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Short communication

# Rapid identification of inhibitors that interfere with poliovirus replication using a cell-based assay

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## Abstract

A small molecule library containing 480 known bioactive compounds was screened for antiviral activity against poliovirus (PV) using a cellular fluorescence resonance energy transfer (FRET) assay for viral protease activity. The infected reporter cells treated with the viral replication-suppressing compounds were examined via fluorescence microscope 7.5 h postinfection. Twelve molecules showed moderate to potent antiviral activity at concentrations less than 32  $\mu$ M during the primary screening. Three compounds, anisomycin, linoleic acid, and lycorine, were chosen for validation. A dose-dependent cytotoxicity assay and a secondary screening using conventional plaque assay were conducted to confirm the results. The developed method can be used for rapid screening for molecules with antiviral activity.

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**Keywords:** Cellular sensor; Protease; Viral replication; FRET

## 1. Article outline

Poliovirus (PV), a member of the genus *Enterovirus* (family Picornaviridae), has been used as a model organism for the study of non-enveloped virus life cycles. Although the detailed molecular basis of PV replication is not fully understood, it is clear that viral replication depends on interactions with host factors for viral gene expression, RNA replication, and virus assembly (Andino et al., 1993; Belov et al., 2003, 2004; Egger et al., 2000). However, the specific functions of these host cellular factors have yet to be determined. Despite decades of studying mechanisms of viral replication, the development of systematic protocols to identify factors modulating the virus life cycle has been minimal. With advances in chemical genomics, having access to chemical libraries of compounds with diverse functions is no longer the limiting factor. The emergence of high-throughput screening (HTS) has called for the development of an accurate and efficient reporter system.

**Abbreviations:** 2A<sup>pro</sup>, 2A protease; FRET, fluorescence resonance energy transfer.

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A cell-based reporter assay for the identification of small molecules suppressing PV replication was established in previous study (Hwang et al., 2006). Cell-based assays are ideal for developing high-throughput methodology because they provide information to address issues raised from chemical-based *in vitro* studies, such as cytotoxicity caused by treatment, drug transport efficiency to the cell, and the effective doses of target compounds, and thus are ideal for developing high-throughput methodologies. However, cell-based platform available to elucidate the roles of host factors in viral replication remains limited. In an attempt to fulfill the demand for an effective screening protocol of inhibitors against virus replication, we established a cellular platform to exploit the potential application for HTS.

We have recently developed a cellular reporter system using fluorescence resonance energy transfer (FRET) for viral protease activity as an indication of active viral reproduction (Hwang et al., 2006). FRET is a phenomenon involving two fluorochromes with overlapping emission and excitation spectra. The energy from the donor fluorophore is partially transferred to the acceptor if the distance between the two fluorophores is less than 60 Å (Jares-Erijman and Jovin, 2003). Buffalo green monkey kidney (BGMK) (ATCC, Rockville, MD) cells were stably transfected with the plasmid encoding an enhanced cyan fluorescent

