

Fission yeast as a HTS platform for molecular probes of HIV-1 Vpr-induced cell death

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Abstract: HIV-1 viral protein R (Vpr) plays an important role in the viral life cycle and pathogenesis of HIV-1. Its activities associate with activation of viral replication, suppression of host immune responses and depletion of CD4⁺ T-lymphocytes. In particular, Vpr induces cell death through apoptosis that may contribute to the depletion of CD4 T cells, which is a hallmark of HIV-1 infection. Currently, there are no anti-Vpr drugs or adequate assays for high throughput screening to identify selective and potent inhibitors of Vpr activities. In this report, we developed a simple fission yeast-based High Throughput Screening (HTS) system that allows us to screen small molecule compounds that specifically inhibit HIV-1 Vpr. This HTS system includes an absorbance-based primary growth assay, a secondary assay using a semi-quantitative colony-forming dot test, and a fluorescence-based LIVE/DEAD yeast viability assay, which is used as the counter screen tests. We further present here results of a pilot study using a Microsource Spectrum Collection Library, which contains 2,000 biologically active and structurally diverse compounds of known drugs, experimental bioactives, and pure natural products. One compound Benfotiamine showed >50% inhibitory concentration (IC₅₀) and 100% inhibition at 20 and 100 μ M, respectively. Interestingly, Benfotiamine is in the same family as thiamine, which was used in this system to control Vpr production through transcriptional inhibition. Even though transcriptional suppressor is not what we are looking for, finding Benfotiamine through the described HTS system from a drug library nevertheless demonstrated the feasibility of the screening assay.

Keywords: HIV-1 viral protein R (Vpr), fission yeast (*Schizosaccharomyces pombe*), high throughput screening (HTS), small molecules, drug library, Benfotiamine

Introduction

HIV/AIDS is one of the most devastating diseases in the world. The cocktail antiretroviral therapy (ART) is currently the most successful antiretroviral strategy to reduce HIV-1 in infected patients. However, the rapid emergence of viral drug resistance often renders ineffective ART. Therefore, there is a great need to develop additional drugs ideally with different drug targets.

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr), a virion-associated protein of about 12.7 kilodaltons (kD), is highly conserved among HIV, simian immunodeficiency virus (SIV) and other lentiviruses.^{1,2} A tertiary structure of Vpr proposed on the basis of NMR analysis consists of an α -helix-turn- α -helix domain in the amino-terminal half from amino acids 17 to 46 and a long α -helix from a 53 to 78 in the carboxy-terminal half.^{3,4} These three α -helices are folded around a hydrophobic core in a structure which allows interaction of Vpr with different cellular proteins.^{5,6}

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This conformational structure of Vpr could potentially be useful in characterizing the interaction of Vpr with small molecules that are identified in the proposed HTS.

Increasing evidence suggests that Vpr plays an important role in the viral life cycle and the pathogenesis of HIV-1. For example, Vpr is required both *in vitro* and *in vivo* for efficient viral infection of nondividing cells such as monocytes and macrophages.⁷⁻⁹ Extracellular addition of Vpr to latently-infected T-lymphocytes markedly increased HIV-1 replication.¹⁰ Our recent evidence further suggested that Vpr promotes viral replication in nondividing cells through a hHR23 A-mediated interaction with the 26S proteasome.⁹ Consistent with the important roles that Vpr plays during the viral life cycle, infections with Vpr-defective viruses in rhesus monkeys, chimpanzees or human subjects seem to correlate with low viral load and slow disease progression, and some of the point mutant *vpr* could revert back to the wild-type phenotype in the viral genome, which further supports the importance of Vpr *in viral* survival.¹¹⁻¹⁵

Vpr plays multiple roles during the viral life cycle and displays several distinct activities in host cells. These Vpr specific activities include cytoplasmic-nuclear shuttling,^{7,8} induction of cell cycle G2 arrest^{16,17} and cell killing.¹⁸ The cytoplasmic-nuclear shuttling is believed to participate in nuclear transport of the viral PIC.^{7,19,20} The cell cycle G2 arrest induced by Vpr is thought to suppress human immune functions by preventing T cell clonal expansion²¹ and to provide an optimized cellular environment for maximal levels of viral replication.¹¹ In addition, Vpr induces apoptosis, which may contribute to the depletion of CD4 T cells in HIV-infected patients.^{15,22,23} Since the Vpr-specific activities have been linked to such clinical manifestation of AIDS as activation of viral replication,¹⁰ suppression of host immune responses²¹ and depletion of CD4+ T-lymphocytes,^{15,18} identification of new molecular probes that can inhibit the Vpr activities could potentially provide a new approach to reduce Vpr-mediated detrimental effects in HIV-infected patients and thus prolong patient's lives. Currently, there are no anti-Vpr drugs or adequate assays for high-throughput screening (HTS) to identify selective and potent inhibitors of Vpr activities.

Our laboratory was the first in developing the fission yeast (*Schizosaccharomyces pombe*) model system for the Vpr studies. We have been very actively exploring the use of fission yeast as a model system to study the Vpr activities over the past 15 years (For reviews of this topic, see).^{17,24-28} Objective of this study was to adapt a number of fission yeast assays that we have developed over the years for the study of

HIV-1 Vpr-induced cell death and apoptosis for the purpose of developing a new HTS system. Such a HTS system could then be used to screening small molecule inhibitors against HIV-1 Vpr.

For the measurements of HIV-1 Vpr-induced cell death, we have previously demonstrated that inducible gene expression of *vpr* via an *nmt1* promoter prevents cell growth and colony formation in fission yeast.¹⁷ Further examinations showed that Vpr actually induce cell death after prolonged cell growth arrest. Induction of cell death induced by Vpr was confirmed by use of a commercial LIVE/DEAD yeast viability test.²⁹ It should be mentioned that Vpr induces cell death through apoptosis and Vpr-induced cell death in fission yeast is reminiscent of apoptotic process shown in mammalian cells.^{30,31} Thus use of fission yeast as a HTS to screening small molecules against HIV-1 Vpr should have direct relevant to their effects in mammalian cells. A number of Vpr suppressors were identified from fission yeast and were also shown subsequently have the same effect in mammalian cells.³⁰⁻³³ Altogether, we have established that fission yeast can be used as a reasonable model system to study Vpr-induced cell death and apoptosis.^{25,34}

In this report, we described adaptation and validation of a set of fission yeast assays that can be used in HTS as the primary, secondary and counter screen assays for determination of Vpr-induced cell death. Results of a pilot study using a small drug library are also presented.

