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Development of a homogeneous screening assay for automated detection of antiviral agents active against severe acute respiratory syndrome-associated coronavirus

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Abstract

The severity and global spread of the 2003 outbreak of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) highlighted the risks to human health posed by emerging viral diseases and emphasized the need for specific therapeutic agents instead of relying on existing broadly active antiviral compounds. The development of rapid screening assays is essential for antiviral drug discovery. Thus, a screening system for anti-SARS-CoV agents was developed, which evaluated compound potency, specificity and cytotoxicity at the initial screening phase. Cell lines were engineered to constitutively express an enhanced green fluorescent protein (EGFP) and used to detect (1) antiviral potency in SARS-CoV infection tests; (2) antiviral specificity in tests using the porcine coronavirus transmissible gastroenteritis virus (TGEV); and (3) cytotoxicity in the same assays without virus challenge. The assay system involves minimal manipulation after assay set-up, facilitates automated read-out and minimizes risks associated with hazardous viruses. The suitability of this assay system in drug discovery was demonstrated by screening of 3388 small molecule compounds. The results show that these assays can be applied to high-throughput screening for identification of inhibitors selectively active against SARS-CoV.

Keywords: SARS-CoV; High-throughput screening; Antiviral activity; EGFP expression

1. Introduction

SARS is an infectious disease with a high mortality rate caused by the human coronavirus SARS-CoV (Ksiazek et al., 2003). The severity and global spread of the 2003 SARS outbreak has led to the search for specific antiviral compounds against SARS-CoV. Results from both in vitro studies and case reports from the clinic have been published, but no consensus treatment has yet been established. Major efforts are being made to understand the mechanism of SARS-CoV replication in order to identify drug targets of specific antiviral compounds (Chu et al., 2004; Liu et al., 2004; Thiel et al., 2003; Vastag, 2003; Wu et al., 2004). Coronaviruses are enveloped positive-strand RNA viruses that initiate infection of susceptible cells by binding to human angiotensin converting enzyme-2 (ACE-2) on the host cell membrane via the viral S protein (Li et al., 2003; Xu et al., 2004). Following receptor binding and membrane fusion, the infectious genomic RNA is delivered into the cytoplasm and viral gene expression is initiated resulting in the translation of two replicase/transcriptase polyproteins. These two enzymes are responsible for the replication of the viral genome as well as the generation of a multiple subgenomic mRNAs including those encoding the structural proteins S (spike), M (membrane), E (envelope) and N (nucleocapsid) (De Haan et al., 2002). A complex set of regulatory mechanisms controls the viral gene expression, and additional virus-encoded enzymes, such as helicase and proteases, are crucial for the production of infectious virus particles (Thiel et al., 2003). Progeny

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virions are assembled in the endoplasmatic reticulum-golgi apparatus and released from infected cells by exocytosis. The viral entry and assembly processes as well as the enzymatic steps involved in viral replication constitute potential targets for antiviral therapy.

Understanding the mechanism of viral replication is not the only key step towards identification of effective drugs against a virus. Development of rapid screening assays is also essential for antiviral drug discovery. The present standard infectivity assay for SARS-CoV is based on viral infection of VeroE6 cells followed by visual monitoring of cytopathic effects (CPE; Ksiazek et al., 2003). The low-throughput nature of this assay format limits its use for efficient screening of large chemical libraries. This study describes the development of a stable cell line susceptible to SARS-CoV that constitutively expresses EGFP, which can be used for the automated screening of inhibitors of virus activity. A high degree of correlation between virus replication and reduced EGFP signal is expected for highly cytopathic viruses such as SARS-CoV where the background EGFP signal is rapidly reduced by extensive cell death. The specificity and selectivity of compounds active in this SARS-CoV screening assay were determined by parallel screening for cytotoxicity and inhibition of a porcine coronavirus using similar assay formats. This set of assays was used in the screening of a test panel of 3388 randomly chosen chemical compounds to demonstrate its application to drug discovery.