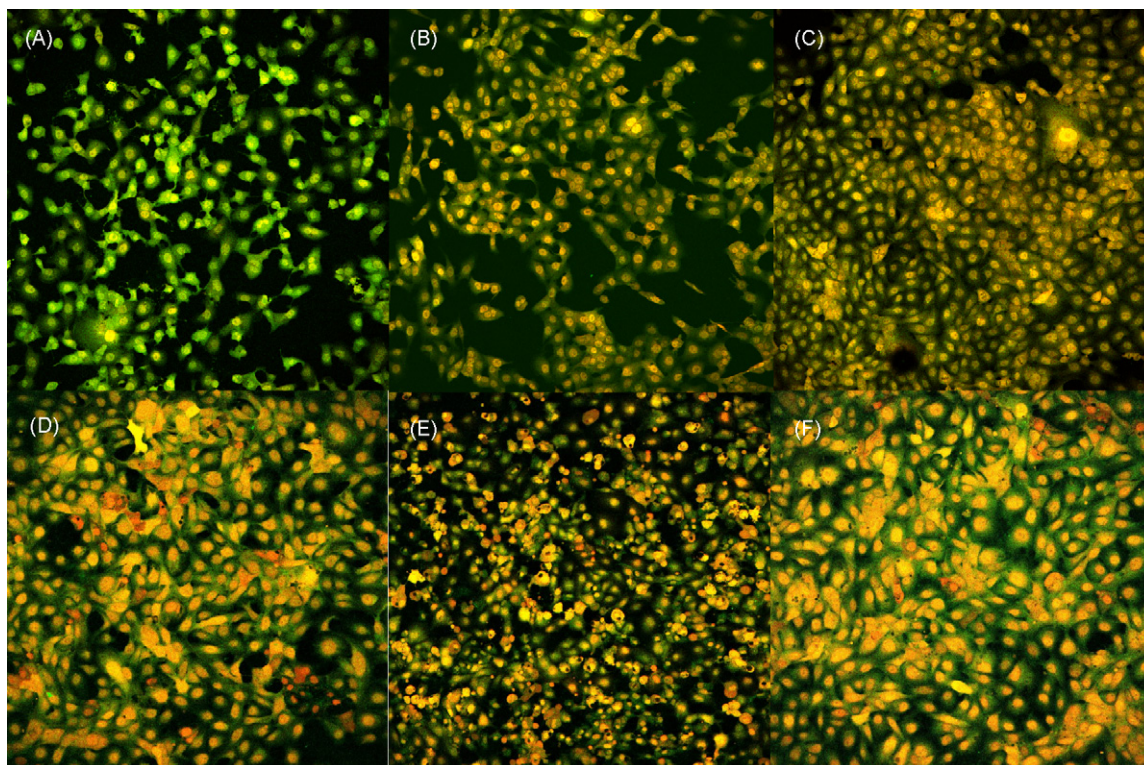


Fig. 1. Cell-based FRET assay for the detection of PV replication. Effects of the inhibitor on the reporter cells challenged with PV1 were tested. Samples were fixed 7.5 h postinfection and then the images were acquired via a fluorescence microscope (Nikon Eclipse TE2000-U) at magnification of 200 $\times$  with CFP (excitation of 436 nm and emission of 460 nm) and YFP (excitation of 500 nm and emission of 535 nm) filter set, respectively. Cells with active 2A<sup>pro</sup> activity are shown in green, while cells without proteolysis are in yellow. The cells were mock infected (C) or infected with PV1 at an input MOI of 10 and treated with DMSO only (A), 1 mM MPCMK (B), 31.4  $\mu$ M anisomycin (D), 1.7  $\mu$ M linoleic acid (E), or 25.7  $\mu$ M lycorine (F). For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

protein (ECFP) and an enhanced yellow fluorescent protein (EYFP) FRET pair linked with the 2A<sup>pro</sup> cleavage sequences (Hwang et al., 2006). The PV 2A<sup>pro</sup>, which is produced at an early stage of the replication cycle (Cao et al., 1993), cleaves the linker peptide flanking the FRET pair; the separation of the fluorescent protein pair was monitored using fluorescence microscopy (Fig. 1).

A chemical library with 480 bioactive small molecules was chosen to demonstrate the potential usage of the established cellular FRET assay for high-content screening. Since most of the compounds in the chosen library target specific cellular functions, this approach is suitable to map cellular factors or pathways contributing to PV replication. For the primary screening, the reporter cells were seeded in 384-well plates with the culture medium and infected with PV 1 at a multiplicity of infection (MOI) of 10. The culture medium was removed by aspiration and the virus suspension in DMEM with one of the bioactive small molecules was added to each well. *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MPCMK) (Sigma–Aldrich), a known 2A<sup>pro</sup> inhibitor, was included as a positive control (Fig. 1B). Samples were incubated for 7.5 h at 37 °C, and then fixed with 3.7% formaldehyde in PBS at room temperature for 5 min. The samples were examined under an inverted fluorescence microscope (Nikon Eclipse TE2000-U) with CFP (excitation wavelength of 436 nm and emission wavelength of 460 nm) and YFP (excitation wave-

length of 500 nm and emission wavelength of 535 nm) filter set.