Material and methods

Yeast strains, media and drug treatment

The RE007 strain (*h-ade6-216 leu1-32 ura4-294::vpr (NL4-3)::ura4+*), derived from a commonly used wild type fission yeast strain SP223 (*h-ade6-216 leu1-32 ura4-294*), was used in this study. RE007 carries a single integrated copy of the *vpr* gene under the *nmt1* promoter in the *ura4* locus of chromosome and it stably produces high level of Vpr protein upon induction with very low spontaneous mutation rate, ie, $<1/10^6$ (data not shown).³²

Fission yeast strain was grown in YES complete media or Pombe Glutamate (PMG) minimal medium supplemented with adenine and leucine using standard culture techniques.³⁵

Thiamine was dissolved in water with final concentration of 20 mM or other indicated concentrations; other drugs were dissolved in pure DMSO with final concentration of 10 mM or less. A final concentration of 1% DMSO was used in all cultures.

Cell growth and HIV-1 *vpr* gene induction in fission yeast

Induction of the HIV-1 *vpr* gene under the *nmt1* promoter in fission yeast has been previously described.^{17,36} Specifically, cells carrying plasmids with the *nmt1* promoter were maintained selectively in PMG minimal medium supplemented with 20 μ M of thiamine to prevent *nmt1*-mediated transcription. To induce gene expression, cells were first grown to mid-log growth phase in the presence of 20 μ M thiamine. Cells were then washed three times with distilled water and diluted to a final concentration of $1\text{--}4 \times 10^4$ cells/mL in PMG supplemented with no thiamine to fully induce the gene expression (Vpr-On). $1\text{--}4$ nM of thiamine was used to partially induce gene expression, or 20 μ M of thiamine to complete block gene expression (Vpr-Off). All cells were normally grown at 28°C with or without constant shaking at 300 rpm for HTS.

Measurement the fission yeast cellular growth and the suppressing effect of HIV-1 Vpr

To measure the suppressing effect of HIV-1 Vpr on fission yeast cellular growth, cells with or without *vpr* gene expression were prepared the same way as described above. Each cell culture with the volume of 40–50 μ L was added to the micro wells in a 96- or 384-well plate. The plates were incubated at 28°C without agitation. Cell growth was measured at indicated time intervals by the optical density (OD) using an ELISA plate reader at 650 nm.

Measurement of Vpr-induced cell death by colony-forming dot test

Measurement of fission yeast colony-formation with serial dilution of known amount of cells can be used to evaluate cell survival in a semiquantitative fashion.³⁷ Briefly, cell cultures were prepared the same way as described in the yeast viability assay. After 48 hours of incubation, 1.5×10^6 cells were collected and resuspended into 30 μ L of water to get the final concentration of 5×10^7 cells/mL. A serial 10-fold dilution was performed. An aliquot of 2 μ L cell suspension was dropped onto the YES complete agar plate. Plates were incubated at 30°C before counting for cell survival.

Yeast viability assay

S. pombe cell viability was determined with a commercial LIVE/DEAD yeast viability kit (Cat. No. L-7009; Invitrogen,

Carlsbad, CA) as we described previously.²⁹ Briefly, cell culture in the logarithmic phase was washed three with distilled water to remove thiamine from the medium, diluted to a final concentration of 4×10^4 cells/mL, and re-suspended them into the PMG minimal medium supplemented with or without 20 μ M thiamine to suppress or induce HIV-1 *vpr* gene expression. To test the potential suppressive effect of the identified drug, the final concentration of 0, 0.1, 1.0, 10, or 100 μ M was added to the *vpr*-expressing cells. All cells were normally grown at 28°C with constant shaking at 300 rpm. Cells were collected at 48 hours after *vpr* gene induction. They were then resuspended into the GH solution (2% D-(+)- glucose, 10 mM Na-HEPES, pH 7.2). FUN-1 solution was prepared by diluting 10 mM stock of FUN-1 solution into the GH solution to a final concentration of 80 μ M. A 50 μ L aliquot of FUN-1 solution was added to an equal volume of yeast suspension. After 45 min incubation at 30°C, A 3 μ L aliquot of the yeast suspension was then dropped onto a glass slide, which was then covered with a coverslip and sealed with wax.

Cells were examined by using a Leica DM fluorescent microscopy using 11001v2 long path Chroma filter cube. Actively respiring cells are typically marked clearly with orange-red fluorescent structures at the maximum wavelength of approx. 590 nm, while dead cells exhibit bright, diffuse, green-yellow fluorescence at the maximum wavelength of approx. 540 nm.²⁹ FUN1 stained cell images were collected at the excitation wavelength of 470 ± 20 nm with red, green and blue filter set to get color picture after merging them. The Leica DM fluorescence microscope (DM4500B; Leica Microsystems, Frankfurt, Germany) is equipped with a high performance camera (Hamamatsu C4742-80-12AG), Micro*Color* Tunable RGB Filter, and the 11001v2 long path Chroma filter cube (>530 nm). The OpenLab software (Improvision, Coventry, UK) was used for imaging analysis.

The same LIVE/DEAD yeast viability assay was also used to quantify level of cellular viability. Specifically, the emission spectrum of FUN1 stained cells described above were measured in a microcuvette with the Cary Eclipse spectrophotometer in a emission range of 500–700 nm with an excitation wavelength of 470 nm. Typically, two clearly distinguishable peaks were seen at max. 590 nm for live cells and max. 540 nm for dead cells.

