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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

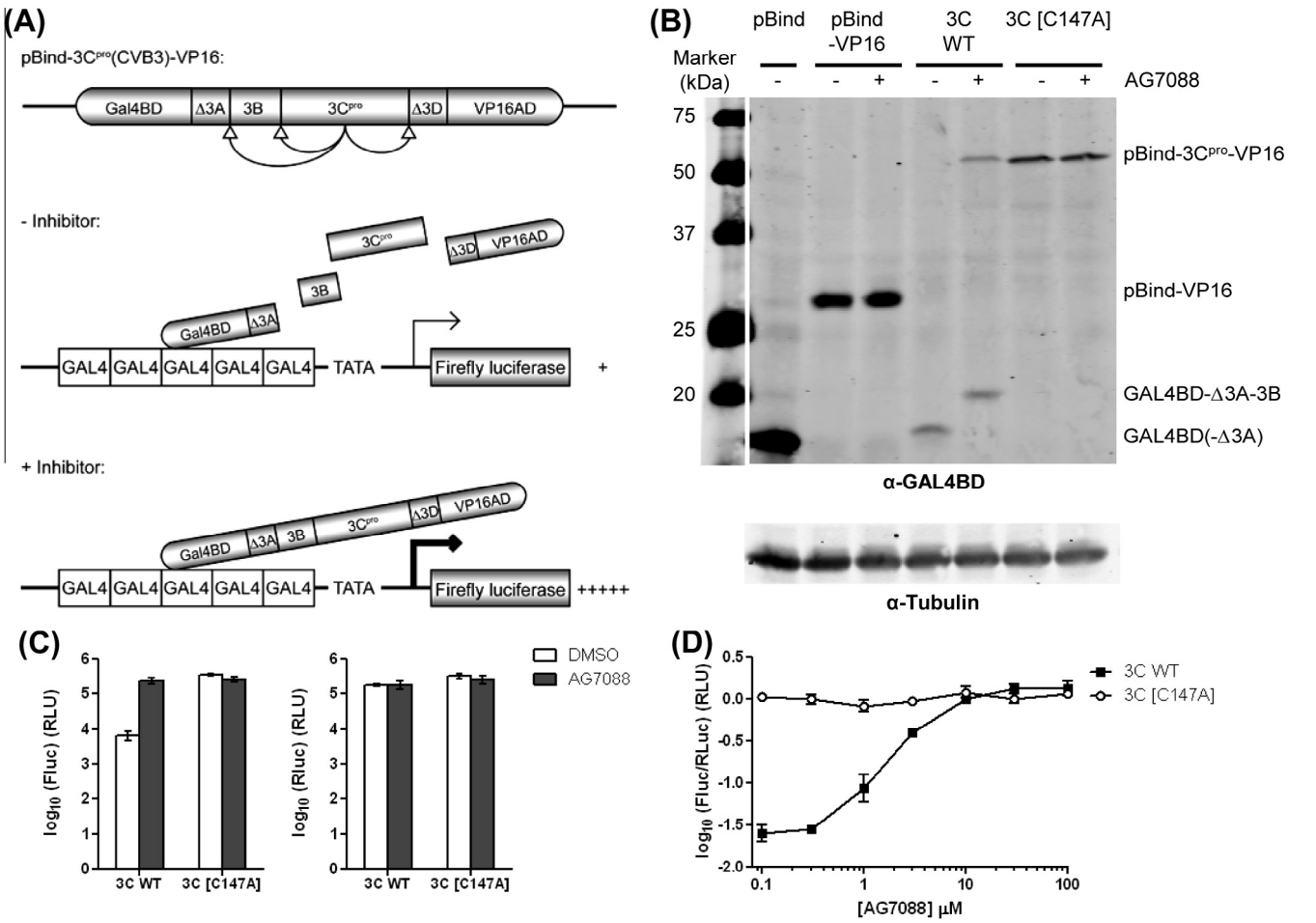

Fig. 1. Principle and evaluation of the cell-based protease assay. (A) A protease expression construct is co-transfected with the pG5luc reporter plasmid into COS-1 cells. The protease construct expresses a chimeric protein which contains a GAL4 binding domain (GAL4BD) and a VP16 activation domain (VP16AD) between which part of the CVB3 polyprotein (15 C-terminal amino acids of 3A, 3B, $3C^{pro}$, and 15 N-terminal acids of 3D) is inserted. Active protease cleaves the chimeric protein at the $3C^{pro}$ cleavage (arrow). If the protease is catalytically inactive, binding of GAL4BD to the GAL4 sequences in the reporter plasmid recruits VP16AD to the transcription start site, resulting in induction of FLuc expression. (B) The fusion protein is $3C^{pro}$-dependently cleaved. Plasmids pBind, pBind-VP16, pBind-$3C^{pro}$(CVB3)-VP16, or pBind-$3C^{pro}$[C147A](CVB3)-VP16 were co-transfected with pG5luc into COS-1 cells. The next day, cells were lysed and the proteins were separated by SDS–PAGE and stained with α-GAL4BD and α-Tubulin antibodies. (C + D) Inhibition of $3C^{pro}$ activity results in induction of FLuc expression. COS-1 cells were co-transfected with protease constructs in combination with the pG5luc reporter and immediately treated with DMSO or AG7088 at 50 μM (C) or at the indicated concentrations (D). At 16 h post transfection, the cells were lysed and FLuc and RLuc were measured. Experiments were performed in triplicate and mean values ± SD are depicted.

Nancy (from infectious clone p53CB3/T7) was tested on Huh-T7 cells and the FMDV strain O_1 Manisa on BHK-21 cells (Goris et al., 2007; Wessels et al., 2005). In short, 20,000 cells seeded in 96-well plates were treated the next day with serial dilutions of compound and infected with virus at low MOI. After three-day incubation, cell viability was measured by MTS assay (AQueous One Solution Cell Proliferation Assay, Promega). The DMSO concentrations used were <0.02% and <4.5% at the EC_{50} values determined for the compounds in the assays on Huh-T7 cells and BHK-21 cells, respectively. These DMSO concentrations have no adverse effects on these cells.

