



A fluorescence resonance energy transfer-based fluorometer assay for screening anti-coxsackievirus B3 compounds

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ABSTRACT

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In view of the need to develop a simple and rapid method to screen for antiviral therapeutic agents, a fluorescence resonance energy transfer (FRET)-based reporter system consisting of engineered mammalian cells expressing a cyan fluorescent protein–yellow fluorescent protein (CFP–YFP) pair linked by a short peptide containing the cleavage site of viral protease 2A (2A^{pro}) was developed. By detecting the 2A^{pro} produced early during the virus infection cycle, the CFP–YFP pair effectively identifies infectious coxsackievirus B3 (CVB3), a picornavirus that causes viral myocarditis in humans. The reporter system was used to screen a library of 2000 drugs and natural products for potential antiviral compounds. The reporter cells were treated with the test compounds, challenged with CVB3, and then examined using a fluorometer at 24 h post-infection. Sixty-four compounds, mostly therapeutic drugs, antimicrobial compounds and compounds with unknown functions, caused at least 50% inhibition of 2A^{pro} activity. Three known antiviral compounds, cosmosiin, ribavirin and baicalein, were also identified in the screening. The developed method is an effective strategy for rapid screening, and identifies compounds that inhibit CVB3 2A^{pro}. This method should be a valuable aid in the antiviral drug discovery effort.

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1. Introduction

The development of robust and reliable assays is critical for antiviral drug discovery. Despite success in treatment of some viral diseases, the search for new antiviral drugs remains an area of active investigation since effective treatment is not available for many viral infections. Moreover, the emergence of drug-resistant variants requires the continuous discovery of new drugs. Methods used for screening and identification of potent and selective antiviral compounds include cytopathic effects (CPE) inhibition assay based on either dye uptake (Jesus et al., 2009; Schmidtke et al., 2001; Takeuchi et al., 1991; Watanabe et al., 1994) or luminescence assay (Gong et al., 2008; Li et al., 2009), flow cytometric analysis (Pavic et al., 1997; Steele-Mortimer et al., 1990), enzyme-linked immunosorbent assay (Leahy et al., 1994; Myc et al., 1999), genomic/subgenomic replicon-based assay (Puig-Basagoiti et al., 2005), and plaque-forming assays (Chern et al., 2004; Gebre-Mariam et al., 2006; Lin et al., 2009). Although reliable for the identification of potent and selective antiviral compounds, these methods are often laborious, time-consuming, the evaluation is subjective, and the results do not correspond well with that of plaque reduction assays (Safrin et al., 1994; Standing-Cox et al.,

1996). Furthermore, not all viruses (e.g., hepatitis A virus) are capable of inducing CPE or producing clear plaques on host cells (Cromeans et al., 1987), making CPE-based and plaque reduction assays ineffective. In view of this, it is necessary to develop improved screening methods with high efficiency and speed.

The utility of fluorescent reporter cells for rapid and sensitive detection of poliovirus infection (Cantera et al., 2010; Hwang et al., 2006) was described recently. The cellular reporter system was based on a fluorescence resonance energy transfer (FRET) approach, a phenomenon wherein the energy from a donor fluorophore is partially transferred to the acceptor fluorophore located in close proximity (Jares-Erijman and Jovin, 2003). In this reporter system, engineered buffalo green monkey kidney (BGMK) cells express a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) joined by a linker peptide containing the viral protease 2A (2A^{pro}) cleavage site. After virus infection, the 2A^{pro} that is produced during viral replication cleaves the linker peptide. Disruption of FRET, as reflected in a change in the CFP and YFP emission ratio, indicates active viral replication within the host cells (Fig. 1B). The use of a FRET-based assay to detect virus infection through the detecting protease activity has also been demonstrated for hepatitis C virus (Sabariegos et al., 2009) and enterovirus 71 (Tsai et al., 2009).