2. Materials and methods

2.1. Cells, viruses and reagents

The porcine kidney cell line LLC-PK1 and the African monkey kidney cell line VeroE6 were kindly provided by Dr. K. Andries (J&JPRD; Beerse, Belgium) and Dr. G. Van Ham (Institute for Tropical Medicine, Antwerp, Belgium), respectively. The LLC-PK1 and VeroE6 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Cambrex BioScience Walkersville, Walkersville, MD) supplemented with 10% fetal calf serum (FCS; Highclone, Logan, UT), 1% L-glutamine (Invitrogen, Carlsbad, CA) and 0.02% gentamycin (Invitrogen). The packaging cell line RetroPack PT67 was purchased from BD Biosciences Clontech (Palo Alto, CA) and grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and 0.02% gentamycin. The retroviral vector pLNCE, containing the EGFP coding sequence under the cytomegalovirus immediate-early promotor, was a kind gift of Dr. E. Kandel (University of Illinois, Chicago, IL). SARS-CoV, strain 200300592, was obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, GA) and TGEV, strain purdue112, was obtained from J&JPRD (Beerse, Belgium). The AF933 antibody directed against ACE-2 was purchased from R&D Systems Europe (Abingdon, UK).

2.2. Generation and characterization of EGFP expressing VeroE6 and LLC-PK1 cell lines

To produce retroviruses for EGFP gene delivery, PT67 cells were transfected with pLNCE using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The supernatant of the transfected PT67 cells was collected and used to transduce LLC-PK1 and VeroE6 cells. The transduced cells were incubated for 3 days at 37 °C and 100 clones from each cell line were screened for homogeneous and high EGFP signal by fluorescence activated cell sorting (FACS). The clones were also analyzed for susceptibility to SARS-CoV or TGEV infection and for growth capacity by seeding 15,625, 25,000, 31,250 or 62,500 cells/ml and monitoring the EGFP signal over 7 days. The constitutively EGFP expressing cell lines were designated VeroE6-EGFP and LLC-PK1-EGFP.

2.3. Virus stock production and titration

All virus culture and screening assays were carried out in a certified biosafety level-3 laboratory. LLC-PK1 cells were inoculated with TGEV and VeroE6 cells were inoculated with SARS-CoV and subsequently incubated in a humidified incubator at 37 °C and 5% CO_2. After 2 days, when the majority of cells showed CPE, the cell culture supernatants were clarified by centrifugation at 2000 × *g* for 5 min and stored at −80 °C until use. The 50% cell culture infectious dose ($CCID_{50}$) was determined by titration on LLC-PK1 or VeroE6 cells depending on virus using 10 serial five-fold-dilution steps. After 5 days incubation at 37 °C, the plates were inspected for CPE.

2.4. TGEV and SARS-CoV screening assays

The test compounds were serially diluted four-fold in cell culture medium and mixed with 200 $CCID_{50}$ of TGEV or 300 $CCID_{50}$ of SARS-CoV and 1000 of LLC-PK1-EGFP or VeroE6-EGFP cells (corresponding to a final concentration of 25,000 cells/ml) in 384-well blackview plates (Corning; Corning, NY). The plates were sealed with gas permeable membrane and incubated in a humidified incubator at 37 °C and 5% CO_2. After 5 days, the wells were examined for EGFP expression using an argon laser-scanning microscope. The microscope settings were excitation at 488 nm and emission at 510 nm and the fluorescence images of the wells were converted into signal values. The results were expressed as EC_{50} values defined as the concentration of compound achieving 50% inhibition of the virus-reduced EGFP signals as compared to the untreated virus-infected control cells.

2.5. Cytotoxicity screening assays

To test for cytotoxicity, cells were incubated with serial compound dilutions as described above but in the absence of virus challenge. The 50% cytotoxic concentration (CC_{50}) was determined by comparing the EGFP signal from treated

wells to that of control wells containing untreated cells and calculated similarly to EC_{50}.

