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Inhibitors of SARS-CoV entry – Identification using an internally-controlled dual envelope pseudovirion assay

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ABSTRACT

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) emerged as the causal agent of an endemic atypical pneumonia, infecting thousands of people worldwide. Although a number of promising potential vaccines and therapeutic agents for SARS-CoV have been described, no effective antiviral drug against SARS-CoV is currently available. The intricate, sequential nature of the viral entry process provides multiple valid targets for drug development. Here, we describe a rapid and safe cell-based high-throughput screening system, dual envelope pseudovirion (DEP) assay, for specifically screening inhibitors of viral entry. The assay system employs a novel dual envelope strategy, using lentiviral pseudovirions as targets whose entry is driven by the SARS-CoV Spike glycoprotein. A second, unrelated viral envelope is used as an internal control to reduce the number of false positives. As an example of the power of this assay a class of inhibitors is reported with the potential to inhibit SARS-CoV at two steps of the replication cycle, viral entry and particle assembly. This assay system can be easily adapted to screen entry inhibitors against other viruses with the careful selection of matching partner virus envelopes.

1. Introduction

SARS-CoV is an emerging pathogen responsible for a global outbreak of atypical pneumonia between 2002 and 2003 (WHO, 2003). Although a number of promising potential vaccines and therapeutic agents for SARS-CoV have been described (reviewed in Golda and Pyrc, 2008), it is unclear if any are likely to become therapeutically useful. In particular, although neutralizing monoclonal antibodies have been identified (Rockx et al., 2008), the diversity of SARS-CoV-like sequences found in the animal reservoirs makes antigen selection for vaccine development difficult prior to a new zoonotic transmission (Deming et al., 2006; Liu et al., 2007).

A number of stages in the SARS-CoV life cycle represent attractive targets for potential anti-SARS-CoV therapeutics. Enzymatic processes such as polymerase and protease activity are often good targets for antivirals due to their specialized mode of action. Trials using small animal models, however, have been less promising with many of these candidates failing to inhibit viral replication *in vivo* (Barnard et al., 2006). Inhibition of viral entry is appealing as a drug target for many viruses including SARS-CoV as it prevents the initiation of any steps towards viral replication. Indeed, virus entry is now a major focus for drug development, with several inhibitors either on the market or in clinical trials. Multiple steps in the entry process are valid targets for inhibition, including attachment, receptor engagement, viral internalization, envelope conformational rearrangements and membrane fusion. The use of pseudovirions to identify small molecule entry inhibitors of difficult to handle and/or highly pathogenic BSL 3/4 viruses such as Lassa virus has recently been described (Garcia et al., 2009; Larson et al., 2008; York et al., 2008). Indeed, using pseudovirions, complemented with live virus, we have previously demonstrated that inhibitors of cysteine proteases, such as leupeptin, act as potent inhibitors of SARS-CoV entry (Simmons et al., 2005). This inhibition is the result of an absolute requirement in cell lines for endosomal processing of the spike glycoprotein by cathepsin L (CTSL) during entry into the target cell (Simmons et al., 2005, 2011). In vivo, the situation is more complicated, with a number of secreted or surface proteases presence in the respiratory tract, such as the cellular type II transmembrane serine protease (TTSP) TMPRSS2,

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able to mediate SARS-CoV entry via non-endosomal routes (Glowacka et al., 2011; Kam et al., 2009).

We describe here a rapid and safe cell-based high-throughput screening (HTS) assay system for specifically screening inhibitors of viral entry, using lentiviral pseudovirions whose entry is driven by SARS-CoV Spike glycoprotein. In preliminary studies, we found that many initial hits identified as potential inhibitors of entry mediated by enveloped, pH-dependent viruses such as SARS-CoV, were mildly toxic in at least some cell lines. These candidate inhibitors were also often able to inhibit many other enveloped viruses, likely due to subtle effects on cellular function such as endocytosis (data not shown). In order to overcome this obstacle at an early stage, a second unrelated viral envelope was utilized in order to reduce the numbers of false positive hits obtained from the primary screen. Both targeted and general small molecule libraries were screened for inhibitors of SARS-CoV entry, and a number of compounds were identified that inhibit SARS-CoV entry and replication.

2. Materials and methods

2.1. Cells and reagents

Human embryonic kidney 293T cells, clone 17 (293T/17) were obtained from the ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% FBS and penicillin and streptomycin (10 U/ml). 293T/17 stably expressing human angiotensin converting enzyme 2 (293T/ACE2) were established by transfecting 293T/17 cells with pcDNA6 (Invitrogen) encoding the ACE2 gene and selecting for stable transformants using blasticidin S (2.5 μg/ml).

Control inhibitors included 3′-azido-2′,3′-dideoxythymidine (AZT; Sigma–Aldrich) as an inhibitor of all pseudovirions, leupeptin and Z-Phe-Phe-FMK (EMD Biosciences) as inhibitors of SARS-CoV entry, ST-193 (Larson et al., 2008) for Lassa, and anti-murine leukemia virus (MLV) antibody for inhibition of MLV (a gift from Dr. Leonard Evans, Rocky Mountain Laboratories).