The compounds were tested at a final concentration of less than 32  $\mu$ M. Internal controls included infected reporter cells as a negative control, infected reporter cells treated with 1 mM MPCMK as a positive control, and uninfected reporter cells for comparison (Fig. 1A–C). The microscopy-based analysis also recognized toxic compounds during the screen on the basis of cellular morphological changes due to the treatment. From the primary screening, we identified 12 compounds that caused the FRET signal in virus-infected reporter cells to be retained, indicating potential antiviral activity (Table 1). The positive compounds included molecules affecting a wide range of host cellular functions, from general protein synthesis inhibitors (e.g., cycloheximide) to calcium channel blockers (e.g., U-50488). The mode of action of each positive compound is summarized in Table 1.

Anisomycin, linoleic acid, and lycorine were chosen to assay for cytotoxicity and followed by dose–response tests to validate the efficacy of the primary screen. A cell proliferation-based MTT assay (CHEMICON, Temecula, CA) was used to examine the level of cytotoxicity due to drug treatment. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, is based on the ability of a mitochondrial dehydrogenase from living cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals that absorb light

Table 1  
Candidate compounds from the primary screen

Name	Mode of action	Concentration ( $\mu\text{M}$ )
AG-879	NGF receptor inhibitor	26.4
Anisomycin	MAP kinase activator	31.4
Brefeldin A	GTP cycling inhibitor	29.7
Ciglitazone	PPAR $\gamma$ agonist	1.7
Cycloheximide	Protein synthesis inhibitor	29.6
FCCP	Mitochondrial uncoupler	11.6
GW-5074	cRaf1 kinase inhibitor	5.6
Linoleic acid	Polyunsaturated fatty acid	1.7
Lycorine	TNF- $\alpha$ production inhibitor	25.7
Ouabain	Na-K-ATPase inhibitor	4.0
Trequinsin	Phosphodiesterase (PDE3) inhibitor	18.9
U-50488	Calcium channels	17.9

at a wavelength of 570 nm. A viral titer reduction assay using conventional plaque assay was conducted to verify the potency of the selected compounds from the primary screening. The tested compounds were dissolved in DMSO with a stock concentration of 10 mM, and the working solutions were diluted in DMEM to achieve desired concentrations. Each compound was tested at four concentrations, 1.25, 2.5, 5, and 10  $\mu\text{M}$ , in triplicate. BGMK cells were seeded in 24-well plates (Corning) and infected with PV 1 at a MOI of 10. After adsorption, 1 ml of an appropriate concentration of the test compound was added to each well. The supernatant from the wells containing infected cells was collected after 8 h of incubation and the viral titer of each sample was determined using plaque assay.

Anisomycin achieved 2  $\log_{10}$  unit reduction of virus titer at 1.25  $\mu\text{M}$  with negligible cytotoxicity (Fig. 2A). Anisomycin, originally discovered as a peptidyl transferase inhibitor secreted by *Streptomyces* spp., is also a potent activator of mitogen-activated protein (MAP) kinase cascades (Barros et al., 1997; Grollman, 1967; Zinck et al., 1995). Anisomycin showed strong inhibition against PV replication in this study (Fig. 2A) as well as in prior reports (Ehrenfeld and Manis, 1979; Hazzalin et al., 1998). Linoleic acid, a polyunsaturated fatty acid, showed only 0.52  $\log_{10}$  unit reduction of virus titer at 10  $\mu\text{M}$  (Fig. 2B). Lycorine caused 1  $\log_{10}$  unit reduction of virus titer at 2.5  $\mu\text{M}$  with no signs of cytotoxicity (Fig. 2C). Earlier studies have demonstrated that lycorine, a TNF- $\alpha$  production inhibitor found in plant species *Lycoris* and *Narcissus* (Vrijzen et al., 1986) exhibits antiviral against PV replication, herpes simplex virus and severe acute respiratory syndrome coronavirus (Ieven et al., 1982; Li et al., 2005; Renard-Nozaki et al., 1989). Both anisomycin and lycorine exhibited strong antiviral activity against PV replication, with anisomycin being the most potent compound among all that were tested; however, linoleic acid was not a significant PV inhibitor at the condition tested (Fig. 2).