In an attempt to fulfill the demand for an effective screening protocol for inhibitors against viral infections, and to determine if the protocol is feasible for antiviral drug screening, we constructed a

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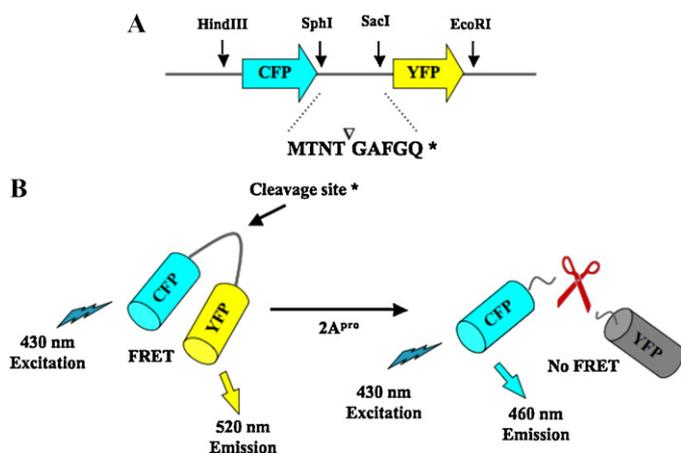


Fig. 1. (A) DNA fragment encoding the substrate peptide for CVB3 2A^{pro} was inserted between the coding sequences of CFP and YFP in pcDNA3.1(+). Zeocin (Invitrogen). The plasmid was introduced into buffalo green monkey kidney (BGMK) cells using Lipofectamine Plus (Invitrogen). Stably transfected BGMK-C3 were selected using Zeocin, and sorted using fluorescent-activated cell sorter (BD FACSAria). (B) Principle of FRET.

reporter cell line that responds to coxsackievirus B3 infection. Coxsackievirus B3 (CVB3), a non-enveloped single positive-strand RNA virus belonging to the *Picornaviridae* family, was chosen for this study. The genome of CVB3 is translated into a long polyprotein precursor that undergoes proteolytic cleavages to produce functional viral proteins. Cleavage of the polyprotein mediated by the viral proteases 2A (2A^{pro}) and 3C (3C^{pro}) is essential for the production of new infectious virions (De Clercq et al., 1991; Tong, 2002; Zell et al., 2004). CVB3 is the primary causative agent of viral myocarditis in humans (McManus et al., 1993; Xiong et al., 2002), a heart disease that leads to dilated cardiomyopathy, which may result in sudden death in children and adolescents or end-stage congestive heart failure in adults (Martino et al., 1994). Although much is known about this pathogen, there is no effective antiviral therapy for prevention and treatment of CVB3 infection currently available, and the only definitive treatment currently available for the viral-induced disease is heart transplantation.

The use of the reporter system for screening of potent compounds against CVB3, and validation of the activity of the selected compounds against CVB3 infection using the plaque reduction assay are described herein. The protocol can be applied as a robust and effective method for high-throughput screen of anti-coxsackievirus compounds.

2. Materials and methods

2.1. Cell culture and viruses

Buffalo green monkey kidney (BGMK) (ATCC, Rockville, MD) cells were cultured in Eagle's minimum essential medium with Earle's salts (EMEM) supplemented with 0.075% NaHCO₃, 2 mM L-glutamine, 10 mM nonessential amino acids, 100 µg/ml penicillin, 100 U/ml streptomycin, 8% (v/v) fetal bovine serum, 20 mM HEPES (pH 7.4), at 37 °C in 5% CO₂. Engineered BGMK cells were grown and maintained in EMEM containing 150 µg/ml Zeocin. Virus strains used in this study were obtained from the American Type Culture Collection. Coxsackievirus B3 strain Nancy (CVB3), Coxsackievirus B6 (CVB6), Echovirus 11 (EV11), Echovirus 17 (EV17), Echovirus 19 (EV19) and Poliovirus type 1 (strain LSc) (PV1) were cultivated in BGMK cells. Hepatitis A strain HM-175 (HAV) was cultivated in fetal rhesus monkey kidney (FRhK-4) cells. Virus stocks were prepared by the freeze-thaw method, followed by chloroform extraction.

Virus titers were determined using the plaque assay in either BGMK or FRhK-4 (HAV) cells. Briefly, 10-fold serial dilutions of virus stocks were distributed on confluent cell monolayers, and samples were incubated for 30 min at 37 °C. After adsorption, the cell monolayer was overlaid with maintenance medium containing 1% (w/v) carboxymethylcellulose (Sigma–Aldrich) and 2% FBS, and incubated at 37 °C for 48 h. Plaques were visualized after cell fixation and staining using 0.8% crystal violet–3.7% formaldehyde solution in PBS.