2.2. Plasmids and gene constructs

Pseudotyped viruses were generated from two plasmids, one encoding the envelope and the other encoding the viral backbone with a reporter gene. Plasmids encoding SARS-CoV spike glycoprotein, Ebola GP, vesicular stomatitis virus (VSV)-G, chikungunya virus (CHIKV), as well as MLV envelope, have been described (Gilbert et al., 1994; Salvador et al., 2009; Simmons et al., 2002, 2004, 2005). The Lassa GP gene was synthesized based on the sequence of strain GA391, and subcloned into pcDNA3.1.

The envelope-deficient HIV reporter construct, pNL4-3 Luc-R^-E^- (pNL-luc), encodes a replication-incompetent variant of the HIV-1 molecular clone NL4-3, in which the nef gene has been replaced by a firefly luciferase (luc) reporter, and the env and vpr genes were inactivated, as previously described (Connor et al., 1995). Similarly, pNL4-3.ren.R^-E^- was constructed by swapping the firefly luciferase gene for *Renilla* luciferase using *Not*I and *Xho*I.

2.3. Pseudovirion production and titration

Pseudovirions were produced essentially as previously described (Simmons et al., 2004). Briefly, 293T/17 cells were transfected with 30 μg of viral envelope encoding plasmid and 10 μg of pNL4-3 reporter backbone per 10-cm dish by using calcium phosphate. The next day, expression was induced with sodium butyrate (10 mM) for 6 h before washing once. Forty hours after transfection, supernatant was filtered through a 0.45 μm-pore-size filter and frozen at −80 °C. Resulting reporter viruses were classified according to reporter system and viral envelope, for example HIV-luc(SARS-CoV S) or HIV-ren(Lassa GP). Virus was titrated using the same conditions described below for the screening assays.

2.4. Libraries and compounds

A 2000+ compound library of diverse candidate cysteine protease inhibitors collected from a range of academic and industry sources and curated at UCSF Sandler Center for Drug Discovery as previously described (Goetz et al., 2007) was screened. A general small molecule compound library purchased from ChemDiv and curated at UCSF Small Molecule Discovery Core was also screened.

WRR-182, WRR-183 and WRR485-WRR496 were synthesized as previously described (Goetz et al., 2007; Roush et al., 1998). Each synthetic inhibitor had a purity of >95%, as determined by NMR analysis, except for WRR-493 that was isolated and tested as an inseparable mixture of epoxide diastereomers.

2.5. 96-well and 384-well screening assays

High-throughput screens for inhibitors of SARS-CoV-mediated entry were performed in either 96-well or 384-well formats, using white tissue culture plates (NUNC). Initial 100% dimethyl sulfoxide (DMSO) stocks of compounds at 20 mM were used to prepare dilution plates at 1 mM using 100% DMSO. In the 96-well assay format, compounds were then further diluted in DMEM with 10% FBS to give a concentration of 80 μM (8% DMSO). Positive inhibition controls of 80 μM AZT and negative controls of medium alone were also adjusted to contain 8% DMSO. Ten μL of test compound, AZT or medium alone were transferred to blank and assay wells using a Biomek FX-P (Beckman-Coulter). A mixture of the target virus HIV-luc(SARS-CoV S) and the control virus [i.e., HIV-ren(Lassa GP) or HIV-ren(MLV Env)] was made, with the relative does of each derived empirically to give similar robust levels of reporter expression. Thirty μL of reporter virus mix was added to each well using a Matrix WellMate (Thermo Scientific) on the fast setting with small-bore tubing. 293T/ACE2 cells were removed from tissue culture dishes using versine, washed and resuspended at 500,000 cells per milliliter in a sterile bottle with gentle agitation using a magnetic stirrer. Using the WellMate, 40 μL of cells were then added to all wells on the medium setting and standard-bore tubing. Thus, compounds were used at a final concentration of 10 μM (1% DMSO) with 20,000 cells per well. Plates were incubated for two days at 37 °C/5% CO_2 and firefly and *Renilla* luciferase reporter expression was determined using the Dual-Glo Luciferase Assay System (Promega), by sequential addition (WellMate) and luminescence determination (POLARstar, BMG Labtech) of first 40 μL of luciferase reagent and then 40 μL of stop-and-glo.

The assay was performed similarly for the 384-well assay format, except that 10 μL of compounds and controls were delivered at 50 μM in 5% DMSO, together with 10 μL of reporter virus mix and 30 μL of 293T/ACE2 cells at 170,000 cells per milliliter. Levels of viral infection were determined with 30 μL of each component of the Dual-Glo system. Test runs to determine assay robustness were performed as above with medium containing DMSO to give a final assay concentration of 1%.

2.6. Dose response curves

Assays for dose response curves were performed in 96-well white tissue culture plates (NUNC). Compounds were serially diluted in medium maintaining a concentration of 8% DMSO. Ten μL of the test compounds, or medium alone were transferred to assay wells, followed by 30 μL of either a single virus or a two reporter virus mixture, depending on the purpose of the assay, and