Results

The primary assay

HIV-1 Vpr blocks cell proliferation followed by rapid cell death.^{17,29} Thus no cellular growth is observed as soon as the

vpr gene expression is fully induced in about 16 hours. Based on this principle, we have developed an absorbance-based determination that is designed for large-scale screening of inhibitors against Vpr-induced cell death. Specifically, a fission yeast strain RE007 that stably produces high level of Vpr protein upon induction, was used in this assay.³² The stable Vpr protein production was achieved through inducible induction of *vpr* expression from the fission yeast *nmt1* promoter in minimal liquid medium.^{17–36} Briefly, fission yeast RE007 cells containing the *vpr*-carrying plasmid, will first be grown in a small volume to late log phase in the presence of 20 μ M thiamine. Cells were then washed three times with distilled water, diluted to a final concentration of approximately 1×10^4 cells/mL in the desired volume for large scale screening. The PMG minimal medium was used to culture cells either with 1nM or without thiamine to induce low level of full level induction of *vpr* gene expression. The control culture includes use of the same PMG medium but containing either 1 nM of thiamine to reduce Vpr production or 20 μ M of thiamine to prevent Vpr production. A total

volume of 50 μ L cell culture was added to each micro wells in a 96-well plate and cells were incubated at 28°C. Cellular growth was examined at indicated time intervals or 72 hours after gene induction by using an ELISA reader. Under this experimental condition, ie, growing cells in microtiter plate without agitation, the OD_{650 nm} reading was approx. 0.12 for the no Vpr control cells; whereas little or no readable OD_{650 nm} was seen in *vpr*-expressing cells (Figure 1A). We further miniaturized the system to 384-wells. As shown in Figure 2A-a,b, both 96-well and 384-well formats worked well, in which Vpr killed more than 98% of the cells (the Vpr-On culture); whereas addition of 20 μ M of thiamine completely reversed the cell killing effect (the Vpr-Off culture). To sensitize the system for drug screening, 1 nM of thiamine was added to the growth medium that showed small about 20% recovery of the cell survival (Vpr-On 1 nM). To evaluate the performance of these assays, the Z-factor values were calculated based on the formula of

$$Z\text{-factor} = 1 - 3 \times (\sigma_p + \sigma_n) / |\mu_p - \mu_n|$$

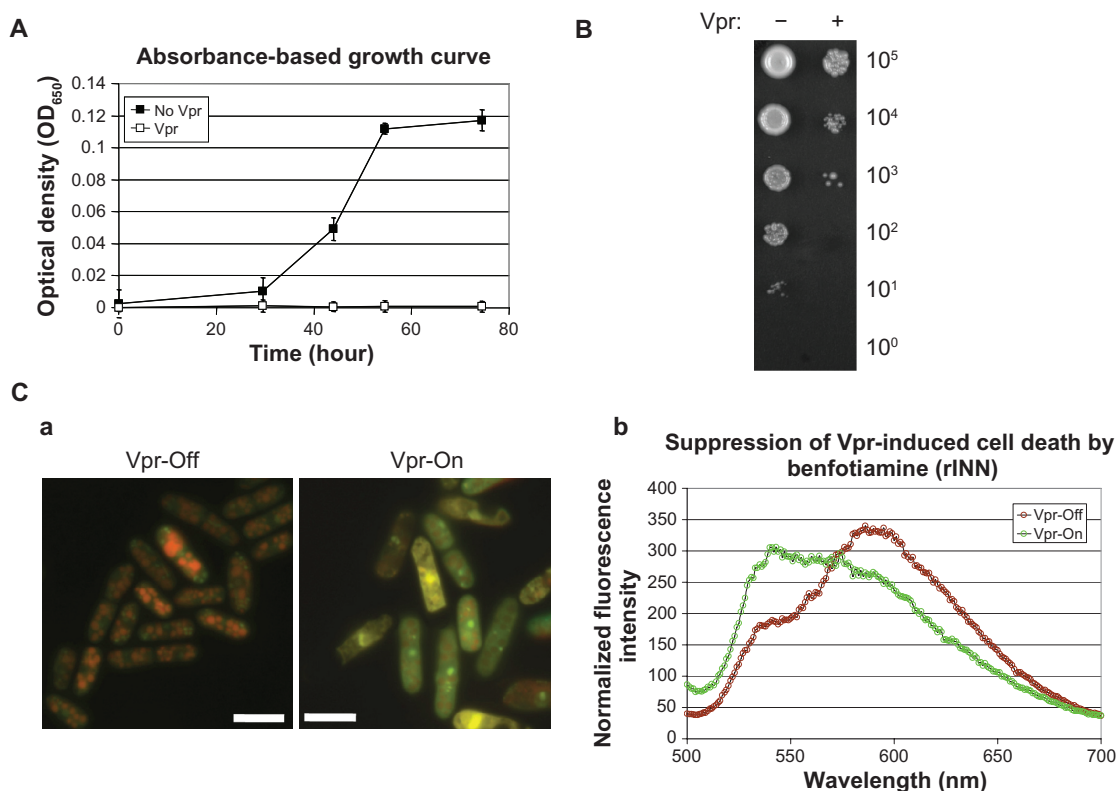


Figure 1 A) The primary assay. An absorbance-based determination of the Vpr effect on cellular growth. No Vpr, cells without *vpr* gene expression, ie, grown in a repressing liquid media; Vpr culture are cells, in which *vpr* is expressed that resulted in little or no cellular growth. **B)** The secondary assay. A colony-forming based dot assay that is used to show Vpr prevents colony formation on agar plate. Vpr-Off (-) or Vpr-On (+) cells grown in liquid medium were collected 48 hours after gene induction. 10-fold series of dilution were plated on a repressing complete YES agar plate. **C)** The counter screen assay. **C-a)** Qualitative observation. Viable yeast cells (Vpr-Off) that are metabolically active and have an intact plasma membrane can convert the fluorescent substrate FUN-1 from a diffuse green fluorescent color to a compact orange-red fluorescent metabolite within cells (left). After 48 hours induction of Vpr (Vpr-On) cells failed to convert FUN-1 thus remain diffuse green-yellow indicating the cell death (right). Scale bar represent the size of 10 μ m. **C-b)** Quantitative measurement. two clearly distinguishable peaks at max. 590 nm and 540 nm are live cells and dead cells, respectively.