A dose-dependent response curve of anisomycin further confirmed the robustness of the developed protocol (Fig. 3). The 50% inhibitory concentration ( $\text{IC}_{50}$ ) of PV replication was determined using the viral titer reduction assay described previously. The infected reporter cells were incubated with 10-fold serial dilutions (from  $10^{-2}$  nM to 1  $\mu\text{M}$ ) of the compounds in order to

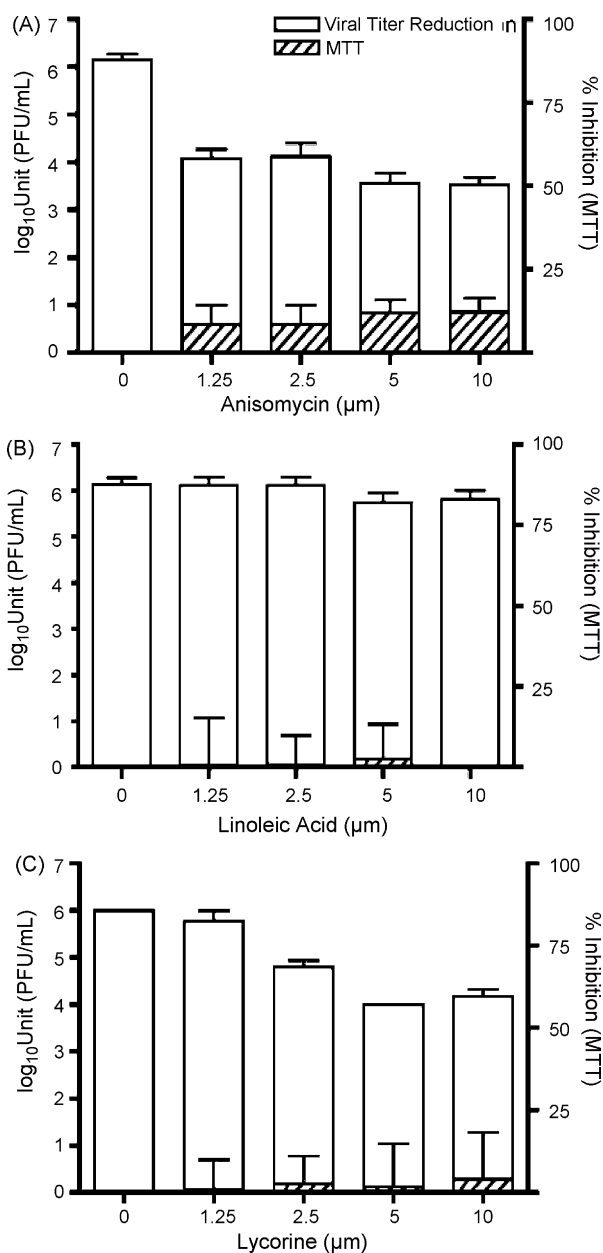


Fig. 2. Selected positive compounds from the cellular screen. Anisomycin (A), linoleic acid (B), and lycorine (C) were chosen for detailed dose-dependent tests of viral titer reduction and MTT assay for cytotoxicity levels. Each data point was collected from two independent experiments with two replicates within each experiment and the error bar represents standard deviation. Left y-axis, viral titer in  $\log_{10}$  unit (PFU/ml). Right y-axis, percentage inhibition of cellular proliferation.