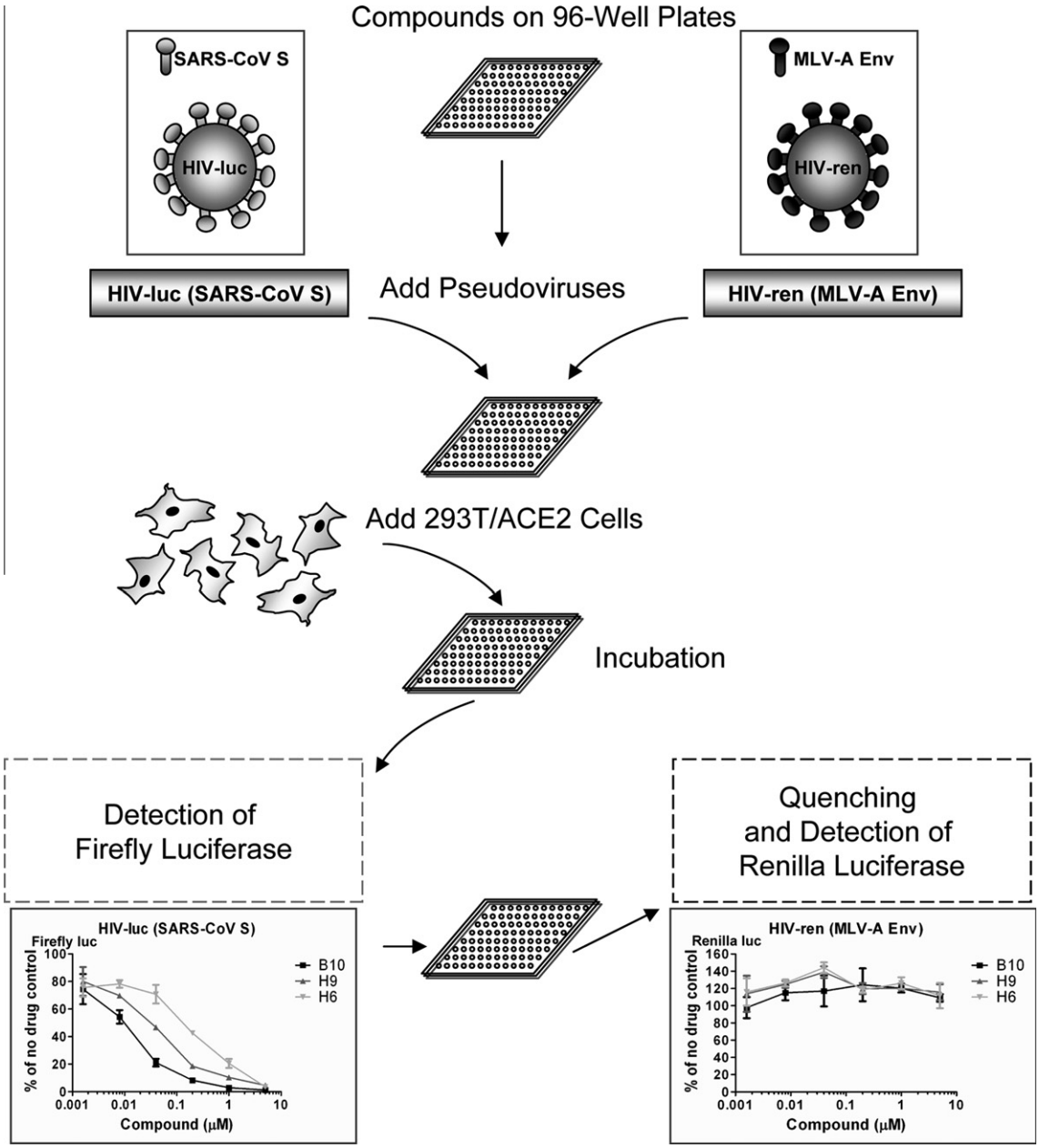

Fig. 1. Diagram of experimental design. The assay uses simultaneous infection including a control pseudovirion encoding *Renilla* luciferase and incorporating an irrelevant envelope such as MLV envelope. Thus, toxicity and non-specific inhibition can be controlled. Cells were pretreated with inhibitor, followed by equal volumes of HIV-luc (SARS-CoV S) and HIV-ren (MLV-A Env) and 293T/ACE2 cells. After 48 h, firefly luciferase and *Renilla* and were analyzed sequentially using a two-step detection system (Promega, Medison, WI).

then 40 μL of cells (500,000 cells per milliliter) to all wells. Plates were incubated for two days at 37 °C/5% CO_2 and firefly and renilla luciferase reporter expression was determined using the Dual-Glo Luciferase Assay System (Promega), or detection of firefly luciferase reporter expression using the Bright-Glo™ Luciferase Assay System (Promega).

2.7. Cytotoxicity screening

For cell viability assays, cells were seeded in 96-well black tissue culture plates (Costar) coated with compounds at a final concentration of 1% DMSO. The quantity of the ATP present in metabolically active cells was determined with CellTiter-Glo® Luminescent Cell Viability Assay Systems (Promega, Madison, WI).

2.8. Data analysis

A standard method of assessing the quality of HTS assays is the dimensionless calculation, Z prime (Z′). Statistical calculations were performed in excel and made as follows: $Z' = 1 - [(3 \times \text{standard deviation (SD) of the maximum signal control} + 3 \times \text{SD of the minimum signal control})/|(\text{mean of the maximum signal control} - \text{mean of the minimum signal control})|]$. $\%CV = 100 \times (SD/\text{mean})$ (Zhang et al., 1999). A Z′ value for each plate of greater than 0.5 is generally a good indication of an adequate assay (Zhang et al., 1999). Compounds from the primary screens were considered inhibitory with the luciferase readings of SARS-CoV, but not the internal control pseudotyped viruses, fell below the pre-defined cut-off, mean-3×SD (m-3SD).