2.7. Molecular docking

All molecular-docking studies were performed by using the flexible docking program Fleksy (Nabuurs et al., 2007). The crystal structure of FMDV $3C^{pro}$ complexed with a peptide substrate (Zunszain et al., 2010, PDB ID: 2WV4) solved at 2.5 Å resolution was used as the receptor structure. The structure was prepared for docking by removing the peptide from the complex. Subsequently, hydrogen atoms were added to the structure, and their positions were optimized by using the Yasara program (http://www.yasara.org). Otherwise, default parameters as described previously were applied (Nabuurs et al., 2007).

3. Results

3.1. Principle of the protease assay

The principle of the protease assay is the induction of firefly luciferase (FLuc) expression from a reporter plasmid in the absence of proteolytic activity of the viral protease (Fig. 1). Expression of this reporter plasmid, pG5luc, is regulated by a transcription factor composed of the DNA-binding domain of Gal4 (Gal4BD) and the transactivating domain of VP16 (VP16AD). In between these domains, we inserted part of the CVB3 polyprotein containing $3C^{pro}$ and neighboring cleavage sites (Δ3A-3B-$3C^{pro}$-Δ3D). Proteolytical activity of $3C^{pro}$ leads to cleavage of the chimeric protein at the $3C^{pro}$ cleavage sites and as a consequence, the transcription factor lacks its VP16AD domain and no Fluc is expressed. However, in the case of an inactive protease no proteolytic cleavage occurs and the full-length fusion protein transactivates expression of FLuc. Renilla luciferase (RLuc), independently expressed from a different promoter on the plasmid expressing the protease construct, serves

as a measure for transfection efficiency and indirectly for cell viability.

