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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 μM 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.

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.

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...
protein (ECFP) and an enhanced yellow fluorescent protein (EYFP) FRET pair linked with the 2Apro cleavage sequences (Hwang et al., 2006). The PV 2Apro, 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 PV1 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 2Apro 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 wavelength of 500 nm and emission wavelength of 535 nm) filter set.

The compounds were tested at a final concentration of less than 32 μM. 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 (μM) |
|---------------|--------------------------------|--------------------|
| AG-879        | NGF receptor inhibitor         | 26.4               |
| Anisomycin    | MAP kinase activator           | 31.4               |
| Brefeldin A   | GTP cycling inhibitor          | 29.7               |
| Ciglitazone   | PPAR γ agonist                 | 1.7                |
| Cycloheximide | Protein synthesis inhibitor     | 29.6               |
| FCCP          | Mitochondrial uncoupler        | 11.6               |
| GW-5074       | cRaf1 kinase inhibitor         | 5.6                |
| Linoleic acid | Polysaturated fatty acid       | 1.7                |
| Lycorine      | TNF-α 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 μM, 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 log10 unit reduction of virus titer at 1.25 μM 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 log10 unit reduction of virus titer at 10 μM (Fig. 2B). Lycorine caused 1 log10 unit reduction of virus titer at 2.5 μM with no signs of cytotoxicity (Fig. 2C). Earlier studies have demonstrated that lycorine, a TNF-α production inhibitor found in plant species Lycoris and Narcissus (Vrijsen et al., 1986) exhibits antiviral against PV replication, herpes simplex virus and severe acute respiratory syndrome coronavirus (Leven 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 \( IC_{50} \) 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 μM) of the compounds in order to obtain an accurate \( IC_{50} \). 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 \( \left( \frac{C_0 - C}{C_0} \right) \times 100\% \). The calculated \( IC_{50} \) 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...
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). Crotty 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 (PPARγ 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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References

Andino, R., Rieckhof, G., Achacoso, P., Baltimore, D., 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. EMBO J. 12, 3587–3598.
Barros, L., Young, M., Saklatvala, J., Baldwin, S., 1997. Evidence of two mechanisms for the activation of the glucose transporter GLUT1 by anisomycin: p38 (MAP kinase) activation and protein synthesis inhibition in mammalian cells. J. Physiol. 540, 517–525.
Barton, D., Morasco, B., Flanagan, J., 1999. Translating ribosomes inhibit poliovirus negative-strand RNA synthesis. J. Virol. 73, 10104–10112.
Belov, G., Romovana, L., Tolskaya, E., Kolesnikova, M., Lazebnik, Y., Agol, V., 2003. The major apoptotic pathway activated and suppressed by poliovirus. J. Virol. 77, 45–56.
Belov, G., Lidsky, P., Mikitas, O., Egger, D., Lukyanov, K., Bienz, K., Agol, V., 2004. Bidirectional increase in permeability of nuclear envelope upon poliovirus infection and accompanying alterations of nuclear pores. J. Virol. 78, 10166–10177.
Cao, X., Kuhn, R., Wimmer, E., 1993. Replication of poliovirus RNA containing two VPg coding sequences leads to a specific deletion event. J. Virol. 67, 5572–5578.
Chao, C., Gekker, G., Sheng, W., Hu, S., Peterson, P., 2001. U50488 inhibits HIV-1 expression in acutely infected monocyte-derived macrophages. Drug Alcohol Depend. 62, 149–154.
Cherry, S., Kunte, A., Wang, H., Coyne, C., Rawson, R., Perrimon, N., 2006. COPI activity coupled with fatty acid biosynthesis is required for viral replication. PLoS Pathog. 2, 900–912.
Crotty, S., Saleh, M., Gitlin, L., Beske, O., Andino, R., 2004. The poliovirus replication machinery can escape inhibition by an antiviral drug that targets a host cell protein. J. Virol. 78, 3378–3386.
Egger, D., Teterina, N., Ehrenfeld, E., Bienz, K., 2000. Formation of the poliovirus replication complex requires coupled viral translation, vesicle production, and viral RNA synthesis. J. Virol. 74, 6570–6580.
Ehrenfeld, E., Manis, S., 1979. Inhibition of 80S initiation complex formation by infection with poliovirus. J. Gen. Virol. 43, 441–445.
Grollman, A., 1967. A Inhibitors of protein biosynthesis. II. Mode of action of anisomycin. J. Biol. Chem. 242, 3226–3233.
Hazzalin, C., Le Panse, R., Cano, E., Mahadevan, L., 1998. Anisomycin selectively desensitizes signalling components involved in stress kinase activation and fos and jun induction. Mol. Cell. Biol. 18, 1844–1854.
Hwang, Y., Chen, W., Yates, M., 2006. Use of fluorescence resonance energy transfer for rapid detection of enteroviral infection in vivo. Appl. Environ. Microbiol. 72, 3710–3715.
Jeven, M., Vlietinck, A., Vandenv Berge, D., Totte, J., Dommisse, R., Esmans, E., Alderweireldt, F., 1982. Plant antiviral agents. III. Isolation of alkaloids from Clivia miniata Regel (Amaryllidaceae). J. Nat. Prod. 45, 564–573.
Jares-Erijman, E., Jovin, T., 2003. FRET imaging. Nat. Biotechnol. 21, 1387–1395.
Kapadia, S., Chisari, F., 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc. Natl. Acad. Sci. U.S.A. 102, 2561–2566.
Latruffe, N., Vamecq, J., 1997. Peroxisome proliferators and peroxisome proliferator activated receptors (PPARs) as regulators of lipid metabolism. Biochimie 79, 81–94.
Li, S., Chen, C., Zhang, H., Guo, H., Wang, H., Wang, L., Zhang, X., Hua, S., Yu, J., Xiao, P., Li, R., Tan, X., 2005. Identification of natural compounds...
with antiviral activities against SARS-associated coronavirus. Antiviral Res. 67, 18–23.
Nedergaard, J., Golozoubova, V., Matthias, A., Shabalina, I., Ohba, K., Ohlson, K., Jacobsson, A., Cannon, B., 2001. Life without UCP1: mitochondrial, cellular and organismal characteristics of the UCP1-ablated mice. Biochem. Soc. Trans. 29, 756–763.
Renard-Nozaki, J., Kim, T., Imakura, Y., Kihara, M., Kobayashi, S., 1989. Effect of alkaloids isolated from Amaryllidaceae on herpes simplex virus. Res. Virol. 140, 115–128.
Starkuviene, V., Peppertkok, R., 2007. The potential of high-content high-throughput microscopy in drug discovery. Br. J. Pharmacol. 152, 62–71.
Vrijsen, R., Vanden Berghe, D., Vlietinck, A., Boeyé, A., 1986. A Lycorine: a eukaryotic termination inhibitor? J. Biol. Chem. 261, 505–507.
Zinck, R., Cahill, M., Kracht, M., Sachsenhaier, C., Hippskind, R., Nordheim, A., 1995. Protein synthesis inhibitors reveal differential regulation of mitogen-activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1. Mol. Cell. Biol. 15, 4930–4938.