IC_{50} (50% inhibitory concentration) and IC_{90} values were calculated using non-linear regression analysis based on the sigmoidal dose response equation using PRISM software applied to the percent inhibition and concentration data.

3. Results

3.1. Assay optimization and validation

We have developed a novel dual envelope pseudovirion (DEP) Assay for screening inhibitors of viral entry. This dual envelope strategy allows more efficient screening of libraries, and introduces internal controls into the primary screen (Fig. 1). A wide-range of parameters were examined and optimized, including plate types, dispensing methods, timing of cell addition and cell number, pseudovirion and DMSO concentration (data not shown).

It is possible that interference between the two envelopes could occur, either to inhibit entry or to overcome inhibition. In order to verify that this was not the case entry and inhibition of both pseudoparticle variants were tested separately and in combination (Table 1). Assays were carried out with different dual pseudovirus combinations, one encoding a test viral envelope with firefly luciferase reporter [HIV-luc(viral Env)] and the other encoding a control viral envelope with renilla luciferase [HIV-ren(viral Env)] and then compared to the individual virus alone. After 48 h, infection of the pseudovirion particles in terms of relative light units for firefly and *Renilla* luciferase were analyzed sequentially. These studies determined that some envelopes, such as VSV-G are unsuitable for use in the dual envelope assay. Use with HIV-luc(VSV-G) led to an almost universal increase in infection by HIV-ren viruses (Table 1). Similarly, most HIV-luc viruses were enhanced by HIV-ren(VSV-G). This was possibly due to the highly fusogenic nature of VSV-G. It is notable that a number of other more specific combinations of pseudotyped viruses also appear to be incompatible (Table 1). For example, the combination of HIV-luc(MLV Env) and HIV-ren(SARS-CoV S) yielded only 62.3% and 81.5% infectivity compared to the single virus controls, respectively, although the inverse was, respectively, 82.1% and 105.4%. Moreover, the mix of the same virus with both firefly and *Renilla* luciferase reporters also showed decreased infection compared to the single virus alone, especially for pseudoviruses with SARS-CoV S, MLV Env or Ebola GP. This is likely due to competition for low levels of receptors between the viruses with the two different reporters.

We utilized known inhibitors to determine whether inclusion of a second virus would adversely affect the ability of inhibitors to specifically block infection. Cysteine protease inhibitors, such as leupeptin, are highly effective against SARS-CoV entry in cell lines (Simmons et al., 2005), and ST-193 has recently been identified as an inhibitor of arenavirus entry (Larson et al., 2008). The dose response curves for these two inhibitor classes were super-imposable for mixed or individual pseudovirion infections (Fig. 2A and B). Leupeptin exhibited dose-dependent inhibition of SARS-CoV infection for both HIV-luc(SARS-CoV S) and HIV-ren(SARS-CoV S), regardless of whether the partner Lassa GP pseudovirion was present or not. Thus, the presence of an irrelevant envelope does not interfere with inhibition by a specific inhibitor. The dose response curves of SARS-CoV spike with other envelope combinations were also assessed and found to give similar results to SARS-CoV spike and Lassa GP, including envelopes from MLV (Fig. 2C and D) and CHIKV (data not shown). These results indicate that the dual pseudovirion systems with SARS-CoV, Lassa GP, MLV or CHIKV were suitable to be used in HTS against inhibitors of SARS-CoV-mediated entry. These studies also demonstrated that there were no issues with signal quenching in the dual assay, such as carry-over of false signal or loss of true signal. Before proceeding to HTS, we analyzed the performance of our assay system without adding any compounds. An example of assay evaluation with the combination of HIV-luc(SARS-CoV S) and HIV-ren(Lassa GP) five 96-well plates indicated that HIV-ren(Lassa GP) the inter-plate coefficient of variation (CV) was 3.10%, while the Z′ was 0.91. Similarly, for HIV-luc(SARS-CoV S), the CV equaled 6.08% with a Z′ of 0.82. Similar results were observed with the 384-well format [$CV_{HIV\text{-}ren\ (Lassa\ GP)} = 9.93\%$; $Z' = 0.78$ and $CV_{HIV\text{-}luc\ (SARS\text{-}CoV\ S)} = 7.55\%$; $Z' = 0.78$].

3.2. Screening of a compound library for inhibitors of SARS-CoV-mediated entry

HTS for inhibitors of SARS-CoV-mediated entry was performed with 96-well or 384-well NUNC plates. Assays were optimized with final concentration of compounds at 10 μM in 1% DMSO and a reporter virus mix of [HIV-luc (SARS-CoV S)+HIV-ren (Lassa GP) or HIV-luc (SARS-CoV S)+HIV-ren (MLV Env)], and 293T/ACE2 cells at 2×10^4 cells in each well for 96-well or 5.1×10^3 cells per well for 384-well plates.

