific positioning of sulfate group may be the major impact on the cell-based assay, which may also be the reason for that no non-specific inhibition was observed in ADS-J1 and XTT formazan.

3.4. Application of the mammalian two-hybrid assay in high-throughput screening

To screen for novel small molecular fusion inhibitors, a HTS campaign was launched using a library of natural and synthetic small molecule compounds. All of the compounds were tested at a final concentration of 10 μM and added 2 h after transfection. Here, we present a set of representative primary data of 1624 natural products from herbal screens in 96-well plates. The compounds with cytotoxicity were excluded before screening. Fig. 7A shows the inhibitory activity of these compounds in the primary screen. Four compounds showed inhibition greater than 50%. After confirmation screening and false-positive detection, only one compound, tannin acid, was identified as a positive hit, with an IC_{50} value of 0.8 μM (Fig. 7B). Tannin acid is a polyphenolic compound that reportedly has the potential to inhibit the formation of the gp41 six-bundle core and interrupt the interaction between NHR and CHR (Liu et al., 2002; Lü et al., 2004). Interestingly, in our chemical library, there are a serial of polyphenolic compounds (Fig. 7C) with similar chemical structure to tannin acid, such as pavetannin A, cinnamtannin B1 and aesculitannin B, but only tannin acid proved positive in the novel cell-based assay. Thus, the further study on their structure–activity relationship may have significance for discovery of new gp41 inhibitors.

Fig. 7D shows the S/B values from the 24 screened plates of the 1624 compounds. The average S/B values were 9.2 and 6.7, respectively, against the background of transfection with pVP16-NHR or with pGAL4-CHR. Fig. 7E shows the Z' factors obtained from the 23 screened plates, the average Z' factor value was 0.56. Typically, a Z' factor value greater than or equal to 0.5 indicates that an assay is favorable for HTS (Zhang et al., 1999).

3.5. Miniaturization of the assay in a 384-well format

High density microplates are commonly used in HTS to satisfy the requirement for cost-effectiveness. To investigate the potential of the assay to meet the requirements for higher throughput, the transfection was miniaturized into a 384-well microplate format. As shown in Fig. 8A, the assay in the 384-well format still has an average S/B value of over 7.4 and a Z' factor of 0.52, and the reference compound ADS-J1 exhibited dose-dependent inhibition of luciferase activity with an IC_{50} of 4.7 μM, which was similar to that observed in the 96-well format. These results suggested that the sensitivity and robustness of the assay could be maintained in the 384-well format plate.

In conclusion, the high S/B, acceptable Z' factors and feasible miniaturization suggest that the mammalian two-hybrid system assay is suitable for HTS. The IC_{50} values of the two reference compounds (ADS-J1 and XTT) and the positive hit (tannin acid) obtained from this assay are similar to those in the literature (Table 2), indicating that the novel cell-based assay is sensitive and specific for identifying small-molecule HIV-1 fusion inhibitors targeting gp41.

To date, based on the mechanism of gp41 trimer-of-hairpins (six-helix bundle) conformation, several *in vitro* methods for detecting gp41 inhibitors have been developed (Liu and Jiang, 2004), such as an enzyme-linked immunosorbent assay (ELISA) (Jiang et al., 1999), a fluorescence enzyme-linked immunosorbent assay (FLISA) (Liu et al., 2003), a fluorescence resonance energy transfer (FRET) assay (Xu et al., 2007), and a fluorescence intensity (FI) assay (Cai and Gochin, 2007). Compared with these *in vitro* methods, the cell-based assay offers several specific advantages: (1) in addition to the more physiologically relevant environment provided by the assay (Luo et al., 1997), the permeability and toxicity of a compound can be measured synchronously by the assay. (2) All these *in vitro* assays are based on the protein expression and purification, preparation of antibodies or the protein labeling, while this assay is more convenient and easier to develop and can be finished in two weeks because of abolishing these well-known time-consuming and labor-intensive processes. (3) The protocol of the assay is not more complex than the other assays, the manipulation is routine and can be automatic. (4) Although the assay spans two days, and manipulation time is limited (not longer than 1.5 h).