2.6. Selectivity indices

Two selectivity indices (SI) were calculated. The cytotoxicity/antiviral activity SI provides a measure of the range where a compound is effective without being cytotoxic and was calculated as the ratio CC_{50}/EC_{50}. The TGEV/SARS-CoV SI represents the specificity of a compound in inhibiting SARS-CoV over TGEV and was calculated as the ratio TGEV EC_{50}/SARS-CoV EC_{50}.

2.7. Validation and characterization of screening assays

The validity of reduced EGFP expression as a marker for virus activity was determined by titration of a SARS-CoV stock on VeroE6-EGFP cells and by calculating titers based on both EGFP signal and CPE. Similar experiments were performed using TGEV and LLC-PK1-EGFP cells.

To determine the sensitivity of the assays, defined as the lowest virus titer resulting in a detectable decrease in EGFP signal, serially diluted SARS-CoV or TGEV stocks were added to their respective indicator cells and the EGFP signals were recorded as described for the screening assay.

To determine intra- and inter-experiment reproducibility, 25,000 VeroE6-EGFP cells/ml were added together with 300 $CCID_{50}$ of SARS-CoV into 192 wells of a 384-well plate and an additional 192 wells of the same plate were seeded with 25,000 uninfected VeroE6-EGFP cells/ml as controls. The EGFP signal of each well was recorded 5 days post plating as described for the screening assay. The experiment was repeated on three separate days and intra-experimental variation was determined by calculating mean EGFP signals for the virus-infected and control cells plated within the same day. Inter-experimental variation was determined by calculating the mean EGFP signal for the experiments performed on three separate days. Standard deviation (S.D.), coefficient of variation (CV), and signal ratio for control versus virus-infected cells were calculated for both intra- and inter-reproducibility experiments.

To demonstrate the specificity of the assay, defined as the ability to detect selective SARS-CoV inhibitors, serial dilutions of an ACE-2 antibody were prepared in phosphate buffered saline (PBS; Sigma, St. Louis, MO) and analyzed in parallel in the SARS-CoV, TGEV and cytotoxicity screening assays as described above.

2.8. Selection and preparation of compounds

To identify potential classes of pharmacological agents active against SARS-CoV, a range of previously described antiviral compounds was tested (Table 1). The antiviral agents included compounds targeting both viral and cellular factors. Ribavirin (So et al., 2003) and AZT (Furman et al., 1986; Zhang et al., 2001) were included as representatives of the widely used class of nucleoside analogs inhibiting viral polymerases. Selected antiviral protease inhibitors have been implicated as being active against the SARS-CoV (Yamamoto et al., 2004; Wu et al., 2004; Chu et al., 2004), meriting the inclusion of lopinavir/ritonavir. A range of non-specific antiviral reagents was included such as aurintricarboxylic acid (ATA), dextran sulfate 5000 (DS5000), and Novaron. Glycirrhizin and *S*-nitroso-*N*-acetylpenicillamine were included due to previously reported activities against SARS-CoV by unclear mechanisms of action (Cinatl et al., 2003; Keyaerts et al., 2004). Additionally, a variety of antiviral compounds acting on cellular targets were included such

Table 1
Reference compounds tested in the screening assay

Compound	Source	Reported activity	Reference
Ribavirin	Sigma	Nucleoside analog active against a diverse range of virus	So et al., 2003
AZT	Sequoia Research Products	HIV and HTLV-1 RT inhibitor	Furman et al., 1986; Zhang et al., 2001
lopinavir/ritonavir[a]	Sequoia Research Products and Toronto Research Chemicals	HIV PR inhibitor	Mangum and Graham, 2001
ATA	Sigma	Broadly acting with reported binding to multiple viral targets	Zhang et al., 1999; Cushman and Sherman, 1992; Schols et al., 1990
DS5000	Specs and biospecs	Non-specific virus entry inhibitor	Zhang et al., 1999; Schols et al., 1990
Novaron	Milliken chemical	Broadly acting microbiocide with SARS-CoV activity	Toagosei Press Release, 2003; Yoshida et al., 1999
Glycirrhizin	TCI Europe	SARS-CoV replication inhibitor	Cinatl et al., 2003
S-Nitroso-*N*-acetylpenicillamine	Sigma	SARS-CoV replication inhibitor	Keyaerts et al., 2004
P38 MAP kinase inhibitors #1–6	J&J PRD	Possible suppression of HIV and HCV infection and symptoms	Balasubramanian et al., 2003
TGF-β inhibitors #1–3	Scios Inc.	Possible suppression of HCV symptoms	Ray et al., 2003
ACE inhibitors #1–21	J&J PRD	Inhibition of human ACE	Internal screening data