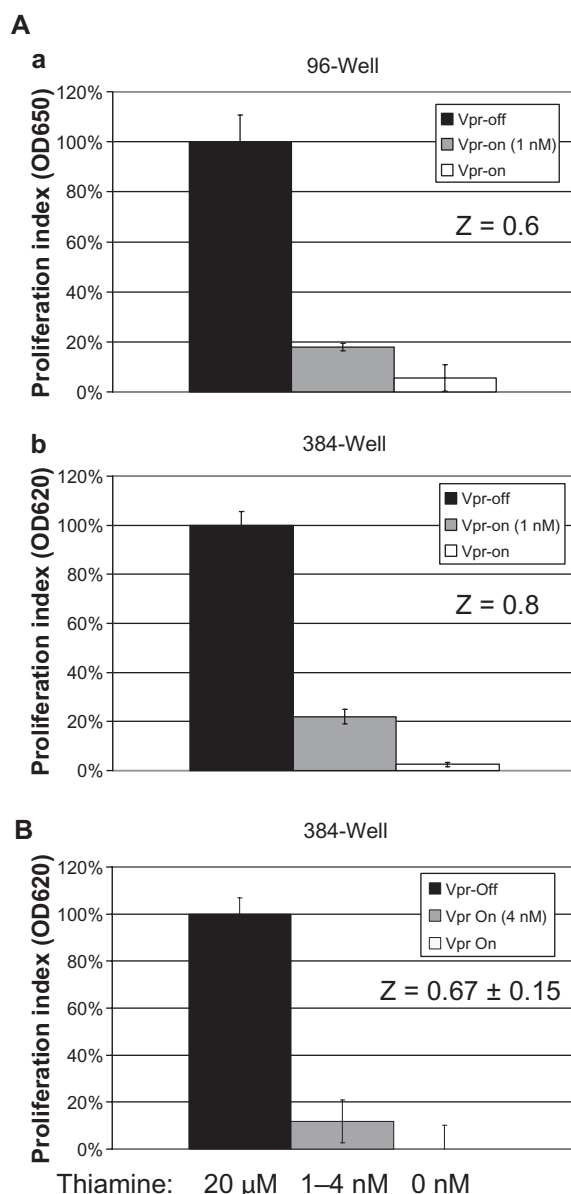


Figure 2 Performance of the primary assay. **A)** Measurement of HIV-I Vpr-induced growth arrest in 96-well (**a**) and 384-well (**b**) format in the laboratory. 1 nM of thiamine was added to reduce Vpr-induced growth arrest. Cell growth were measured by optical density of 650 (OD₆₅₀) 72 hours after gene induction using a spectrophotometer (Molecular Devices, UVmax). Cell growth is expressed as proliferation index here because data are normalized to Vpr-off cells. **B)** Measurement of HIV-I Vpr-induced growth arrest in 384-well format using automated liquid-handling system at the university HTS core facility. Cell growth were measured by optical density of 620 (OD₆₂₀) after 72 hours gene induction with a spectrophotometer (BMG Labtech, POLARstar). Note that two different spectrophotometers were used in our laboratory and in the HTS core facility with two different OD wavelengths. The assay was optimized under each condition with the given optical wavelength. That is why 4 nM was used in the HTS assays instead of 1 nM we originally optimized in the laboratory conditions with the wavelength of 650.

where the means and standard deviations of both the positive (p; Vpr-off) and negative (n; Vpr-on) controls are μ_p , σ_p , and μ_n , σ_n . The calculated results showed Z-values of 0.6 and 0.8 in 96-wells and 384-wells, respectively. Since these Z-values are ≥ 0.5 , together this assay is considered as an

excellent assay. Generally, duplicate or triplicate tests are not necessary for HTS.

To further adapt this absorbance-based assay into the high throughput format by using an automated liquid handling system, the adapted 384-well plate format was further evaluated for configuration and verification of the described assays into the HTS formats. The developed 384-well system was interfaced with the Biomek FX software Systems for control of the assay performance including liquid handling and incubation. Performance of the assay was then further evaluated in cell culture media with or without 1% DMSO. A total volume of 40 μ L was used in each microtiter well for all runs. Out of 5 independent experimental runs, the average of Z-value was 0.67 ± 0.15 or 0.65 ± 0.18 for cells containing none or 1% of DMSO (Table 1). These data suggest that this primary assay is not affected by DMSO and is also a highly reproducible assay for HTS. Note that the developed fission yeast-based primary HTS assay is a very simple “mix and measure” assay and requires no complicated preparation. All culture reagents are common chemicals without need of any key or critical reagents. Since there are no centrifugation, filtration or extraction steps during the HTS process, this assay can be readily carried out for automated large-scale HTS.

The secondary assay

A secondary assay was also developed to confirm the primary assay and to quantify possible dose-dependent suppression of Vpr-induced cell death. This is achieved by using a semi-quantitative colony-forming dot-assay that we have described previously.³⁷ In this assay, a different endpoint from the primary assay is used, ie, to measure cell survival in the form of colony formation on a solid agar plate. Specifically, *vpr* gene was first induced for about 48 hours and cells with various treatments and control were plated on the complete YES medium agar plates with serial 10-fold

Table 1 Performance of the primary assay in 384-wells as determined by average OD reading, standard deviation and Z-values

	0% DMSO			1% DMSO		
	X	SD	Z-values	X	SD	Z-values
Exp. 1	1.505	0.031	0.76	1.502	0.032	0.83
Exp. 2	1.477	0.039	0.76	1.467	0.039	0.84
Exp. 3	1.048	0.045	0.50	1.133	0.047	0.50
Exp. 4	–	–	na	1.083	0.039	0.59
Exp. 5	–	–	na	1.083	0.039	0.47
X ± SD			0.67 ± 0.15			0.65 ± 0.18

Abbreviations: na, not applicable; X, average OD in +T media; SD, standard deviation.

dilutions of cells from 10^5 to 10^0 cells per spot. Cell growth was determined 3–4 days after incubation at 30°C . As shown in Figure 1B, Vpr-suppressing RE007 cells grown well and formed expected numbers of colonies on all of the dilutions; in contrast, there were about 100-fold growth reduction in the Vpr-producing cells.