2.2. Construction of reporter cell system

Engineered BGMK cells expressing the fluorescent protein pair were constructed as described previously (Hwang et al., 2006). Briefly, a short DNA fragment encoding the substrate peptide containing the CVB3 2A^{pro} cleavage site (MTNT/GAFGQ; nucleotide position 3284–3310, accession number M88483) was inserted between the CFP and YFP coding sequences of pUC19.FRET (Fig. 1A) as described (Hwang et al., 2006). The coding region for CFP-CVB3-YFP was transferred into pcDNA3.1(+). Zeocin (Invitrogen, Carlsbad, CA) (generating pCFP-C3-YFP) for expression in mammalian cells. The resulting plasmid was introduced into BGMK cells by lipofection using Lipofectamine Plus reagent (Invitrogen) following the manufacturer's recommendations. The transfected cells were selected for Zeocin resistance in regular growth medium consisting of 200 µg/ml Zeocin (Invitrogen). Stably transfected BGMK cells (designated as BGMK-C3) were sorted using a fluorescent-activated cell sorter (FACS, BD FACSAria cell sorting system) and maintained in regular growth medium containing Zeocin (150 µg/ml).

Expression, cleavage and specificity of the substrate were verified by Western blot using GFP variant-specific antibodies (JL-8, Clontech) employing standard procedures. Cleavage of the protein substrate was visualized using a confocal microscope equipped with CFP (436ex/480em) and FRET (436ex/520em) filters.

2.3. Fluorometer assay

BGMK-C3 cells were seeded in a 96-well black plate (Corning, Lowell, MA); at approximately 90–95% confluence, they were infected with different dosages of CVB3 in a 50-µl volume. After 30 min adsorption at 37 °C, the virus suspension was replaced by 100 µl of MEM with 2% FBS. The infected cells were incubated at 37 °C for 2–24 h post infection (hpi). After incubation, cells were fixed with 1% formaldehyde. The fluorescence intensity of the CFP and YFP was measured using a fluorometer (SynergyTM 4, BIOTEK[®] Instruments, Inc., Winooski, VT) at excitation and emission wavelengths of 430 nm and 460 nm for CFP, and 430 nm and 520 nm for YFP from CFP excitation.

2.4. Plaque reduction assay

BGMK cells in 24-well plates were incubated with 80–100 plaque-forming units (PFU) of CVB3. After virus adsorption, the infecting medium was removed and the cell monolayer was overlaid with 1% carboxymethylcellulose in MEM containing different amounts of test compounds. After 40–48 hpi, cells were fixed and stained with a crystal violet/formaldehyde solution.

2.5. Screening for 2A^{pro} inhibitors and validation

The control compounds anisomycin and glyceryl trinitrate were obtained from Sigma–Aldrich (St. Louis, MO) and AccuStandard (New Haven, CT), respectively. The library tested was the Spectrum Collection 2000 (Microsource Discovery, Inc., Gaylordville, CT), and contained 2000 compounds that are primarily Food and

Drug Administration (FDA)-approved known drugs, bioactive compounds or natural products (www.msdiscovery.com/spect.html). The compounds are supplied as 10 mM solutions in dimethyl sulfoxide (DMSO).

Twenty μM of each compound from the Spectrum Collection were added to BGM-C3 in 96-well black plates using a Biomek[®] FXP Laboratory Automation Workstation. Three wells on each plate served as cell controls (no test compound added) and three wells served as virus controls. In addition, the 50% and 100% plaque inhibitory concentrations of anisomycin (3 wells each) were included as positive controls in each microtiter plate. After 30 min, CVB3 was added into each well at a Multiplicity of Infection (MOI)=2. After 24 h, cells were fixed with 1% formaldehyde and analyzed using the fluorometer. The viral titer of cells without added compound was defined as the original virus concentration, C_0 , and C was the virus concentration of compound-treated cells. The percentage of viral growth inhibition was calculated as $(C_0 - C)/C_0 \times 100\%$. Compounds that reduced FRET intensity relative to uninfected control cells by more than 50% were identified as positive compounds. The quality of the screening assay was evaluated from the Z'-factor (Zhang et al., 1999, 2000) generated from fluorescent signals from infected and uninfected cells. For the cell toxicity assay, 20 μM of each compound was added to BGMK cells in 96-well clear-bottom plates, and analyzed in parallel using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Plumb, 2004).

The IC_{50} and CC_{50} of compounds were determined from dose–response curves determined using plaque reduction and MTT assays, respectively.