obtain an accurate  $\text{IC}_{50}$ . A non-linear regression was applied to fit the curve using SigmaPlot (Systat Software, San Jose, CA). The viral titer of cells without treatment was defined as the original virus concentration,  $C_0$ , and  $C$  represented the virus concentration of anisomycin-treated cells. The percentage of viral growth inhibition was calculated as  $(C_0 - C)/C_0 \times 100\%$ . The calculated  $\text{IC}_{50}$  of anisomycin is 2.35 nM, which has a negligible effect on host protein synthesis (Grollman, 1967).

Some of the positive compounds from the primary screening are cellular function-specific molecules that have been reported

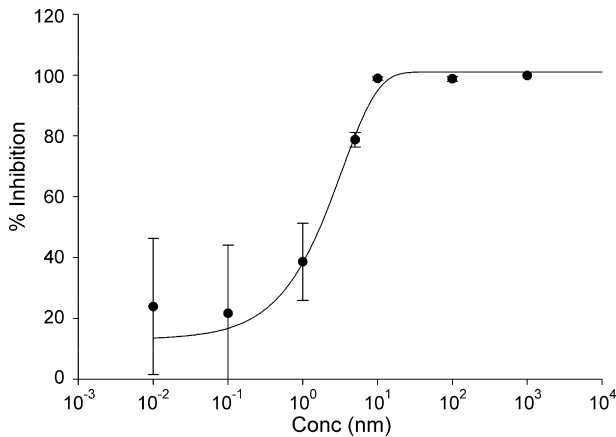


Fig. 3. IC<sub>50</sub> curve of anisomycin. The infected reporter cells were treated with anisomycin and the viral titer reduction assay was performed to determine the virus concentration. The percentage of viral growth inhibition represents the difference in viral titer between cells treated with and without anisomycin divided by virus concentration of the untreated cells. Each data point was collected in triplicate within one experiment.

to have antiviral activity according to previous research. It has been reported that cycloheximide, a translational elongation inhibitor, blocks general virus replication due to the fact that the host protein synthesis is impaired upon drug treatment, thus the host cells fail to support virus growth (Barton et al., 1999). A potent PV replication inhibitor, brefeldin A, which blocks GTP cycling and results in the disruption of the Golgi complex, also scored positive in the screening (Crotty et al., 2004). Chao et al. (2001), has shown that U-50488, a calcium channel inhibitor, inhibits HIV-1 protein expression. Lipid metabolism is thought to be associated with the modulation of virus replication (Kapadia and Chisari, 2005; Cherry et al., 2006). We identified three compounds, ciglitazone (PPAR  $\gamma$  agonist), FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, mitochondrial uncoupler), and linoleic acid, that target the peroxisome proliferators-activated receptors (PPAR) signaling pathway, which is involved in the signal transduction cascade of fatty acid and lipid metabolism (Nedergaard et al., 2001; Latruffe and Vamecq, 1997). In addition, this study also identified other small molecules involved in pathways that are novel for their association with PV replication, such as AG-879, GW-5074, ouabain, and trequinsin (Table 1). Further investigation of the roles of these compounds in PV replication is needed to advance the understanding of the mechanisms involved.

In summary, we describe a cellular reporter system targeting PV replication, which should enable the rapid identification of PV-specific inhibitory molecules. It requires 48 h to observe the plaque forming units via plaque assay, while the incubation time of the fluorescent assay is 7.5 h. Preliminary data has shown the change in ECFP/EYFP ratio can be measured using a fluorescence plate reader with a  $z$ -factor of 0.64. This assay is also amenable to high-content high-throughput microscopy with automation of sample preparation, image acquisition and analysis, and data generation and mining (Starkuviene and Peppertkok, 2007).

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