The counter screen assay

We have further adapted a qualitative and quantitative LIVE/DEAD[®] yeast viability assay, which could be used as the counter screen assays and to further verify dose response of the identified small compounds to Vpr-induced cell death.²⁹ Unlike the primary and secondary assays, this assay does not measure cell growth or survival. Instead, it determines the intracellular metabolic status as an endpoint for cell viability. By using direct fluorescence microscopic observation, it allows rapid detection of live or dead cells by observing changes of the fluorescent signals.

As shown in Figure 1C-a, fission yeast cells without *vpr* gene expression (Vpr-Off) stained with orange-red (left) but the *vpr*-expressing (Vpr-On) cells remained mostly as green fluorescent color (right) indicating metabolically inert cells. Measurement of the fluorescent emission wavelength change could also be used as a semi-quantitative method to verify or dispute results of the primary and secondary assays. As shown in Figure 1C-b, two clearly distinguishable peaks were seen at max. 590 nm for the *vpr*-repressing cells indicating live cells and max. 540 nm for the *vpr*-inducing cells indicating dead cells, respectively. The relative level of the fluorescent emission intensity at each peak could be used as a semi-quantitative marker for testing the dose response.

Pilot runs with a commercial drug library

Pilot runs were carried out to test the developed HTS system at the University of Maryland Baltimore's HTS core facility. A drug library that includes 2,000 biologically active and structurally diverse compounds (the Microsource Spectrum Collection), which contains known drugs, experimental bioactives, and pure natural products, was used. To prepare cell culture for the HTS of this drug library, the fission yeast strain RE007 was grown in selective PMG minimal medium to mid-log growth phase ($\sim 1 \times 10^7$ cells/mL) in the presence of thiamine to prevent Vpr from expression. Cells were then prepared as described in the Materials and Methods to a final concentration of approximately 1.5×10^4 cells/mL in bulk volume (~ 600 mL) in the PMG minimal medium. After 15 hours shaking at 28°C to allow initial Vpr production, testing drugs

were added to measure the potential suppressing effect on Vpr. The small molecule compounds from the drug library were initially stored in 10 mM of DMSO. Each drug was dispersed into the wells of microtiter plates in an orientation as shown by the plate map (Figure 3A). A total of $7\frac{1}{4}$ of 384-well plates were used in each test run. Note that although 384-well plates were used in the test runs, the controls and testing drugs were added to each well by the automated liquid handling system in a 96-well format each time, ie, it took 4 automated liquid transfers to complete the filling of each plate. The positive assay control included growing testing cells with 20 μM of thiamine, which completely shut-down Vpr production thus resulting full cellular growth (Figure 3A, in black squares); the negative assay control was cells grown in the same medium without thiamine (Figure 3A, white squares). All drug effects were tested in RE007 cells with 4 nM of thiamine (Figure 3A, grey squares). Thus the assay baseline control were the same cells in 4 nM of thiamine but without adding drugs (Figure 3A, the 2 right columns). The drug-containing fission yeast cells were incubated at 28°C little or no agitation and the growth intensity was measured by spectrophotometer at OD_{620} 72 hours after *vpr* gene induction.

Two independent HTS with two duplicated parallel runs (SC#1H, SC#1 L, SC#2 and SC#3) were carried out with the Microsource Spectrum Collection drug library. The SC#1H, SC#2 and SC#3 runs were screened against drug concentration at 100 μM ; the SC#1 L was against drug concentration of 20 μM . Note that $\frac{3}{4}$ of the microwells on the 7th 384-well plate were left empty in each run. To use up those spaces, we filled those wells with additional drugs, which duplicated part of that run, eg, SC#2a vs SC#2b shown in Table 2. The suppression level of $>90\%$ was used as the cutoff for the HTS in the 100 μM drug runs; and 70% suppression level was used in the 20 μM drug screening for the initial cutoff. Examples of the screening results in either 20 or 100 μM drug concentrations are illustrated in Figure 3B. Only those compounds that showed $\geq 90\%$ suppression in the 100 μM screenings and at least 70% suppression in the 20 μM screening in all 4 runs were selected for further analysis. Note that these cutoffs were arbitrary. Our intention was to limit the number of "hits" that were showing signals that are significantly beyond the background OD readings. We used more stringent cutoff (90%) for high drug concentration and a relative low cutoff (70%) for low drug concentration. Five compounds fulfilled such criteria and are listed in Table 2. The actual levels of Vpr suppression by compound 08-B11 is shown in Figure 4A, which showed the strongest

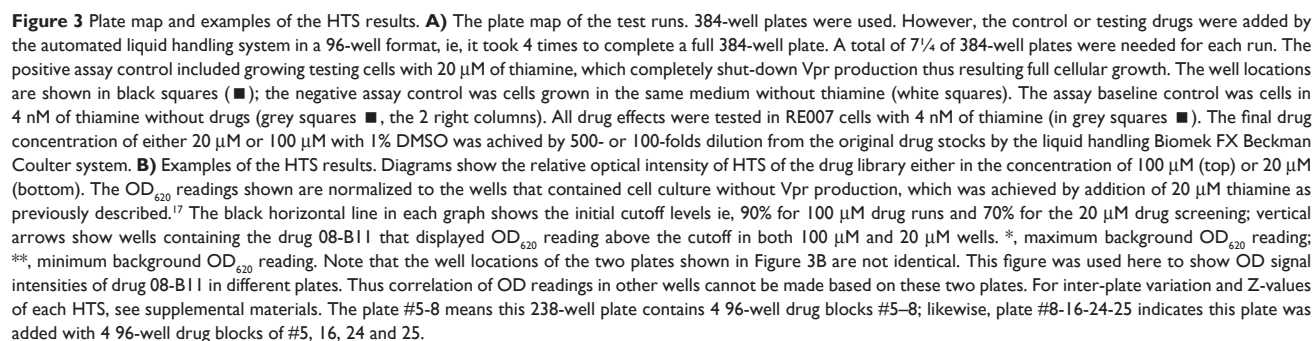


Table 2 Results of the 5 compounds that were found to fulfill the initial selection criteria

Well position	Drug name	% survival based on OD ₆₂₀ reading					X ± SD
		20 μM	100 μM				
		SC#1L	SC#1H	SC#2a	SC#2b	SC#3	
08-B11	Benfotiamine	88 (77)	100	100	99	99	99.5 ± 0.6
15-G2	3β-Acetoxydeoxyangolensic acid, methyl ester	69 (38)	90	92	82	91	88.8 ± 4.6
17-F7	Acetal Isogambogic acid	55 (9)	94	94	nd	92	93.3 ± 1.2
19-H3	Sappanone A trimethyl ester	64 (29)	92	98	nd	99	96.3 ± 3.8
25-D8	Diflubenzuron	75 (47)	111	124	nd	109	114.7 ± 8.1

Notes: Numbers shown are % of suppression based on the OD₆₂₀ reading; numbers in parentheses are % after subtracting the background reading.