3. Results

3.1. Development of the reporter cell and cleavage of the fluorescent substrate

A fluorescent reporter cell system that detects CVB3 $2A^{\text{pro}}$ activity was developed as described (Hwang et al., 2006). A vector, pCFP-C3-YFP, which encodes the CFP–YFP fusion protein linked by a short peptide (9 amino acids in length) containing the cleavage motif for CVB3 $2A^{\text{pro}}$ was generated (Fig. 1A). Using this vector, a stably transfected cell line (designated BGM-C3) was developed by transfecting pCFP-C3-YFP into BGMK cells, to minimize errors associated with transient transfection and to permit the continuous utilization of the reporter cells. To demonstrate the cleavage of the fluorescent substrate by CVB3 $2A^{\text{pro}}$ *in vivo* and to determine if the reporter cells will respond to proteases from enteric viruses other than CVB3, the fluorescent reporter cells were challenged with the same dosages of CVB1, CVB3, CBV6, EV11, EV17, EV19, PV1 and HAV and subjected to Western blot analysis (Fig. 2). As expected, $2A^{\text{pro}}$ -mediated cleavage resulted in the separation of CFP and YFP and the formation of ~ 27 kDa fragments from CVB3-infected cells (lane 2). Results also showed that the fluorescent substrate responded to CVB1, CVB6, EV11 and EV17 $2A^{\text{pro}}$ (lanes 1, 3, 5 and 6) but not to EV19, HAV and PV1 (lanes 7–9), although CPE was also observed in EV19- and PV1-, but not in HAV-challenged cells. This result further suggests the specificity of the fluorescent substrate and the viral $2A^{\text{pro}}$, probably due to variations in the amino acid sequences at the $2A^{\text{pro}}$ cleavage sites as in the case of EV19 and PV1 (data not shown), or lack of infection of cells as in HAV.

To monitor the dynamics of $2A^{\text{pro}}$ activity in intact cells, BGM-C3 cells were infected with CVB3 at a Multiplicity of Infection (MOI) of 4 and observed under a confocal microscope at 0, 3 and 8 h post-infection (hpi). Upon excitation at 436 nm for the CFP, a gradual increase in the CFP emission at 480 nm was observed (Fig. 3, upper level); while a gradual decrease in the YFP (FRET) intensity

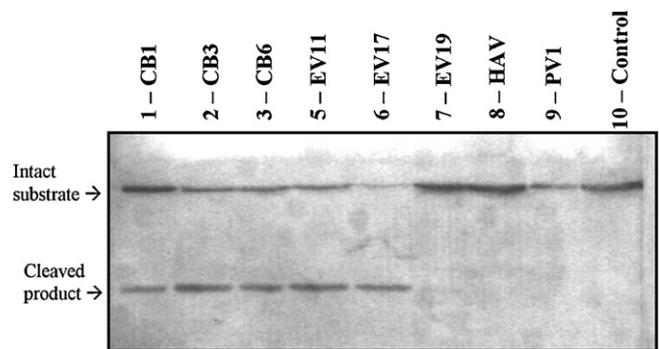


Fig. 2. BGM-C3 cells were infected with the same dosage of Coxsackievirus B1 (CB1), Coxsackievirus B3 (CVB3), Coxsackievirus B6 (CB6), Echovirus 11 (EV11), Echovirus 17 (EV17), Echovirus 19 (EV19), Hepatitis A virus (HAV) and Poliovirus 1 (PV1) at an MOI of 0.1. After 48 hpi, cells were harvested, boiled, then run in 10% acrylamide gel. Western blot was performed using anti-GFP as primary antibody. $2A^{\text{pro}}$ -mediated cleavage resulted in the separation of CFP and YFP. The reporter cells responded only to CB1, CVB3, CB6, EV11 and EV19.

(436ex/520em) was detected as the infection progressed. Overlaid images of the two fluorescent intensities showed a clear shift toward the CFP emission with increasing infection time (green).

3.2. Quantitation of the infection process using a fluorometer

To determine if changes in FRET intensity due to CVB3 $2A^{\text{pro}}$ activity could be detected using a fluorometer, the BGM-C3 cells were infected with CVB3 of various dosages ranging from MOI of 0.03–2. As a negative control, BGM-C3 cells were infected with EV19 at various dosages similar to CVB3. The degree of infection was represented by the FRET ratio defined as the CFP intensity over the FRET intensity when excited at 430 nm. As expected, the CVB3-infected cells exhibited a progressive decline in YFP/CFP ratio in a dose- and time-dependent fashion (Fig. 4A), consistent with those from the imaging analyses (Fig. 3). In contrast, EV19-infected cells did not result in any measurable decrease in the FRET ratio regardless of the titers and infection durations (Fig. 4B). It should be noted that no further decrease in the FRET ratio was observed between 24 and 36 hpi for cells infected with an MOI of 2, suggesting that maximum cleavage was achieved after 24 hpi.

