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Application of a cell-based protease assay for testing inhibitors of picornavirus 3C proteases

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ABSTRACT

Proteolytical cleavage of the picornaviral polyprotein is essential for viral replication. Therefore, viral proteases are attractive targets for anti-viral therapy. Most assays available for testing proteolytical activity of proteases are performed *in vitro*, using heterologously expressed proteases and peptide substrates. To deal with the disadvantages associated with *in vitro* assays, we modified a cell-based protease assay for picornavirus proteases. The assay is based on the induction of expression of a firefly luciferase reporter by a chimeric transcription factor in which the viral protease and cleavage sites are inserted between the GAL4 binding domain and the VP16 activation domain. Firefly luciferase expression is dependent on cleavage of the transcription factor by the viral protease. This biosafe assay enables testing the effect of compounds on protease activity in cells while circumventing the need for infection. We designed the assay for 3C proteases (3C^{pro}) of various enteroviruses as well as of viruses of several other picornavirus genera, and show that the assay is amenable for use in a high-throughput setting. Furthermore, we show that the spectrum of activity of 3C^{pro} inhibitor AG7088 (rupintrivir) not only encompasses enterovirus 3C^{pro} but also 3C^{pro} of foot-and-mouth disease virus (FMDV), an aphthovirus. In contrary, AG7404 (compound 1), an analogue of AG7088, had no effect on FMDV 3C^{pro} activity, for which we provide a structural explanation.

1. Introduction

Picornaviruses are small positive-sensed RNA viruses. The single open reading frame is divided into three regions: P1, encoding the structural proteins, and P2 and P3, encoding the nonstructural proteins. In some viruses, the P1 region is preceded by a small leader (L) protein. The viral polyprotein is cleaved by viral proteases resulting in the release of the viral proteins and some stable precursors. In all picornaviruses, the 3C protease (3C^{pro}) is responsible for the majority of the cleavages within the viral polyprotein. Some picornavirus genera encode an additional protease. 2A^{pro}, expressed by members from the genus Enterovirus, cleaves between the P1 and P2 regions. L^{pro}, expressed by Aphthovirus and Erbovirus members, autocatalytically cleaves itself from P1 at its C terminus.

In addition to their function in polyprotein processing, viral proteases target a variety of host proteins for efficient virus replication. These include proteins involved in translation, transcription, immune signaling, or nucleocytoplasmic traffic. E.g., enterovirus 2A^{pro} and aphthovirus L^{pro} impair cap-dependent translation through cleavage of initiation factor eIF4G, leading to a translational host shut-off (Devaney et al., 1988; Krausslich et al., 1987; Lloyd et al.,

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1988; Medina et al., 1993). Cleavage of eIF4AI by FMDV $3C^{pro}$ and poly(A)-binding protein by enterovirus $2A^{pro}$ and $3C^{pro}$ also contribute to the host shut-off (Joachims et al., 1999; Li et al., 2001).

Viral proteases provide an attractive target for anti-viral therapy against picornaviruses because of their essential role in the virus replication cycle and the absence of cellular homologues (Tong, 2002). As yet, no anti-viral therapy has been approved to treat picornavirus infections. Most human pathogens can be found in the genus Enterovirus, e.g., poliovirus (PV), coxsackievirus, enterovirus 71 (EV71), and human rhinovirus (HRV). These viruses cause a variety of diseases including acute flaccid paralysis, aseptic meningitis, respiratory infections, and hand-foot-and-mouth disease. The highly contagious foot-and-mouth disease virus (FMDV), an Aphthovirus member, is one of the most important animal pathogens, causing outbreaks among livestock with enormous economical impact.

The compound AG7088 (also known as rupintrivir) was developed as a potent inhibitor of HRV $3C^{pro}$ (Dragovich et al., 1999). AG7088 is an irreversible peptidomimetic with an α,β-unsaturated ester. Further studies revealed that AG7088 was also able to inhibit replication of other enteroviruses (De Palma et al., 2008b; Lee et al., 2008; Patick et al., 1999; Tsai et al., 2009). However, the clinical development was halted because of limited activity in clinical trials with natural HRV infections (Patick et al., 2005). AG7404 (also known as compound 1) is an analogue of AG7088 with improved oral bioavailability (Dragovich et al., 2003). AG7404 displays anti-viral activity *in vitro* and is safe and well-tolerated in vivo, but clinical development was discontinued (Patick et al., 2005). Recently, we reported the synthesis of a series of $3C^{pro}$ inhibitors, which, like AG7088, are peptidic α,β-unsaturated esters (Tan et al., 2013). Of these series, the compound SG85 was the most potent inhibitor, with anti-viral activity against EV71, PV, echovirus 11, and HRV (Tan et al., 2013).