Abbreviations: nd, not determined; SC#, screening number; X, average; SD, standard deviation.

suppression in all of the runs. Averages of the relative % of cellular survival in wells treated with all five compounds to the positive experimental controls are shown in Figure 4B. Note after subtracting the background OD₆₂₀ reading from the sample OD₆₂₀ reading, only one compound 08-B11 qualifies our selection criteria (Table 2).

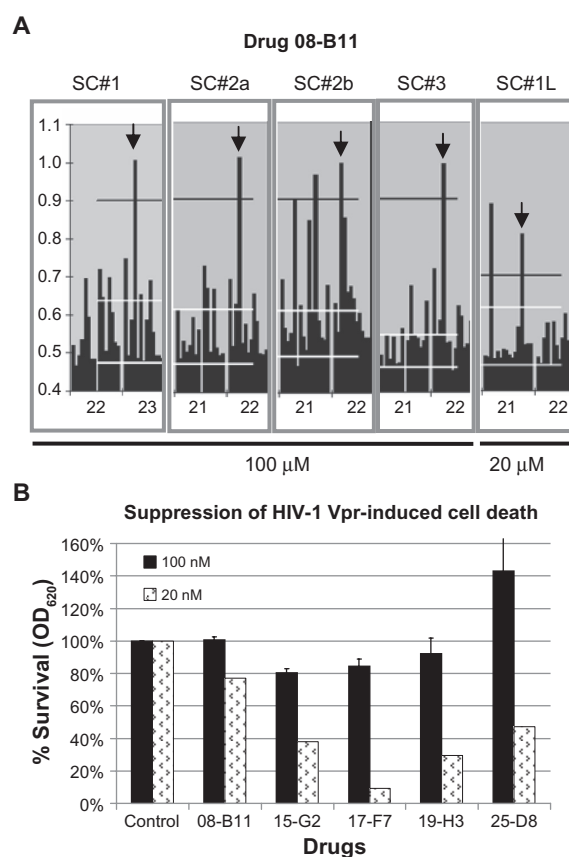


Figure 4 Initial testing profiles of the selected drug candidates. **A)** Percentage of suppression of Vpr-induced cell death by drug 08-B11 in five different runs/plates. SC#, number of the screening runs. The arrows indicate the suppression levels and locations of the drug 08-B11 in different runs/plates. **B)** Summary of the suppression levels by the initially selected five drugs. The average and standard error bars were calculated based on all of the runs. The positive controls were normalized to 100%. Cell survivals were measured by optical density readings 72 hours after gene induction and 57 hours after addition of the drug library during the HTS.

The 08-B11 compound turns out to be a drug known as Benfotiamine (C₁₉H₂₃N₄O₆PS); Mol. Mass: 466.448 g/mol). Its systematic IUPAC (International Union of Pure and Applied Chemistry) name is *S*-[(2*Z*)-2-[(4-amino-2-methylpyrimidin-5-yl)methyl] (formyl)amino}-5-(phosphonoxy)pent-2-en-3-yl] benzenecarbothioate.

To further evaluate possible dose-dependent suppression of Benfotiamine on Vpr-induced growth arrest, 10-fold serial dilution of Benfotiamine in the range of 1 to 100 µM was tested by using the same absorbance-based assay as we did in the HTS. As shown in Figure 5A, 1.0 µM of Benfotiamine partially suppressed Vpr-induced growth arrest; both 10 µM and 100 µM showed complete suppression, which are demonstrated by the same growth kinetics as the Vpr-Off cell culture. Consistent with the results shown in Figure 5A, suppression of Vpr-induced cell death by Benfotiamine was also seen in the range of 0.1–100 µM when the secondary colony-forming dot assay was used (Figure 5B).

The counter-screening LIVE/DEAD yeast viability was also used to verify the suppressing effect of Benfotiamine. While 0.01, 0.1 and 1 µM of Benfotiamine showed no obvious switch from green to red, 10 µM displayed a clear profile that coincided with the live cell spectrum (Figure 6A). This observation is consistent with the results shown in the secondary assays that 10 µM of Benfotiamine is sufficient to suppress Vpr-induced cell death. In addition, the relative level of the fluorescent emission intensity was used as a semi-quantitative marker for testing the dose response. Specifically, calculation of the 590/540 nm ratios among different cell treatments suggested that this ratio could be used to differentiate these two types of cells. For examples, Vpr-Off (live) cells showed a ratio of 1.80; while a ratio of 0.93 was seen in the Vpr-On (dead) cells. A dose-dependent increase of this ratio was observed from 0.94, 0.96, 1.07 to 1.82 in *vpr*-expressing cells treated with 0.01, 0.1, 1.0 and 10 µM of Benfotiamine, respectively.

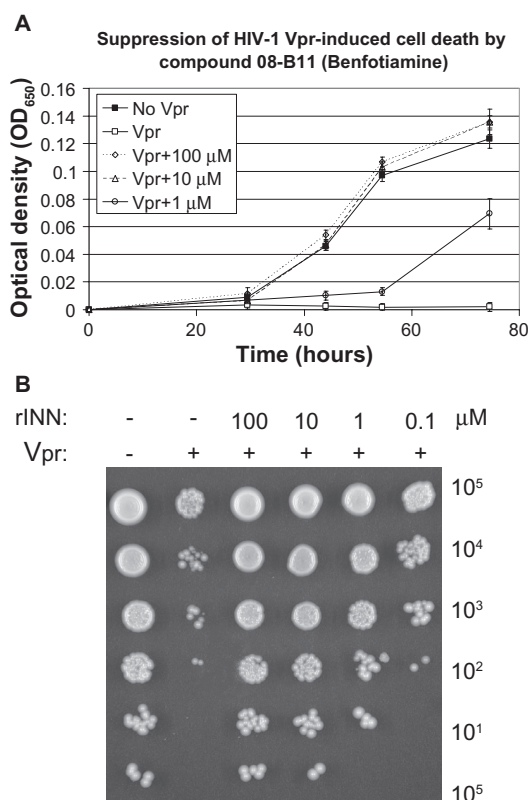


Figure 5 Dose-dependent suppression of Vpr-induced cell death by Benfotiamine (rINN) as determined by **A**) the absorbance-based growth assay or **B**) the colony-forming dot assay. Note that cells plated on the agar plate were at 48 hours after vpr gene induction. Thus not all of the cells were killed by Vpr.