As a demonstration of the utility of the dual envelope strategy, an initial screen of a diverse small molecule library (ChemDiv, San Diego, CA) was conducted at an inhibitor concentration of 10 μM. In Fig. 3A, for example, 31 candidates out of a total of over 1250 compounds were identified as inhibitors of SARS-CoV mediated entry, using a cut-off of the mean minus three standard deviations (m-3SD), which was approximately the equivalent of a 50% reduction in infection. This high hit rate is likely representative of the fact that related compounds were clustered in the library, and indeed a number of related "hits" were identified. Nine of the 31 candidates had corresponding reductions in Lassa GP pseudovirions greater than m-3SD and an additional two candidates had reductions in Lassa GP controls greater than m-2SD. Because we observed less variation in Lassa GP-based pseudovirions in general, and based on past experience with toxicity we utilized the stricter criterion for deciding whether a compound was pursued for further evaluation. Thus, the inclusion of the control pseudovirus

Table 1
Assays with different combinations of two pseudoviruses[a].

	HIV-ren (SARS S)		HIV-ren (Lassa GP)		HIV-ren (MLV Env)		HIV-ren (VSV G)		HIV-ren (Ebola GP)		HIV-ren (ChIKV Env)	
	Luc%[b]	Ren%[b]	Luc%	Ren%	Luc%	Ren%	Luc%	Ren%	Luc%	Ren%	Luc%	Ren%
HIV-luc (SARS S)	46.9	87.7	84.6	100.6	82.1	105.4	332.3	92.3	61.5	73.7	116.5	96.9
HIV-luc (Lassa GP)	83.4	95.0	92.6	98.2	71.7	127.4	117.1	91.2	65.9	89.8	82.6	94.9
HIV-luc (MLV Env)	62.3	81.5	179.1	80.1	5.4	51.8	725.0	78.8	117.5	83.5	384.9	86.7
HIV-luc (VSV G)	91.9	203.1	65.7	116.5	81.8	421.4	42.4	190.0	110.9	138.8	92.7	174.7
HIV-luc (Ebola GP)	75.6	96.8	90.6	97.8	77.4	118.0	83.8	103.3	11.8	65.4	91.7	93.9
HIV-luc (ChIKV Env)	76.4	104.6	99.3	89.2	84.0	388.0	117.8	98.8	91.5	119.0	96.9	94.5

[a] Data is shown as mean of triplicate measurements. A representative experiment of at least three experiments is shown.
[b] Results of the dual results are presented as percentage of luciferase reading of single virus.