3.3. Correlation of fluorometer and plaque reduction assays in determining the IC_{50} of known protease inhibitor

The result of the fluorometer assay was validated using the plaque reduction assay (PA). The PA was performed in measuring the inhibitory concentrations of the previously reported protease inhibitors, anisomycin (Hwang et al., 2008) and GTN (Zell et al., 2004). CVB3-infected reporter cells at MOI of 2 were exposed to different concentrations of either anisomycin or GTN, and the percentage of viral inhibition was determined using both assays. As shown in Fig. 5, the dose–response curves obtained from both assays are virtually identical, with the IC_{50} values of anisomycin and GTN from both assays being comparable. This result confirms that this rapid fluorometer assay can be used to assess the efficiency of viral inhibitors.

3.4. Screening for CVB3 $2A^{\text{pro}}$ inhibitors and potency of the selected positive hit compounds

To demonstrate the potential use of the established fluorometer assay for high-throughput screen (HTS) of antiviral drugs, a chemical library with 2000 bioactive and naturally occurring compounds was screened for CVB3 protease inhibitors. The compounds were

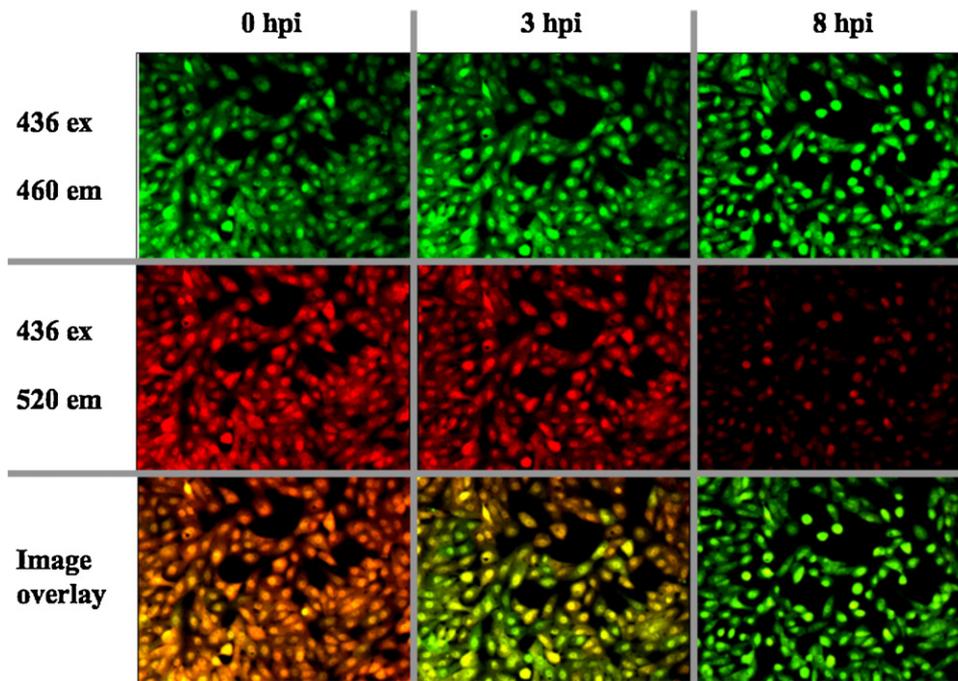


Fig. 3. BGM-C3 was infected by CVB3 at an MOI of 4. Cells were observed at different time points under a confocal microscope equipped with CFP, YFP and FRET filter sets. Upon excitation at 436 nm and emission at 480 nm for CFP, a gradual increase in fluorescence intensity was observed (upper level); while a gradual decrease in intensity in FRET (436ex/520em) was detected as the infection progressed (mid-level). Overlaid image of the two figures (lower level) showed a shift in CFP intensity (green). Similarly, the cytopathic effects, including cell rounding and shrinkage of infected cells, become more distinct along with the infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tested at a final concentration of 20 μ M. Infected BGM-C3 cells were used as a negative control, while uninfected BGM-C3 cells were used as a positive control. The average FRET ratio for the negative control was 2.87 ± 0.36 , and the average for the positive controls was 6.36 ± 0.21 (Fig. 6). The Z'-factor, which indicates the quality of the assay without intervention of the test compounds, was calculated from 60 replicate negative controls and 60 replicate positive controls (Zhang et al., 1999), and had a value of 0.51, suggesting that the assay is of excellent quality.