[a] Lopinavir (obtained from Toronto Research Chemicals) and ritonavir (Sequoia Research Products) was mixed at a ratio of 4:1.

as p38 MAP kinase inhibitors, TGF-β inhibitors, and 21 ACE inhibitors (Table 1). Separately, 3388 randomly chosen compounds from a proprietary chemical library were tested. All compounds were dissolved at 20 mM dimethylsulphoxide (DMSO; Sigma) and then diluted in cell culture medium to a final DMSO concentration below 0.5 %.

3. RESULTS

3.1. Development of EGFP-expressing VeroE6 and LLC-PK1 cells

To provide convenient indicator cells for the high-throughput screening assays, stable cell lines constitutively expressing EGFP were generated. VeroE6 and LLC-PK1 cell lines were transduced with a packaging retrovirus carrying the EGFP gene and 100 clones were isolated for each cell line for subsequent characterization studies. The cells were subjected to FACS analysis of EGFP expression to select those clones exhibiting a profile of high mean fluorescence intensity and homogeneous signal (Fig. 1). The EGFP expressing clones were further evaluated for susceptibility to virus infection, showing all clones were susceptible to either SARS-CoV or TGEV as measured by CPE induction after virus challenge (data not shown). The growth characteristics of the VeroE6-EGFP and LLC-PK1-EGFP clones were similar to the parental VeroE6 and LLC-PK1 cell lines (data not shown), indicating that the EGFP expression did not affect cell proliferation. As expected, the transduced cells showed an exponential growth that was dependent on the initial cell density (a representative example is shown in Fig. 2). Moreover, the growth experiments showed that a final seeding density of 25,000 cells/ml was suitable for keeping the cells in the exponential growth phase throughout a 5-day assay period. Taking all selection criteria into account, clone 21 of VeroE6-EGFP and clone 45 of LLC-PK1-EGFP (data not shown) were selected as indicator cells to be seeded at a density of 25,000 cells/ml in the screening assays.

3.2. Validation of screening assays

To ensure a reliable screening platform, the reduced EGFP expression was validated as a marker for virus-induced cytopathic activity and the robustness and specificity of the assays were determined. The validity of using quantification of a reporter gene such as EGFP to measure viral activity was determined by virus titration experiments calculating titers based on both CPE and EGFP measurements. The titration experiments showed a virus-concentration dependent loss of EGFP expression and that virus titers scored by both methods were in good agreement (Table 2), confirming that EGFP expression can serve as a marker for activity of these two viruses.

The assay sensitivity was determined by the lowest virus titer resulting in an at least two-fold decrease in EGFP signal.