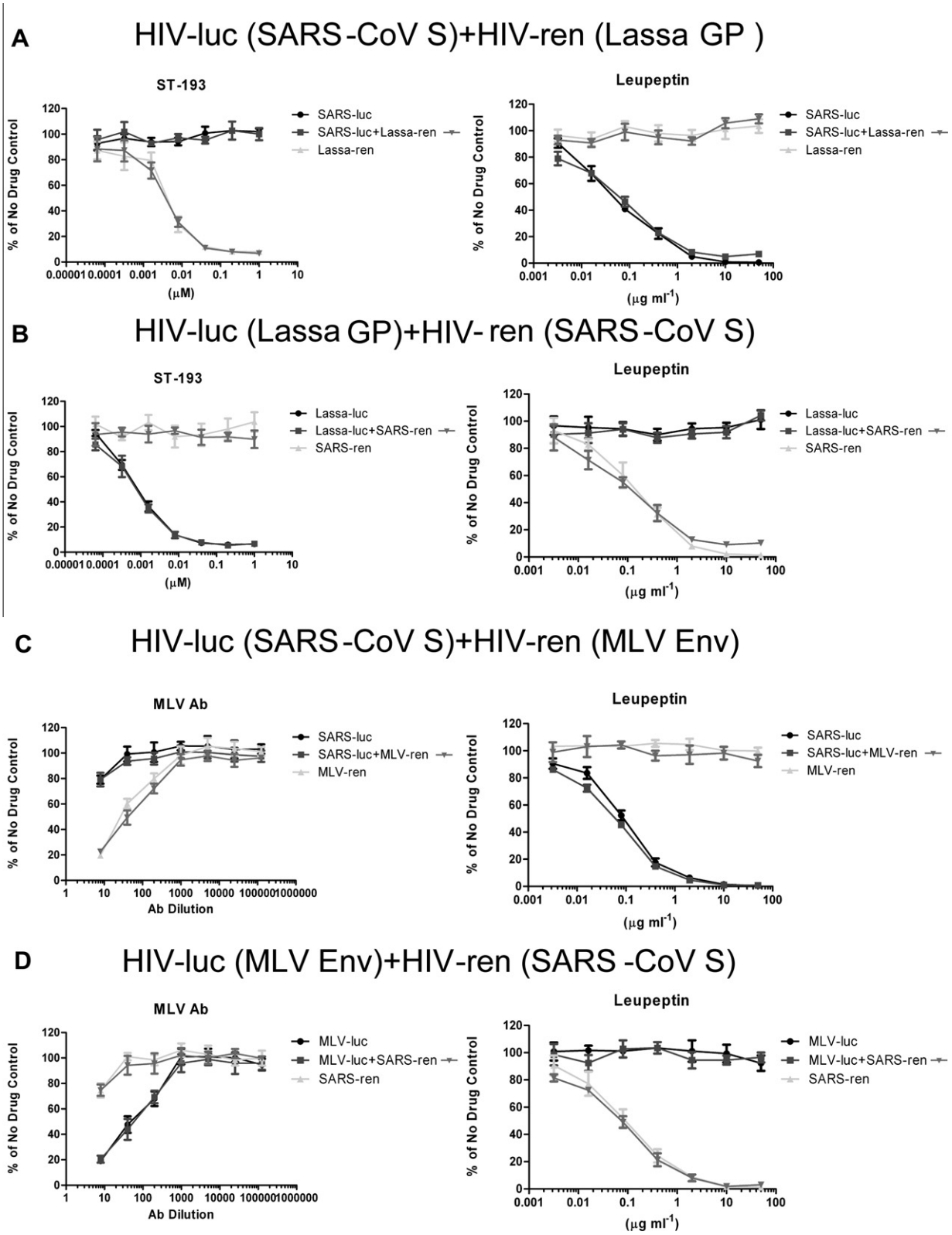

Fig. 2. Inhibition of dual infection assays. Dose response curves for leupeptin (panels A–D), ST-193 (panels A and B) or MLV antibody (panels C and D) with SARS-CoV spike pseudovirus with or without combination with Lassa GP/MLV Env pseudovirus. Results are presented as percentage of luciferase reading of no drug controls and shown as mean ± SD of triplicate measurements. A representative experiment of at least three experiments is shown.

[HIV-ren(Lassa GP)] was found to be useful for avoiding likely non-specific inhibition by promiscuous inhibitors and reduced the hit rate by over a third (11 out of 31 were called false positive) (Fig. 3B).

In addition to performing a random screen for small molecule inhibitors of SARS-CoV entry, we also exploited the fact that we had previously identified specific aspects of coronavirus entry that are amenable to inhibition (Simmons et al., 2005, 2011). Inhibitors

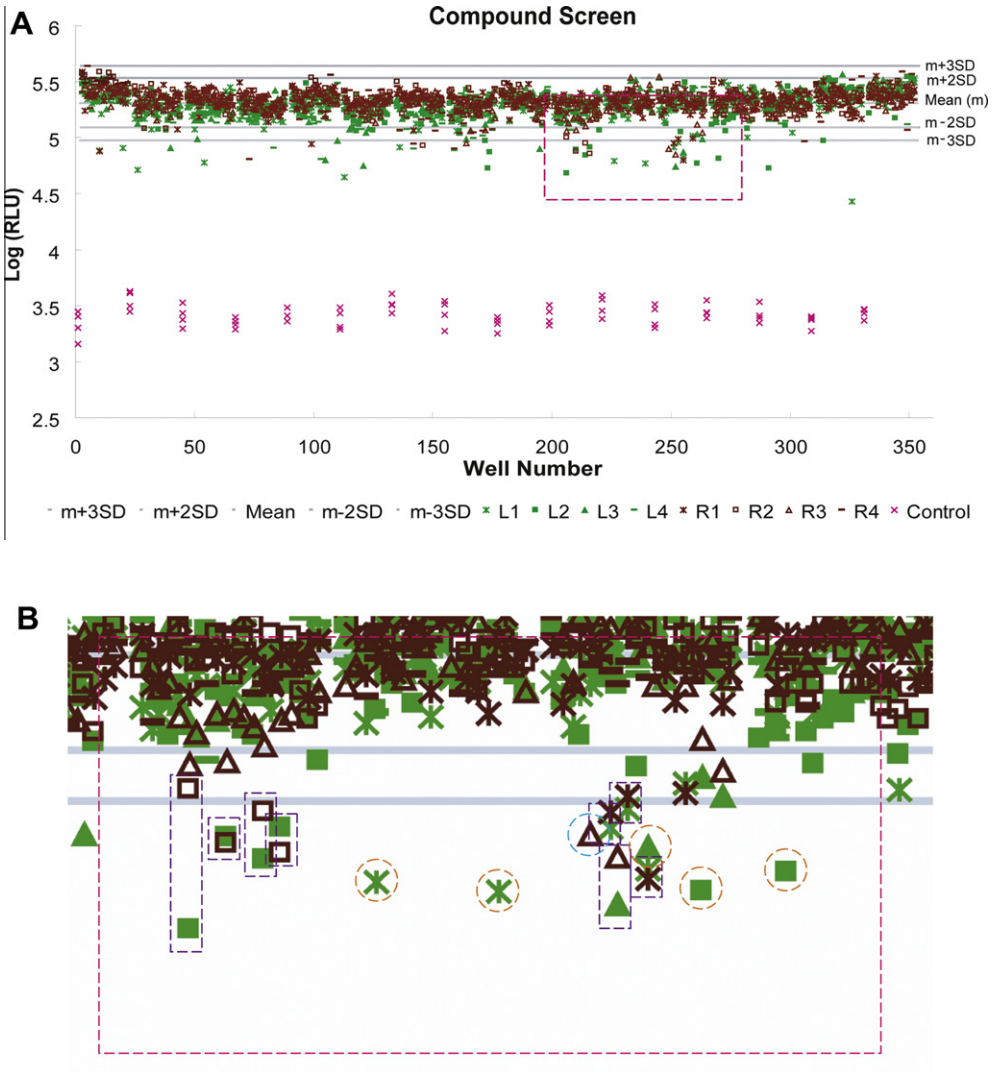

Fig. 3. High-throughput screens for inhibitors of SARS-CoV-mediated entry. (A) An example of screening a small molecule library with the viral combination of HIV-luc (SARS-CoV S) and HIV-ren (Lassa GP). Results are presented as the logarithm (log) of relative light units (luciferase readings) from four 384-well plates. (B) Close-up of the highlighted area in 3A. Eight compounds that reduced both SARS-CoV S (green symbols) and Lassa GP (brown symbols) mediated entry are boxed together. 5 compounds that demonstrate reduction of SARS-CoV alone are highlighted by orange circles, and one compounds that reduces only Lassa GP is highlighted by a blue circle.