3.2. The CVB3 3C^{pro} protease assay

To test whether this CVB3 3C^{pro} protease assay is functional, we analyzed proteins produced upon overexpression of protease constructs by Western blot using α-GAL4BD antibody. In COS-1 cells transfected with controls pBind and pBind-VP16, GAL4BD (reported to run at ~17 kDa (Chen et al., 2012) and GAL4BD-VP16AD (~27 kDa) were detected, respectively (Fig. 1B). When overexpressing pBind-3C^{pro}(CVB3)-VP16, a band migrating slightly slower than GAL4BD appeared, corresponding to fragment GAL4BD-Δ3A produced by cleavage of fusion protein GAL4BD-Δ3A-3B-3C^{pro}-Δ3D-VP16. Upon treatment with AG7088, a band of same molecular mass (i.e., ~53 kDa) was detected as in cells transfected with a 3C^{pro} construct that was rendered inactive by mutation of the catalytic site (C147A) (Lee et al., 2009), demonstrating that this was the uncleaved full-length product. AG7088-treated cells transfected with pBind-3C^{pro}(CVB3)-VP16 also expressed a protein with molecular mass corresponding to the predicted size of GAL4BD-Δ3A-3B, indicating that cleavage is not completely blocked in the presence of 50 μM AG7088. Notwithstanding this, these data show the suitability of this assay for determining 3C^{pro} activity.

To determine whether 3C^{pro} cleavage activity is reflected in luciferase levels produced, we measured FLuc and RLuc activity in cells co-transfected with pBind-3C^{pro}(CVB3)-VP16 and the pG5luc reporter plasmid. Treatment with 50 μM AG7088 resulted in ~40-fold increase in FLuc levels compared to untreated, suggesting that transactivation of the reporter plasmid is reversely correlated to 3C^{pro} activity (Fig. 1C, left panel). The levels of FLuc obtained upon AG7088-treatment were similar to those obtained with the inactive mutant 3C^{pro} [C147A]. RLuc levels were not affected by AG7088-treatment or catalytic activity of 3C^{pro} (Fig. 1C, right panel), so FLuc/RLuc ratios can be used to correct for possible differences in transfection efficiency.

When testing a range of concentrations of AG7088, we observed a dose-dependent increase in FLuc/RLuc ratio (Fig. 1D). With concentrations of ⩾10 μM, FLuc/RLuc ratios were similar to the levels observed with the inactive mutant. The observation that some cleavage occurred in the presence of 50 μM AG7088 (Fig. 1B) indicates that cleavage does not need to be completely inhibited for FLuc levels to be maximal. Nevertheless, the EC_{50} value of 1.7 μM (95% CI: 1.4–2.0 μM) for AG7088 with CVB3 3C^{pro} is in the same range as the EC_{50} value of 0.3 μM (95% CI: 0.2–0.3 μM) that we observed in a CPE-reduction multicycle assay using CVB3 (Table 1), suggesting that this protease assay is a good indicator for the potency of an inhibitor in virus infection.

Together, these results show that our cell-based protease assay is suitable for measuring protease activity and the effect of protease inhibitors or mutations.

3.3. Suitability of the cell-based protease assay for use in high-throughput screening

To assess whether the assay is of sufficient quality for use in a high-throughput setting, we calculated the *Z′*-factor (Zhang et al., 1999). Assays with a *Z′*-factor ⩾ 0.5 are considered excellent candidates for high-throughput screening. To this end, cells were co-transfected with pBind-3C^{pro}(CVB3)-VP16 and pG5luc and treated with DMSO (negative control) or 50 μM AG7088 (positive control). This yielded a *Z′*-factor of 0.57 (using log-transformed FLuc values) or 0.74 (using values corrected per sample for transfection efficiency, i.e., FLuc/RLuc ratios), indicating that the assay is excellent for use in a high-throughput screen (data not shown and Fig. 2A).

Keeping measurement costs low is of great importance for large-scale testing. Therefore, we tested whether it is possible to use GFP as a read-out to eliminate the need for reagents for measuring protease activity. No GFP signal from the pG5EGFP reporter could be detected in cells co-transfected with pBind-3C^{pro}(CVB3)-VP16, whereas GFP was visible upon incubation with AG7088 or upon expression of the catalytic-site mutant of 3C^{pro} (Fig. 2B).

Both results demonstrate that the assay is useful for high-throughput testing.

3.4. The spectrum of activity of AG7088 and SG85

To enable us to determine the spectrum of potential protease inhibitors, we then designed equivalent 3C^{pro} constructs for repre-

Table 1
Potency of 3C^{pro} inhibitors against CVB3 and FMDV (3C^{pro}).