Discussion and Conclusion

In this report, we have described a fission yeast HTS platform that could be used to screen small molecule compounds specifically against HIV-1 Vpr-induced cell death. Specifically, we have successfully adapted a set of fission yeast-based assays, developed in our laboratory in the past, for the proposed large-scale HTS. Based on the results generated from our pilot runs and the subsequent analyses by the secondary tests and the counter screen tests, the proposed HTS system seems to work very well. Thus we believe the developed HTS system can be used in the future large-scale HTS. Note that the developed fission yeast-based HTS assay is a very simple “mix and measure” assay and requires no complicated preparation, centrifugation, filtration, or extraction. All culture reagents are common chemicals without need of any key or critical reagents.

We have also shown the results of a pilot screening by using a Microsource Spectrum Collection Library, which contains 2,000 biologically active and structurally diverse compounds of known drugs, experimental bioactives, and pure natural products. Even though five compounds passed the initial screens with the cutoff of 90% viability at concentration of 100 μ M. However, only Benfotiamine showed IC₅₀ at 20 μ M

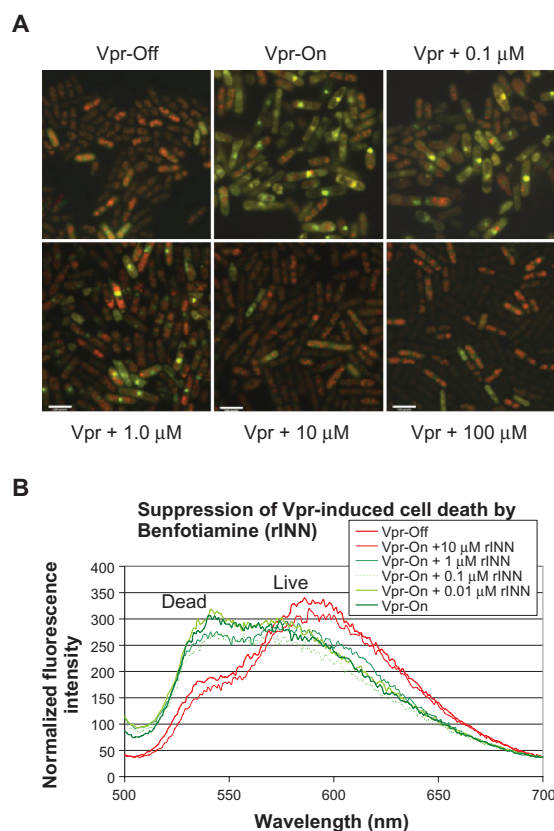


Figure 6 Use of the counter-screen assays to confirm dose-dependent suppression of Vpr-induced cell death by Benfotiamine (rINN). **A**) Results of fluorescent microscopic imaging using the LIVE/DEAD yeast viability assay. Typically, metabolic active cells are orange to red color; metabolic inert cells that are unable to convert the green fluorescent substrate show greenish colors. All cells were shown under the same magnification. Cell images were taken 48 hours after vpr gene induction. Note that Vpr causes gross enlargement of cells as previously reported.¹⁷ Scale bar represent the size of 10 μ m. **B**) Fluorescence spectrum shows dose-dependent suppression of Vpr-induced cell death by rINN. Viable cells emit at the max. wavelength of approx. 590 nm and dead cells at max. 540 nm. An average of three consecutive measurements was used to depict the graph.

(Figure 4; Table 2). Interestingly, Benfotiamine, also known as rINN, or *S*-benzoylthiamine O-monophosphate, is a synthetic S-acyl derivative of thiamine. Recall that thiamine is the compound we used in our screenings as a positive control to inhibitor Vpr production through transcriptional suppression of the *nmt1* promoter.^{17,38} However, there are no prior reports suggesting that Benfotiamine suppresses the *nmt1* promoter in fission yeast. Nevertheless, based on our testing results shown here, we anticipate that the molecular mechanism of suppression by Benfotiamine to Vpr-induced cell death might be very similar to thiamine. However, only additional tests using different Vpr expression systems, such as the use of a different promoter or adenoviral expression of Vpr in mammalian cells, should ascertain this possibility. In summary, even though transcriptional suppressor is not what we are looking for, finding Benfotiamine through our pilot HTS nevertheless demonstrated feasibility of the proposed screening assay.

To carry out future large-scale HTS, we propose the following flow-chart as illustrated in Figure 7. Specifically, an absorbance-based 384-well format HTS system, further miniaturized if it is necessary, should be used as the primary assay for HTS. Two drug doses 100 μM and 20 μM could be used separately in the HTS. The same selection criteria as we used in the pilot studies, ie, >90% suppression in the 100 μM screening and >70% suppression in the 20 μM screening (after subtracting from the background reading), should be used to select the primary hit candidates for further analyses. Assuming a hit rate of about 1/10,000, an approx. number of 50 primary candidates should be appropriate and are within the workable range. Should more “hits” be identified, a more stringent cutoff, eg, >90% for both 100 μM and 20 μM screenings could be used. The identified initial hits should be re-tested by the growth curve analysis as shown in