Current assays available for testing of proteolytic activity are mostly performed *in vitro* using heterologously expressed protease and a peptide substrate. However, such cell-free assays for compound testing have some drawbacks. First, compounds able to inhibit proteolytical activity in these assays may be unable to cross the plasma membrane. Secondly, *in vitro* assays are unable to assess cell toxicity. Thirdly, compounds that require cellular activation will not be identified as a hit in a non-cell-based assay. To deal with these issues, we have adapted a cell-based assay developed previously for EV71 $3C^{pro}$ (Fig. 1) (Lee et al., 2008). We have extended the assay to multiple picornavirus $3C^{pro}$ and applied the assay to test the spectrum of $3C^{pro}$ inhibitors AG7088 and SG85. We demonstrate that our cell-based protease assay is an easy and biosafe assay for testing protease activity and the effect of inhibitors, and that it is amenable for use in high-throughput set-up.

2. Materials and methods

2.1. Cells

COS-1 monkey kidney cells, Hela cells and baby hamster kidney (BHK-21) cells were cultured in DMEM with 10% FCS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. The culture medium was suppplemented with 0.6 mg/ml geneticin (G418 sulphate) for Huh-T7 cells, a derivative of human hepatocellular carcinoma cells that constitutively expresses a T7 RNA polymerase (Schultz et al., 1996).

2.2. Plasmids

Plasmids pBind, pAct and pG5luc were derived from the CheckMate™ Mammalian Two-Hybrid System (Promega). pBind-VP16 was produced by ligating the VP16AD-coding sequence amplified from pAct into the XbaI and NotI sites of the multiple cloning site of pBind. pBind-VP16 was subsequently used for cloning all protease constructs using the SalI and MluI sites between GAL4BD and VP16AD. Mutagenesis was performed using the Quikchange II Site-Directed Mutagenesis Kit (Agilent). pG5EGFP was constructed by inserting the EGFP-coding sequence amplified from pEGFP-N1 (Clontech) into the NcoI and PpuMI restriction sites of pG5luc. Templates and primers used for PCR are shown in the Supplementary Table.

2.3. Compounds

AG7088 and SG85 were synthesized as described previously (Lin et al., 2012; Tan et al., 2013). AG7404 was a kind gift of Pfizer. Compound stocks were dissolved in DMSO and stored at −20 °C.

2.4. Western blot

COS-1 cells were seeded in 12- or 6-well plates. The next day, the medium was replaced with (compound-containing) medium. Cells plated in 12-well plates were co-transfected with 700 ng protease construct and 700 ng pG5luc using 4.2 μl Fugene according to the manufacturer's instructions. For 6-well plates, 1750 ng of each plasmid was transfected using 10.5 μl Fugene. The next day, the cells were harvested and lysis buffer (40 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 1% NP40 supplemented with Complete protease inhibitor cocktail (Roche)) was added to the cell pellet. The cell lysate underwent two rounds of vortexing and 10′-incubations at 4 °C, followed by centrifugation for 10′ at 13,000 rpm. Laemmli was added to the supernatant. The proteins were separated by SDS–PAGE on 12.5% polyacrylamide gel (analysis of fusion protein fragments) or 7.5% polyacrylamide gel (analysis eIF4G cleavage). Proteins were detected using mouse monoclonal anti-GAL4BD antibody (Clontech), rabbit polyclonal anti-eIF4GI antibody (A300-502A, Bethyl Laboratories), or rat anti-tubulin alpha antibody (AbD Serotec) followed by appropriate monoclonal IRDye secondary antibodies (Li-Cor Biosciences). Imaging was done with the Odyssey system.

2.5. Protease assay

COS-1 cells were seeded into 96-well plates. The next day, subconfluent monolayers were transfected with 100 ng protease construct and 100 ng pG5luc reporter plasmid using 0.6 μl Fugene. The transfection mix was added to the cells on which the medium had been replaced with DMSO- or compound-containing medium. After ∼16 h, the cells were lysed with passive lysis buffer and luciferase activities were measured with the Dual-Glo Luciferase Assay System (Promega).

For experiments with the EGFP read-out, the experiment was performed as described above except that pG5EGFP was transfected instead of pG5luc and cells were incubated for ∼48 h before imaging with an EVOS fl digital fluorescence microscope (AMG). Cells were fixed, permeabilized, and stained with α-GAL4BD antibody to check for similar transfection efficiencies.

Graphpad Prism 5.0.3 was used for statistical analysis. EC_{50} values were calculated using non-linear regression setting the values obtained for the inactive mutants and the untreated wt constructs as top and bottom constraints, respectively. The Z'-factor was calculated from 21 values per condition using the following formula: $Z' = 1-3(SD_{DMSO} + SD_{AG7088})/|mean_{DMSO} - mean_{AG7088}|$.

2.6. Multicycle CPE-reduction assay

Multicycle CPE-reduction assays were performed as described previously (De Palma et al., 2008a; Goris et al., 2009). CVB3 strain