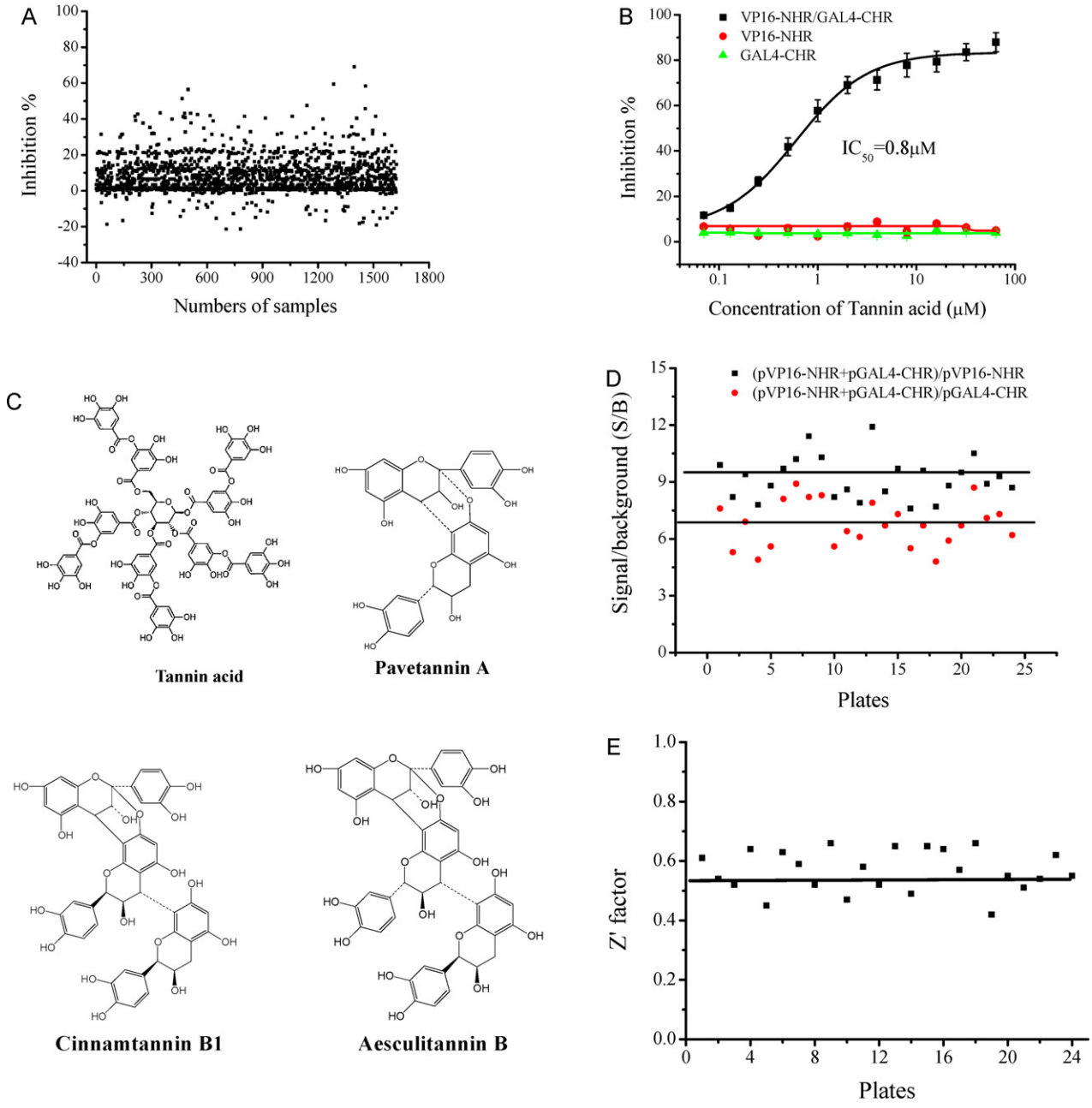

Fig. 7. Representative results of routine screening. Two hours after transfection, 1 μl of test compound (final concentration of 10 μM) or 1 μl of DMSO (0.5%) was added to the cell culture, and the luciferase activity was detected after a 24-h incubation. (A) The primary screening raw data of a subset of 1624 natural herbal products. (B) The inhibitory activity of tannin acid against the luciferase activity in cotransfection of pG5-SV40-Luc with pVP16-NHR and pGAL4-CHR (■), or to that in cotransfection of pG5-SV40-Luc with plasmid pVP16-NHR (●) or pGAL4-CHR (▲) alone. (C) Chemical structure of tannin acid, pavetannin A, cinnamtannin B1, and aesculitannin B. (D) Signal/background ratios form 24 screened 96-well plates. The signal responses over those of the cotransfection with pVP16-NHR (■) or pGAL4-CHR (●) were calculated, respectively. (E) *Z*′ factor values obtained from 24 screened 96-well plates of the 1624 natural products. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Comparison of IC_{50} values (μM) of known gp41 inhibitors obtained from this assay with that in the literature.