EC_{50} (95% CI, in μM)	Protease assay		Multicycle CPE-reduction assay	
	CVB3	FMDV	CVB3	FMDV
AG7088	1.1 (0.9–1.3)	13.9 (12.6–15.2)	0.3 (0.2–0.3)	22.4
AG7404	3.6 (3.0–4.3)	>100	ND	ND
SG85	4.1 (4.0–4.3)	34.4 (31.7–37.3)	0.5 (0.2–0.7)	44.9

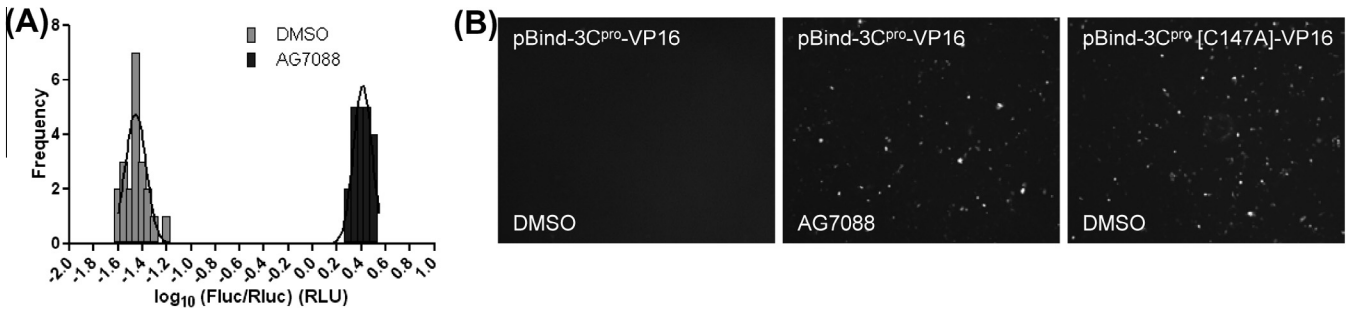

Fig. 2. Suitability of the CVB3 3C^{pro} assay for high-throughput screening. (A) Determination of the *Z′* factor for the CVB3 3C^{pro} assay. COS-1 cells were co-transfected with pBind-3C^{pro}(CVB3)-VP16 and pG5luc, treated with 50 μM AG7088 and luciferase levels were measured the next day. The Gaussia curves displayed were fitted using nonlinear regression. (B) EGFP can also be used as a reporter for protease activity. COS-1 cells were co-transfected with pBind-3C^{pro}(CVB3)-VP16 or the C147A mutant and pG5EGFP, treated with 50 μM AG7088 and EGFP was imaged after 2 days.