From the initial screen, we identified 64 compounds that reduced the FRET ratio relative to uninfected control cells by more than 50% with minimal effects on the cell viability at the tested concentration (using the MTT assay). The positive compounds are mostly therapeutic drugs (e.g., tamoxifen), antimicrobial compounds and a few with unknown functions. Baicalein, a flavonoid that inhibits human cytomegalovirus (Evers et al., 2005), was also identified in the initial screen.

To verify the potency of the selected compounds from the initial screening, the PA was conducted in BGMK cells. Carvedilol tar-

trate, isorotenone and baicalein, were chosen for the MTT assay and the PA to validate the efficacy of the screen, since these compounds scored positive in the initial screen. For comparison, other compounds that scored less than 50% inhibition at the level tested (cosmosiin, ribavirin and rhoifolin) were also included in the analysis to further confirm the assay results. The calculated IC_{50} , CC_{50} and the SI of the tested compounds and their mode of actions are summarized in Table 1. Among the known antiviral drugs, baicalein (34.5) is more potent than ribavirin (15.1). The assay was further validated with two other compounds, carvedilol tartrate and rhoifolin with calculated SI values of 1.4 and 1.8, respectively. Among the three new inhibitory compounds identified, isorotenone is the most potent against CVB3, and also the most toxic for the cells, with IC_{50} , CC_{50} and SI of 0.1, 1 and 10, respectively. However, because of the very low CC_{50} of this compound, it is probable that its antiviral effect may have been due to cell toxicity. Further research is necessary to elucidate its mode of action against CVB3. The inhibition efficiencies of the different compounds obtained from the initial screen and the plaque reduc-

Table 1
Cytotoxicity and antiviral potency of identified antiviral compounds.

Compound	% Inhibition (20 μ M ^a)	IC_{50} (μ M)	CC_{50} (μ M)	SI	Mode of action
Carvedilol	100	16.92	22.82	1.35	Beta adrenergic blocker
Isorotenone	100	0.1	0.95	9.5	NADH dehydrogenase inhibitor
Baicalein	91	29.02	>1000	>34.46	Antiviral
Cosmosiin	22	232.76	>1000	>5.34	Antiviral
Rhoifolin	13	569.05	>1000	>1.76	Unknown
Ribavirin	16	66.39	>1000	>15.06	Antiviral
Controls					
Anisomycin	100	0.17	5.98	35.18	Protein synthesis inhibitor
Glyceryl trinitrate	n.d.	39.16	150.98	3.86	CVB3 2A ^{pro} inhibitor; nitric oxide donor

IC_{50} (50% inhibitory concentration) is the compound concentration reducing the 2A^{pro} activity by 50%, as determined using plaque reduction assay. CC_{50} (50% cytotoxic concentration) is the compound concentration reducing the viability of untreated cell cultures by 50%, as determined by MTT assay. SI (selective index) is CC_{50} divided by IC_{50} . n.d., no data.

^a % Inhibition at 20 μ M concentration as determined by reporter cell assay.