of cysteine proteases, and CTSL in particular, are highly effective at preventing SARS-CoV infection, while being relatively non-toxic *in vitro*. Thus, we screened a unique collection of cysteine protease inhibitors using the 96-well plate format. As expected from the inhibitor profiles, a large number of the library members were able to inhibit entry-mediated by SARS-CoV spike in a specific manner. Candidate inhibitors exhibiting greater than 90% inhibition of the SARS-CoV S mediated entry but no inhibition against the control envelope from the initial screens were further studied and dose response curves were generated (for an example of dose response curves from the protease inhibitor library, see Fig. 1).

3.3. Analysis of the selected inhibitor hits of SARS-CoV-mediated entry

A group of dipeptidyl epoxyketone molecules were identified in the protease inhibitor library screen that demonstrated significant inhibition of SARS-CoV S-mediated infection (Fig. 4). Interestingly, this family of compounds had already been selected from a previous screen of the same library as being potential inhibitors of one of SARS-CoV's own proteases, 3Clpro (Goetz et al., 2007). In particular, WRR 182 and WRR 183 both specifically inhibited live SARS-CoV viral replication by greater than 50% at 10 μM (Goetz et al., 2007), even though only WRR 183 was highly active against the viral protease. In our studies, compound WRR 182 specifically inhibited SARS-CoV entry with an IC_{50} of less than 0.1 nM (Fig. 4A and Table 2), over 40-fold more potent than the inhibitor, Z-Phe-Phe-FMK, which is marketed as being CTSL-specific. WRR 182 is an epoxide-based inhibitor with a P3 L-phenylalanine residue and an R configuration at C-2 of the epoxide group (Table 2). WRR 183, a C-2 (S) epoxide isomer of WRR 182, was a 300-fold less potent inhibitor of SARS-CoV S-mediated entry compared to WRR 182 (Fig. 4A and Table 2). Neither of these compounds demonstrated detectable cytotoxicity, even at 10 μM (data not shown and Goetz et al., 2007).

A second generation of dipeptidyl-derived inhibitors based on the scaffolds of the epoxyketones had already been synthesized as previously described (Goetz et al., 2007) and viral inhibition assays were performed (Table 2). It was hoped that a compound capable of efficiently inhibiting both the viral protease and CTSL could be identified. A number of compounds retained the ability

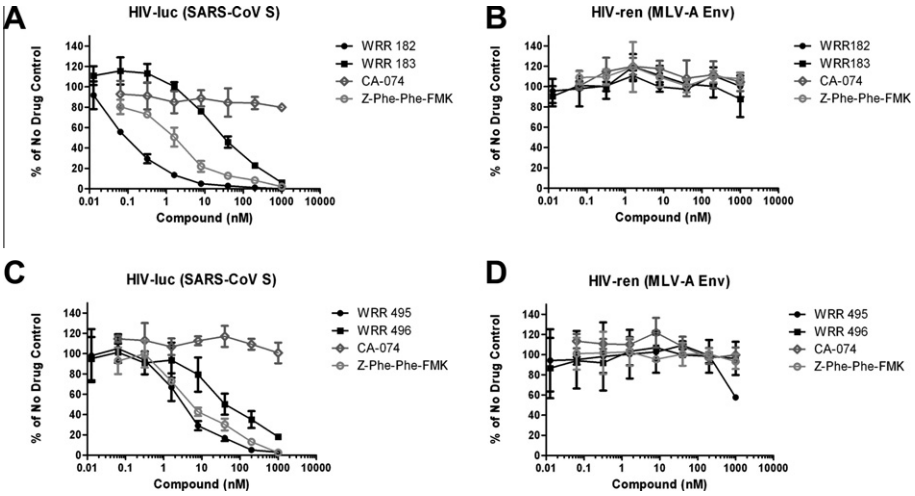

Fig. 4. Epoxyketone inhibition of SARS-CoV (panels A and C) and control envelope entry (panels B and D). Results are presented as a percentage of the luciferase reading of no drug controls and shown as mean ± SD of triplicate measurements. A representative experiment of at least three experiments is shown.

Table 2
WRR182, WRR183 and derivatives[a].

Compounds	IC50 (nM)[b]	IC90 (nM)[c]
WRR182	0.09 ± 0.01	2.97 ± 1.12
WRR183	32.84 ± 3.52	715.07 ± 1.34
WRR485	0.82 ± 0.18	43.41 ± 1.31
WRR486	282.52 ± 20.64	>1000
WRR487	>1000	>1000
WRR488	59.83 ± 8.21	437.48 ± 2.09
WRR492	>1000	>1000
WRR493	>1000	>1000
WRR495	3.36 ± 1.06	54.31 ± 1.99
WRR496	50.57 ± 7.22	>1000
CA-074	>1000	>1000
Z-Phe-Phe-FMK	4.47 ± 1.05	156.22 ± 4.20

[a] Assay were performed in triplicate and the values are representative of three or more independent experiments.
[b] IC50: concentration of the compound that produced 50% decrease in SARS-CoV pseudotyped virus infection in 293T-ACE2 cells, respectively.
[c] IC90: concentration of the compound that produced 90% decrease in SARS-CoV pseudotyped virus infection in 293T-ACE2 cells, respectively.

to inhibit the viral protease, including WRR 495, which was highly potent against 3Clpro (Goetz et al., 2007). WRR 495 was found to inhibit SARS-CoV entry with a respectable IC_{50} of approximately 3 nM, again somewhat better than the CTSL-specific inhibitor, Z-Phe-Phe-FMK (Fig. 4C). However, this compound was found to have greatly increased toxicity with little added anti-viral benefit (Goetz et al., 2007). Even though no ideal lead candidates with dual activity have yet been identified it is hoped that further investigation may yield such a compound.