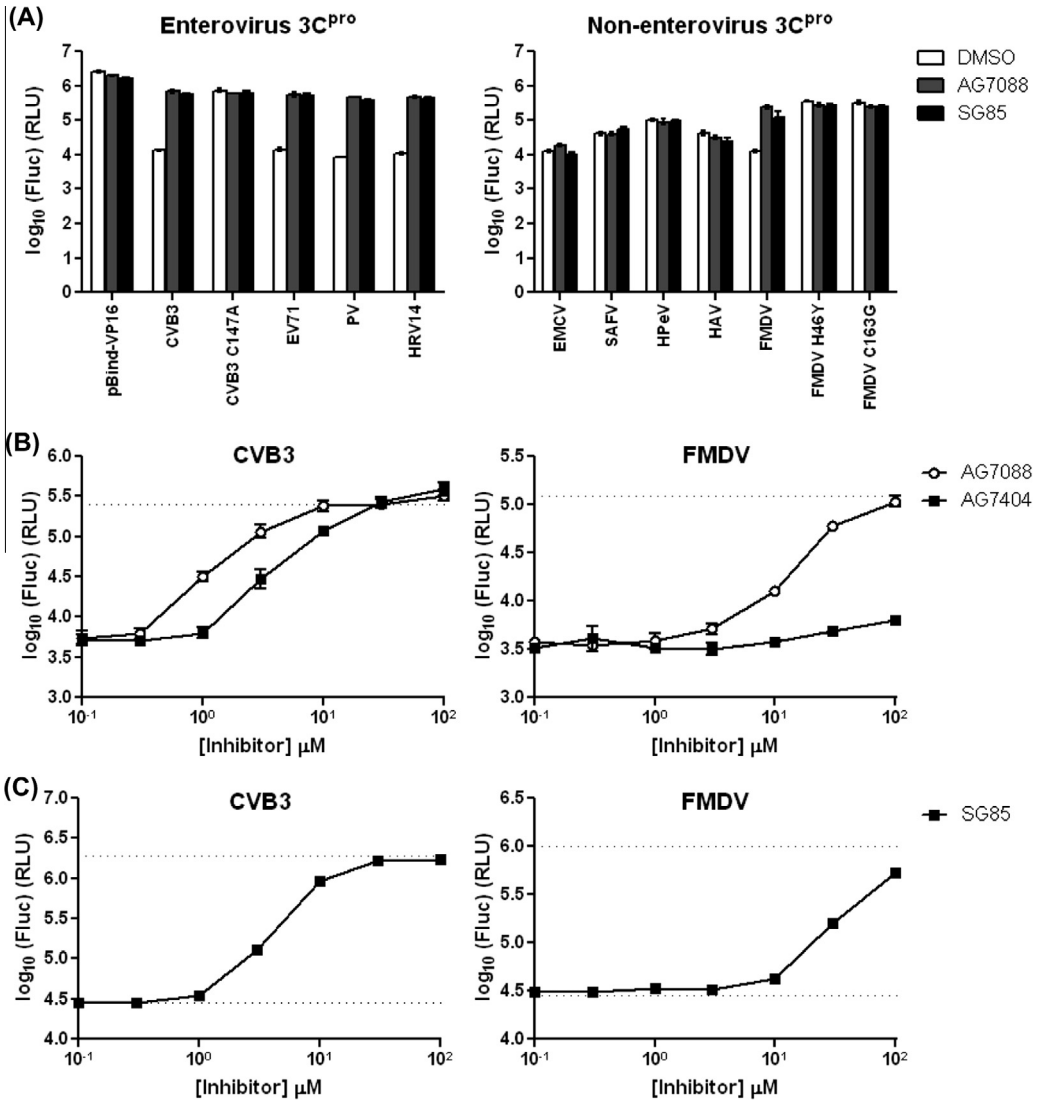

Fig. 3. AG7088 and SG85 inhibit enterovirus $3C^{pro}$ and FMDV $3C^{pro}$. (A) AG7088 and SG85 display activity against enterovirus $3C^{pro}$ and FMDV $3C^{pro}$. Cells co-transfected with the constructs for the indicated proteases and pG5luc reporter were treated with 50 µM AG7088 or SG85 and the next day the luciferase levels were measured. Displayed are the FLuc levels. (B + C) AG7088 and SG85, but not AG7404 inhibit FMDV $3C^{pro}$, albeit with lower potency than CVB3 $3C^{pro}$. Cells co-transfected with pG5luc and the CVB3 or FMDV $3C^{pro}$ constructs were treated with a range of concentrations of AG7088 or AG7404 (B) or SG85 (C). The dashed lines represent the values obtained for the respective inactive mutants (upper dashed line) or the values obtained for the untreated wt constructs (lower dashed line). All experiments were performed in triplicate and values represent the mean FLuc ± SD.

sentative members of different picornavirus genera, including enteroviruses (EV71, PV, HRV14), an aphthovirus (FMDV), cardioviruses (encephalomyocarditis virus, EMCV, and Saffold virus, SAFV), a parechovirus (human parechovirus, HPeV), and a hepatovirus (hepatitis A virus, HAV). Cleavage of the fusion proteins was confirmed by Western blot (data not shown).