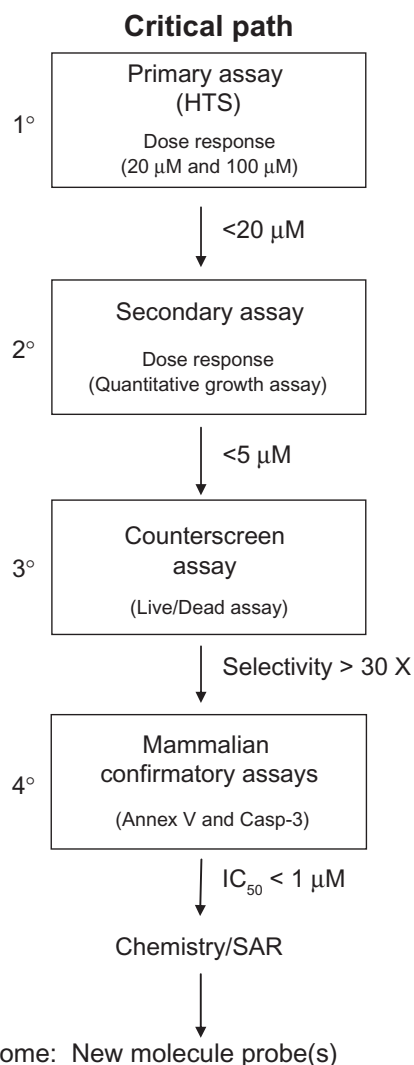


Figure 7 A proposed critical path to screen new probes of HIV-1 Vpr-induced cell death.

Figure 1A and further verified by the secondary quantitative dot-assay for possible dose-dependent responses (Figure 1B). Only those candidates that show >90% suppression at the level of 5 μM should be further tested. To avoid any potential false positives due to transcriptional inhibition of the *nmt1* promoter, additional tests using different Vpr expression systems, such as the use of a CMV constitutive expression promoter or adenoviral expression of Vpr in mammalian cells, as we have described previously,³² should be used. Only those that still suppress Vpr-induced cell death in mammalian cells will be pursued. Note that the number of candidates for the next step analyses depends on the actual numbers of candidates passing the initial tests. The number of candidates should be limited within 30 at this stage, ie, full suppression with <5 μM of drug. However, in the situation where only few or no hits were found at the 5 μM cutoff, a higher cutoff should be considered. This is not unreasonable because fission yeast has a thick cell wall. Higher concentration might be required to achieve the same effect as in human cells. Once the candidates passed the secondary assay, they should then be subject to the counter screen assays as described in Figure 1C, which measures cellular metabolic state as an indication of Vpr-induced cell death. With the possible selectivity of >30X, one or possibly two small molecules could possibly be selected. Since we have shown previously that HIV-1 Vpr-induced cell death is reminiscent of apoptosis in *S. pombe*, the candidate compounds could further be subject to those tests such as detection of phosphatidylserine externalization by Annexin V staining or measurement of mitochondrial membrane potential ($\Delta\psi_m$) using $\Delta\psi_m$ -sensitive dye 3,3'-dihexyloxacarbocyanine iodide (DiOC₆).^{30,31} Finally, the identified compounds could be re-tested in mammalian cells using the same method such as the Annex V staining or determination of Caspase-3 cleavage, which we have used previously to verify our earlier findings from the fission yeast.^{30,31,39} Upon confirmation in mammalian cells, the chemistry and the structure-activity relationship (ASR) of the identified drug should be sought for possible mechanistic insight of the suppression.

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Disclosure

The authors report no conflicts of interest in this work.

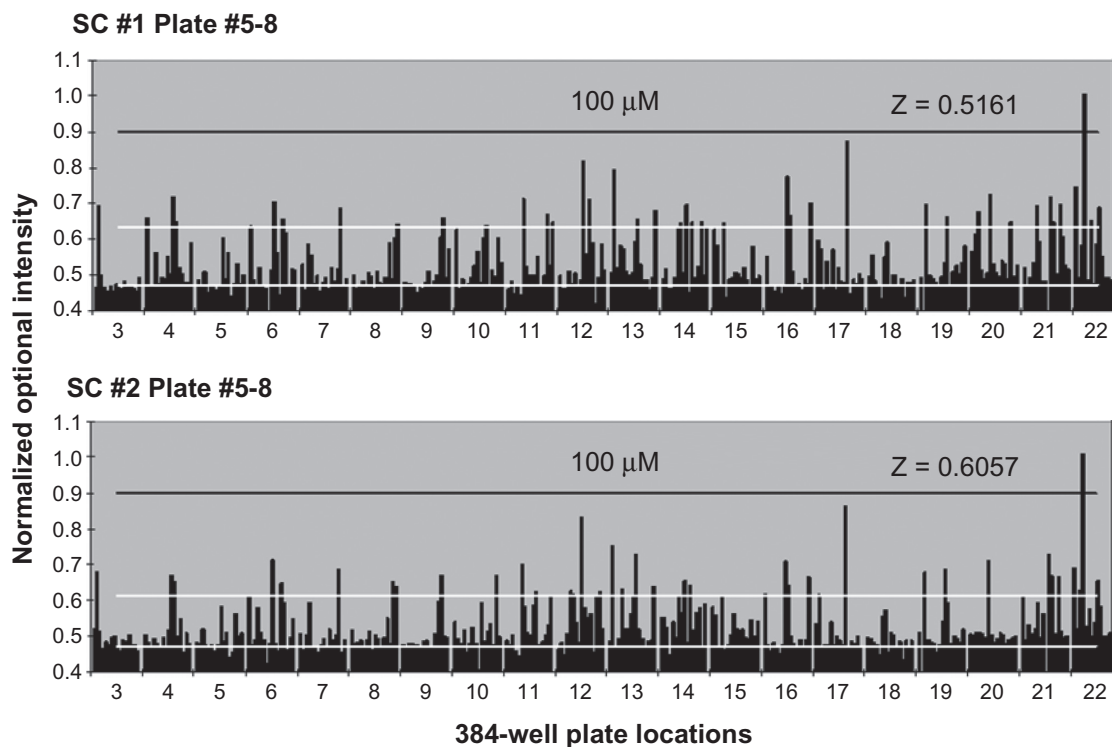
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Supplemental material

Reproducibility of different HTS runs. Intensities of OD620 reading are shown here from two different HTS runs, SC#1 and SC#2, both of which were loaded with the same drugs

at the same locations. Thus correlation of optical intensities of each well between plates could be made. Z-values of each run are also added.



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