	Mammalian two-hybrid	ELISA	Fluorescence intensity	Cell–cell fusion
ADS-J1	4.9	2.55 (19)	0.35 (26)	3.28 (19)
XTT formazan	5.6	7.0 (16)	–	7.28 (16)
Tannin acid	0.8	0.4 (20)	0.18 (26)	5.4–10.2 (21)

–: not detected in this assay.

However, the assay has its limitation in detecting large molecular peptides with poor permeability of the cell membrane. Therefore, this assay is more suitable for screening small molecular fusion inhibitors. Currently, although the discovery of small molecular HIV-1 fusion inhibitors has become a promising alternative therapeutic option, it was proved that discovery of small-molecular gp41 inhibitors is difficult. Up to now, only a very few compounds have been identified as gp41 inhibitors (Debnath, 2006; Liu et al., 2007).

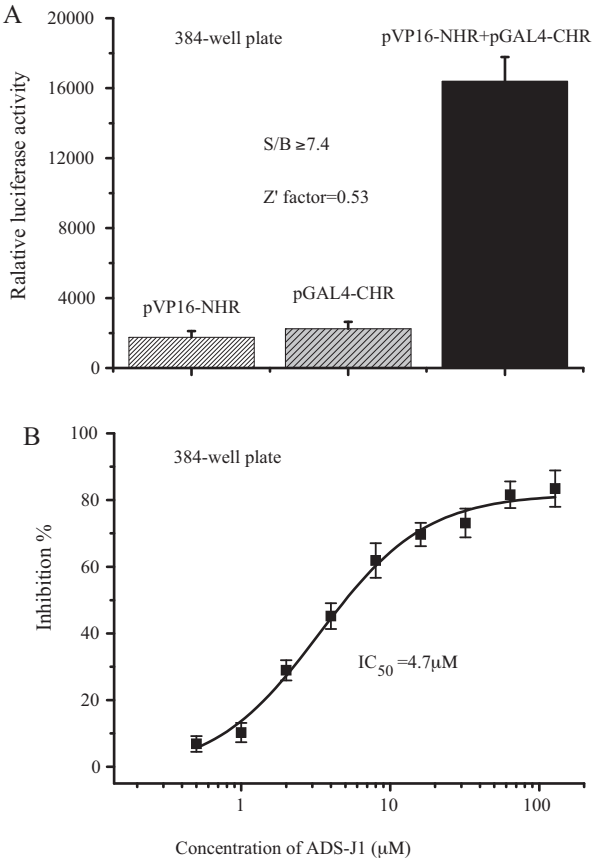

Fig. 8. Miniaturization of the assay into a 384-well format. (A) The signal response of the assay in the 384-well plate. CHO-K1 cells were seeded in 384-well plates at 1×10^4 cells per well one day before transfection. pVP16-NHR, pGAL4-CHR, and pG5-SV40-Luc were cotransfected into cells at a mass ratio of 0.01 μg:0.01 μg:0.1 μg per well. Two hours after transfection, 0.5 μl of the compound was added. The luciferase activity was detected after 24 h incubation. (B) The inhibitory activity of ADS-J1 in the 384-well format plate.

So, novel screening approaches may lead to the discovery of new small-molecular fusion inhibitors.

In addition, protein–protein interactions have become important targets for antiviral drug discovery (Pagliaro et al., 2004; Loregian et al., 2002; Loregian and Palù, 2005), and the identification of protein–protein interaction inhibitors is now a challenge facing the pharmaceutical researchers. Hence, a variety of discovery approaches are needed, and the selection of a tractable protein–protein interaction system is also important. The successful application of the mammalian two-hybrid system in HTS in this study not only demonstrates an alternative method to discover small-molecular gp41 inhibitors but also presents an approach in discovery and development of other new antiviral drugs.

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