We tested the inhibitory activity of $3C^{pro}$ inhibitors AG7088 and SG85 against these picornavirus 3C proteases. As anticipated, expression of FLuc in the absence of compound was comparable to that observed with pBind-$3C^{pro}$(CVB3)-VP16 for most $3C^{pro}$ constructs, although levels were somewhat higher in the case of SAFV, HPeV, and HAV (Fig. 3A). RLuc activity was decreased in cells expressing the FMDV $3C^{pro}$ fusion protein (Supplementary Fig. S1), possibly as result of cleavage of eIF4AI by $3C^{pro}$ (Li et al., 2001). Therefore, FLuc values were used for data analysis instead of FLuc/RLuc ratios.

FLuc expression was induced upon treatment with 50 µM AG7088 or SG85 in cells transfected with all enterovirus $3C^{pro}$ constructs (Fig. 3A, left panel). In contrast, no effect was observed on reporter activity upon expression of constructs for EMCV, SAFV, HPeV, or HAV $3C^{pro}$, suggesting that these proteases are insensitive to AG7088 and SG85. As predicted by the protease assay, 50 µM AG7088 displayed no significant activity against EMCV and SAFV in growth curves of EMCV and SAFV (Supplementary Fig. S2). Remarkably, AG7088 and SG85-treatment increased FLuc expression in cells expressing FMDV $3C^{pro}$ to similar levels as observed for catalytically inactive FMDV $3C^{pro}$ (H46Y or C163G) (Grubman et al., 1995). These data indicate that FMDV $3C^{pro}$ is sensitive to AG7088 and SG85 and that both protease inhibitors have a similar spectrum of activity.

3.5. The effects of AG7088, AG7404 and SG85 on FMDV $3C^{pro}$ activity

A more elaborate testing of the FMDV and CVB3 $3C^{pro}$ constructs with concentration series of AG7088 and SG85 illustrated that both compounds are less potent against FMDV than against CVB3 (Fig. 3B, C and Table 1), in line with them having been originally designed against enteroviruses.