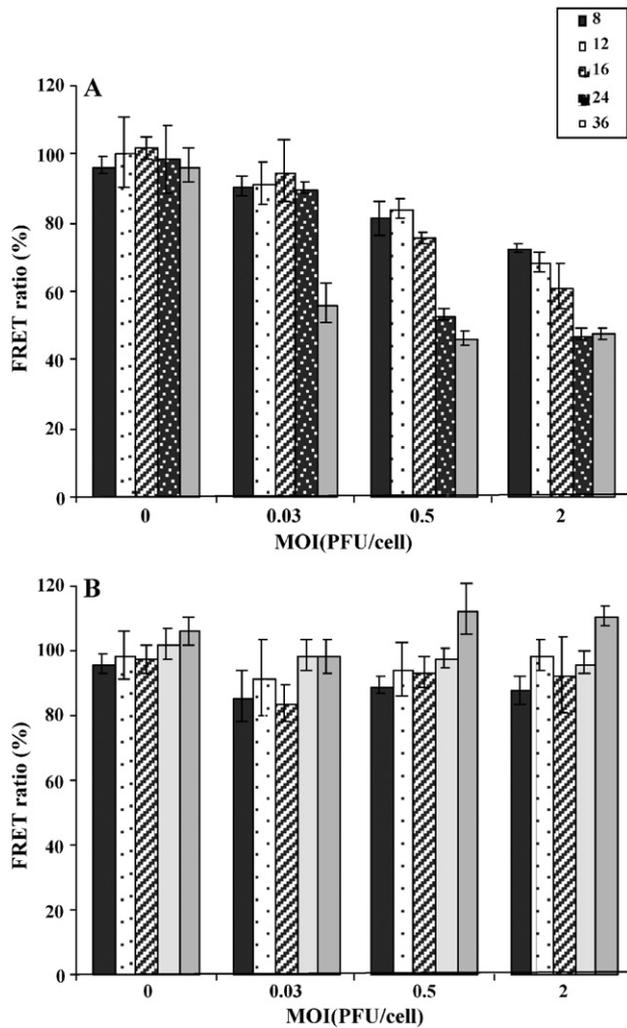


Fig. 4. BGM-C3 cells in 96-well black plates were infected with different dosages of either (A) CVB3 or (B) EV19 (as negative control). Infection was measured in terms of an increase in CFP fluorescence at several time points within 36 hpi. FRET was expressed as a ratio of excited fluorescence emitted by the acceptor (YFP) at 520 nm divided by that emitted by the donor (CFP) at 460 nm at the excitatory wavelength of the donor (430 nm).

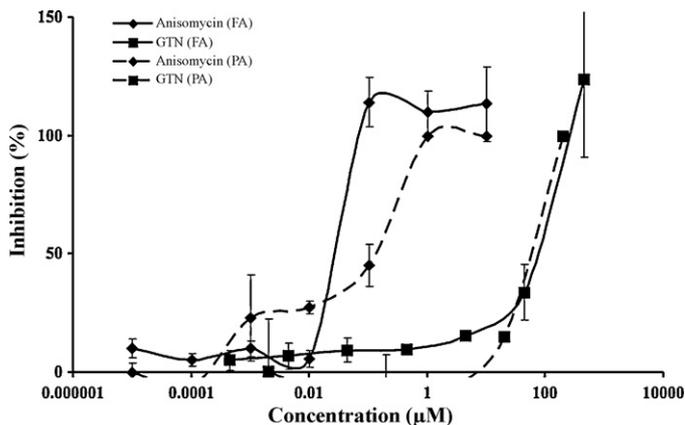


Fig. 5. BGM-C3 cells in either 96-well black plate (for FRET-based assay, FA) or 24-well plate (for plaque reduction assay, PA) were challenged with the same dosage of CVB3. After adsorption, cells were incubated with different concentrations of known protease inhibitors (anisomycin and glyceryl trinitrate (GTN) in maintenance medium).

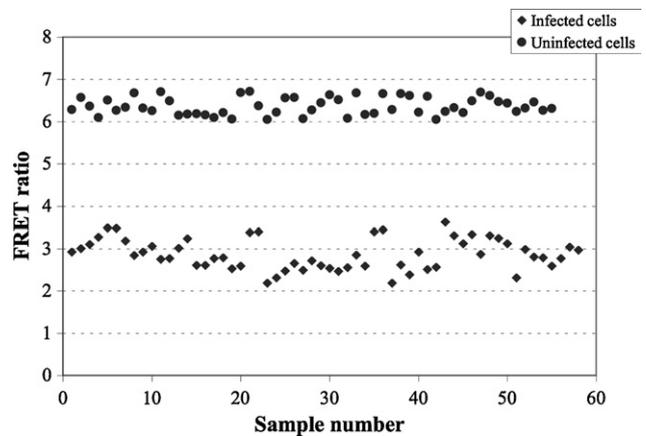


Fig. 6. Typical FRET values from infected (diamond data points) and uninfected (circular data points) cells run in different assay plates.

tion assay are in agreement with each other, again validating the use of the fluorescence screen as a rapid method for viral inhibitor discovery.

4. Discussion

In an effort to search for compounds active against virus infections, a screening protocol was developed using a FRET-based reporter system that responds specifically to the viral 2A protease activity, which plays a crucial role, and is expressed early, in the viral replication cycle. Because of the severity of the disease caused by CVB3 infection in humans which, if not treated properly may progress to end-stage cardiac failure and death, and the lack of effective antiviral therapy for the CVB3-mediated myocardial infections (Why et al., 1994), the protocol was specifically designed to target CVB3-infected cells.