4. Discussion

In this study, we have developed a novel dual pseudovirion-based HTS assay system with lentiviral pseudotyped particles. This system focuses on finding inhibitors of the step of viral entry, and minimizes the risks associated with hazardous viruses, allowing handling at BSL-2, when specialized BSL-3 facilities would otherwise be necessary. The DEP assay also identifies grossly toxic compounds and reduces the number of false positive hits introduced by compounds able to non-specifically inhibit shared cellular functions of many viruses, for example endocytosis. We demonstrate that this system reduces the number of false positive hits significantly, allowing earlier identification of promising inhibitors. Indeed, if envelopes are chosen with care, this assay allows simultaneous screening of two unrelated envelopes of interest that can also act as internal controls for each other.

In earlier screens we found that many promising inhibitors of entry mediated by pH-dependent viruses such as SARS-CoV, were able to inhibit other viruses, likely due to toxic or other more subtle effects on cellular function. These hits were often not cytotoxic in common laboratory cell lines, but showed sufficient signs of toxicity in primary cell types such as hepatocytes not to be considered likely drug candidates (data not shown). Thus, although pan-viral family antivirals are an attractive goal, we have generally avoided such leads as a somewhat unrealistic objective, and the dual envelope assay allows the rapid and early identification and elimination of such compounds. Ironically, notable exceptions to this rule are the protease inhibitors described in this study. These compounds act through a very defined mechanism, blocking envelope processing and/or viral capsid uncoating and can clearly be demonstrated to inhibit filoviruses, coronaviruses, henipaviruses and reoviruses (Chandran et al., 2005; Ebert et al., 2002; Pager et al., 2006; Pager and Dutch, 2005; Schornberg et al., 2006; Simmons et al., 2005). Thus, care must be taken when choosing partner virus envelopes, not to use envelopes with overlapping functions, receptors or specific targets. For example, the use of Ebola GP with SARS-CoV S would have eliminated the detection of the WRR series of compounds, as entry mediated by both viral envelopes is blocked by CTSL inhibitors. Indeed, the assay could be reversed if a common entry mechanism were suspected, in order to deliberately search for specific inhibitors of this mechanism by only selecting hits that reduced both viruses.

Based on this assay system, we successfully initiated screens of two libraries for compounds that would target the viral entry process of SARS-CoV. In particular, inhibitors of the essential step of SARS-CoV spike proteolysis, which, in cell lines at least, occurs in the endosomes of target cells (Huang et al., 2006; Simmons et al., 2005) were identified. It is hoped that compounds identified in these assays to be specifically able to inhibit the endsomal proteolytic processing of SARS-CoV Spike will be useful in animal studies for determining whether the CTSL/endosomal route of entry or surface protease-mediated fusion route is most important in vivo. Many of these hits were dipeptide epoxyketones, and had previously been reported to inhibit one of the coronavirus proteases (Goetz et al., 2007). Two of these epoxyketones, WRR 182 and WRR 183, are isomers of each other. This affects their biological properties, with WRR 183 being 10-fold more potent

against the SARS-CoV chymotrypsin-like protease. Nevertheless, both compounds appear to inhibit SARS-CoV replication in vitro with apparently similar abilities (Goetz et al., 2007). We demonstrate here that WRR 182, but not 183, is highly active against SARS-CoV spike-mediated entry. Thus, the antiviral properties of these two isomers are explained. The R isomer likely acts through the inhibition of CTSL activity. In contrast, the S isomer inhibits replication due to the fact that it binds irreversibly to the 3Clpro protease. Compounds capable of inhibiting both CTSL and 3Clpro would target the virus at multiple steps of the life cycle, thus increasing their efficiency. This would also likely lessen the chances of drug resistance being established. With the WRR 495 compound we demonstrate that this is indeed possible. Unfortunately, this compound, unlike the parental compounds, is highly toxic. It is possible that this is due to WRR 495 becoming broadly active against many cellular proteases, although we have not demonstrated this. Nevertheless, WRR 495 represents the potential that other, less toxic, dually active third generation compounds can be developed.

In conclusion, this highly sensitive, rapid, and reproducible assay system has been successfully applied for the screening of entry inhibitors of SARS-CoV. Moreover, it can be easily adapted to screen for entry inhibitors of other viruses with careful selection of matching partner virus envelopes of no interference. In particular, human coronaviruses together with other respiratory viruses, such as metapneumovirus and parainfluenzavirus, can cause significant morbidity and mortality, particularly among immunocompromised patients. HTS assays for entry inhibitors, such as the DEP assay described here, should provide good lead candidates for antiviral drug development against SARS-CoV and other viruses.

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