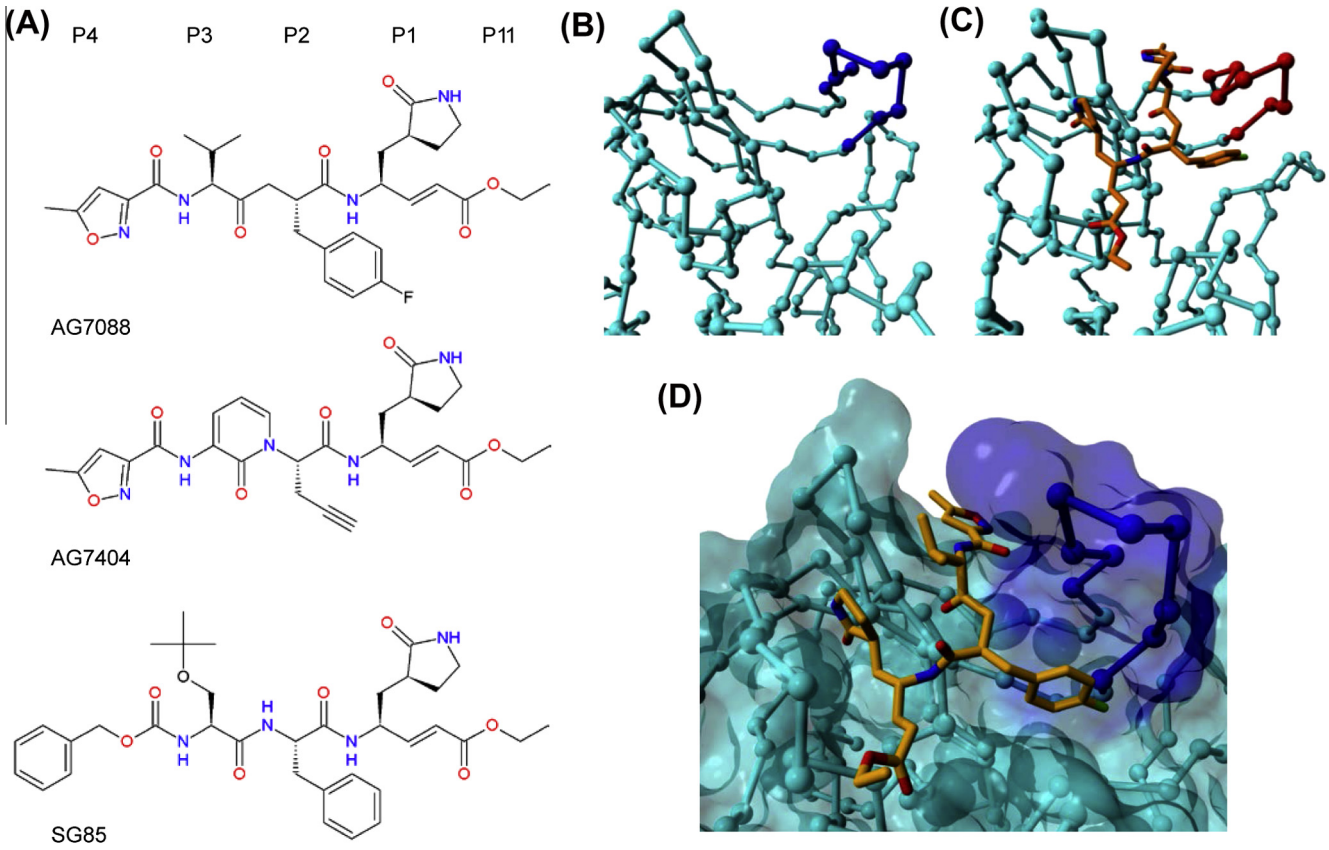

Fig. 4. Rationale for the different potencies of AG7088, AG7404, and SG85 towards FMDV $3C^{pro}$. (A) Chemical structures of AG7088, AG7404 and SG85. The protease pockets targeted by the different chemical moieties are outlined on top. (B) The β-ribbon of FMDV $3C^{pro}$ (highlighted in blue) as observed in a recent crystal structure (Zunszain et al., 2010). (C) HRV2 $3C^{pro}$ in complex with AG7088 (in orange) with the β-ribbon highlighted in red (Matthews et al., 1999). (D) Generated model of FMDV $3C^{pro}$ in complex with AG7088, with the β-ribbon highlighted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AG7088 and SG85 were able to inhibit FMDV-induced CPE on BHK-21 cells in a multicycle CPE-reduction assay with an EC_{50} value of 22.4 and 44.9 μM, respectively, indicating that $3C^{pro}$ inhibition by AG7088 and SG85 impaired replication of FMDV (Table 1).

The sensitivity of FMDV $3C^{pro}$ to AG7088 led us to test whether this protease was also susceptible to AG7404, the orally bioavailable analog of AG7088. Remarkably, this compound displayed little or no inhibitory activity towards FMDV $3C^{pro}$, while the activity against CVB3 $3C^{pro}$ was comparable to that observed for AG7088 (Fig. 3B and Table 1).

3.6. Rationale for the differences in the potencies of AG7088, AG7404, and SG85 towards FMDV $3C^{pro}$

We utilized computational modeling techniques to explain the observed differences in inhibitory activity of AG7088, AG7404 and SG85 (Fig. 4A) towards FMDV $3C^{pro}$. Induced fit docking was used to predict the binding mode of AG7088 (Nabuurs et al., 2007), the most potent inhibitor of FMDV $3C^{pro}$ of the three compounds, to FMDV $3C^{pro}$. A recent crystal structure of FMDV $3C^{pro}$ complexed with a peptide substrate (Fig. 4B) (Zunszain et al., 2010) reveals that the so-called β-ribbon that separates the S2 and S4 subsites of the protease is slightly longer in FMDV $3C^{pro}$ compared to other 3C proteases, as for example seen in the complex of HRV2 $3C^{pro}$ with AG7088 (Fig. 4C) (Matthews et al., 1999; Sweeney et al., 2007). The models we generated suggest interactions with this loop to be mainly responsible for the observed selectivity profile of the three compounds assayed.