During CVB3 infection of the reporter cells, the CVB3 2A^{pro} that were produced during active viral replication cleaved the fluorescent substrate at the protease cleavage site and disrupted FRET, resulting in a change in the CFP/YFP ratio (Figs. 2 and 3). The changes in the fluorescence ratio were successfully quantified using a fluorometer and were shown to correlate directly with the inhibition efficiencies of two known protease inhibitors, anisomycin and GTN (Fig. 5) based on the plaque reduction assay.

When the assay was used to screen for antiviral compounds from a chemical library of known bioactive compounds, approximately 60 compounds from a chemical library that caused at least 50% inhibition of CVB3 2A^{pro} activity with minimal effects on the host cells at 20 μ M concentration were identified (Table 1). Moreover, several known antiviral compounds such as ribavirin, baicalein and cosmosiin were identified in the initial screening. Ribavirin, an inhibitor of human immunodeficiency virus (HIV) reverse transcriptase (Fernandez-Larsson and Patterson, 1990; Patterson and Fernandez-Larsson, 1990), also inhibited CVB3 replication synergistically with alpha interferon (IFN-alpha) (Markland et al., 2000; Okada et al., 1992; Wang et al., 2000), and suppressed infectious virus progeny in human myocardial fibroblasts (Heim et al., 1997). Baicalein, a plant flavonoid that displays a wide spectrum of antiviral activities (Evers et al., 2005), can potentially inhibit CVB3 by blocking virus entry into cells. Cosmosiin, a known antimicrobial compound (Abd-Alla et al., 2009), exhibits antioxidant activity (He et al., 2003; Mikhaeil et al., 2004) and probably helps protect the cells by enhancing interferon response similar to that of ascorbic acid (Siegel, 1974).

More importantly, three other compounds with little or no known antiviral functions were also identified using the devel-

oped protocol. Carvedilol, a nonselective beta-blocker, may protect against viral myocarditis primarily by its antioxidant property and the up-regulation of anti-inflammatory cytokines (Nishio et al., 2003; Tschöpe et al., 2004; Yue-Chun et al., 2008). It is being used to treat dilated cardiomyopathy and is appropriate for post-myocarditis patients (Furberg and Psaty, 2004; Liu et al., 1996; Schwarz et al., 2005). Rhoifolin, a plant flavonoid with no known antiviral action, was also found to inhibit CVB3 infection. Similar to most flavonoids with antimicrobial and antiviral activities (Kaul et al., 1985; Sanchez et al., 2000), its antiviral mechanism may be due to the prevention of virus adsorption onto the cell surface (Evers et al., 2005), inhibition of protein kinase, viral DNA synthesis (Barnard et al., 1993) or virus-associated reverse transcriptase (Chu et al., 2004), among others. Isorotenone, on the other hand, may have exerted its antiviral effect by interfering with the host's electron transport system and inhibition of NADH dehydrogenase (Burgos and Redfean, 1965; Lloyd, 1966), thereby preventing NADH from being converted into usable cellular energy (ATP) upon drug treatment, thus the host cells fail to support virus growth. Further studies on the mechanisms of action of these compounds are needed to better understand their full potential as antiviral drugs.

In summary, the reporter system described herein enabled the rapid screen of compounds with inhibitory activity against CVB3 infection. The method is simple and requires short total assay time. From the time of cell infection and addition of test compounds, the fluorometer-based assay takes about 12–16 h in total including analysis. Moreover, the reporter assay is conducted in a 96-well plate format, which enabled the analysis of a large number of test compounds at the same time, and the fluorometer provided a rapid and objective readout of the results. These make the FRET-based reporter system amenable for use in the routine diagnostic virology laboratory and high throughput screening (HTS) of antiviral compounds. The quality of the assay can be improved further by optimizing the assay conditions using defined cell numbers, virus doses, and optimal incubation time. The signal to noise (S/N) ratio, on the other hand, can be increased by effectively varying the length of the linker peptide to optimize FRET intensity, or by using an improved fluorescent protein pair with narrow spectral overlap. Since the assay does not rely on the formation of CPE, it would be useful in antiviral drug discovery for viruses that do not form plaques or infection that does not lead to CPE (e.g., hepatitis A). Lastly, the compounds validated in this study offer a potentially acceptable alternative as a therapy for CVB3 infections, or may be found useful in the development of novel anti-CVB3 agents.

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