Fig. 4D shows the obtained model of AG7088 bound to FMDV $3C^{pro}$, with the β-ribbon highlighted in blue. The model suggests extensive tight hydrophobic interactions between the P3 position of AG7088 and the β-ribbon of FMDV $3C^{pro}$. The ring closure present at the P3 position of AG7404 is predicted to clash with the β-ribbon of FMDV $3C^{pro}$, providing a rationale for the observed inactivity of AG7404 toward FMDV $3C^{pro}$. SG85, which has intermediate potency compared to AG7088 and AG7404, contains a t-butyl ether of serine as its P3 side chain. This introduces more steric bulk compared to AG7088, but allows for more flexibility when compared to AG7404, potentially explaining the observed intermediate activity of SG85.

4. Discussion

In this study, we developed an assay for the measurement of protease activity of $3C^{pro}$ of various enteroviruses as well as of other picornaviruses (Fig. 1), based on the principle described previously for EV71 $3C^{pro}$ (Lee et al., 2008), and we have applied this assay to test the spectrum of activity of three $3C^{pro}$ inhibitors. A major advantage of this assay is that it is cell-based, in contrast to most protease assays available. As a consequence, compounds that cannot penetrate the plasma membrane or have adverse cellular effects are selected out when searching for protease inhibitors. Another advantage is that the assay depends on an increase in read-out signal upon inhibition of protease activity. This precludes the identification of false-positive hits on the basis of an aspecific reduction in reporter activity because of toxicity. Also, compounds

that require cellular activation will not be accurately evaluated in a non-cell-based assay.

The nature of the cell-based assay allows easy expansion of the range of proteases. In contrast, *in vitro* assays require production of recombinant enzyme which can be time-consuming and potentially cumbersome. The assay could also be applied to proteases of other plus-strand RNA viruses, such as hepatitis C virus, dengue virus, and SARS coronavirus. One example for which our cell-based assay would be advantageous is NS2B/NS3^{pro} of dengue virus (family Flaviviridae). NS3 protease activity is most commonly tested with a construct in which the polypeptide chains of the co-factor NS2B and the protease domain of NS3 are linked by a nonapeptide linker. Optimal activity of this construct *in vitro* requires a high pH of 9.0 potentially affecting the protonation state of compounds and hence false-positive or false-negative hits in screens (Noble et al., 2010).

An assay should preferentially be suitable for use in a high-throughput setting. We showed that the assay is excellent for use in a high-throughput setting, as exemplified by a *Z′*-factor of 0.74 for CVB3 3C^{pro} (Fig. 2D). It would be advisable to also test the assay with reversible protease inhibitors before applying it in a high-throughput screening, to confirm that the assay can also identify such inhibitors. Several adaptations to further optimize the assay for use in large-scale screens may be possible. The assay may be downscaled to a 384-well format to save material. Also, we here provide a proof of principle for use of GFP as read-out instead of FLuc (Fig. 2B). Although this reporter is less sensitive, it is more cost-efficient. In addition, cell lines with stable constructs that are inducible, may be used.

The availability of expression constructs for a wide range of picornavirus 3C^{pro}, enabled us to test the spectrum of activity of 3C^{pro} inhibitors AG7088 and SG85. We showed that both compounds inhibited 3C^{pro} of all enterovirus tested, i.e., CVB3, EV71, PV, and HRV14, but they had no effect on processing by 3C^{pro} of EMCV, SAFV, HPeV, or HAV. A recent publication reported that inhibition of HAV replication by AG7088 is strain-dependent, and also found no effect on the strain we used (HM175) (Debing et al., 2013). Interestingly, both compounds inhibited 3C^{pro} of FMDV, albeit with a lower potency than CVB3 3C^{pro}. Testing in multicycle CPE-reduction assays revealed that our assay accurately predicted that the compounds possessed anti-viral activity. While this study was in progress, AG7088 was shown to inhibit FMDV 3C^{pro} in an *in vitro* FRET assay using fluorogenic substrates with recombinant 3C^{pro} (IC_{50} = 4.21 ± 1.97 μM) (Kim et al., 2012), corroborating our finding. The assay presented and the insights developed here may help as a tool to design potent protease inhibitors of FMDV, a highly contagious zoonotic virus that can cause outbreaks with an enormous economic impact.

In conclusion, we have presented a protease assay that enables measuring protease activity and the inhibitory activity of compounds in a cellular environment. Currently, we are further expanding this assay to proteases of other virus families.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.12.012.

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