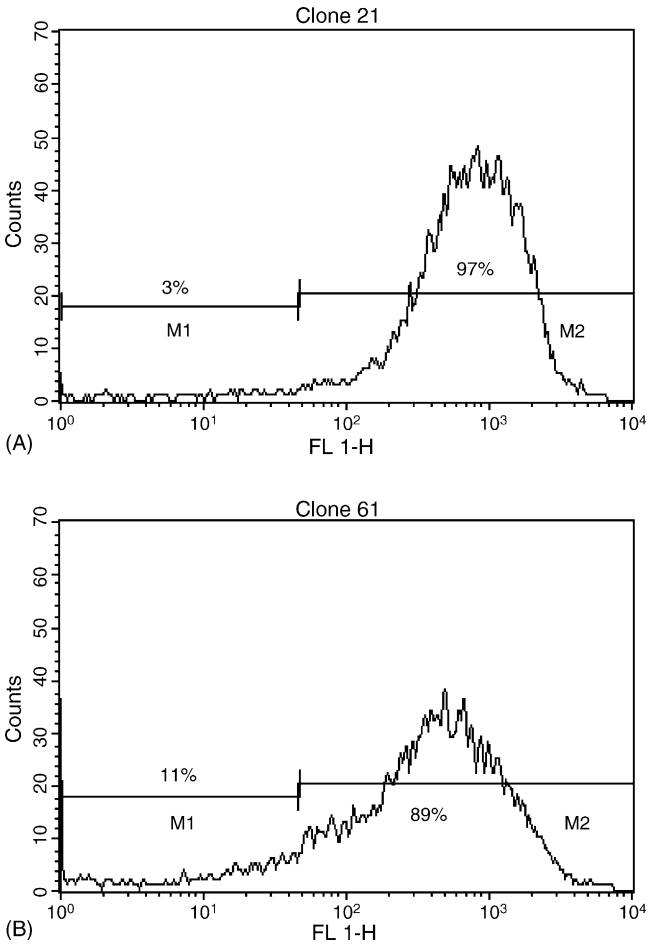

Fig. 1. EGFP expression profiles of VeroE6-EGFP clones. FACS analysis with arbitrarily set gates M1 and M2 showed that cell clone 21 (A) exhibited a preferred EGFP expression pattern as compared to clone 61 (B) as demonstrated by the higher mean fluorescence intensity (925 vs. 672) and higher fraction of bright cells (97% vs. 89% within the M2 gate). Percentages represent the fractions of events recorded within each gate.

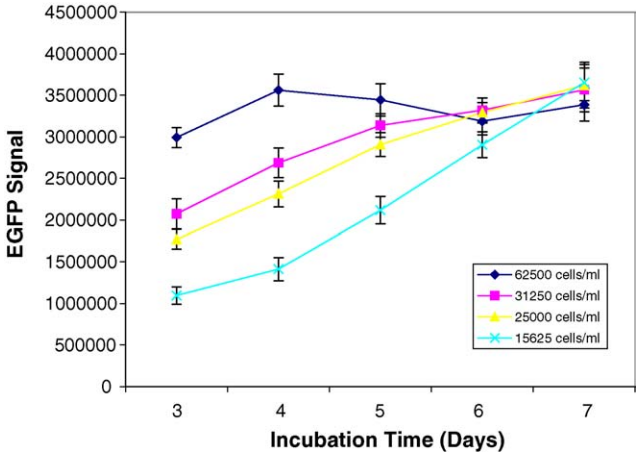

Fig. 2. Growth characteristics of VeroE6-EGFP clone 21. The cells showed an exponential growth that was dependent on the initial seeding density. Data points and error bars represent mean and S.D. obtained in one single experiment using 80 replica wells per cell concentration and time point.

Table 2
Comparative virus titration using CPE and EGFP as read-out

TGEV ($CCID_{50}$/well)		SARS-CoV ($CCID_{50}$/well)	
Titer[a] scored using CPE	Titer[a] scored using EGFP	Titer[a] scored using CPE	Titer[a] scored using EGFP
99,034 ± 8129	116,536 ± 32,715	329,964 ± 38,427	329,964 ± 38,427

[a] Results represent mean ± S.D. of two independent titration experiments.

The indicator cells showed a significant reduction of EGFP signal at virus titers as low as 2.0 $CCID_{50}$ SARS-CoV and 1.2 $CCID_{50}$ TGEV (Fig. 3), confirming that the measurement of EGFP expression in these cells allows sensitive detection of low levels of virus.

Assay robustness or intra- and inter-assay variation was assessed by multiple EGFP measurements on the same day and over several days. The signal ratio between uninfected control cells and virus-infected cells varied between 4.1 and 19.8, suggesting a sufficient dynamic range to measure antiviral activity of test compounds (Table 3).

The specificity of the assay, defined as the ability to detect selective SARS-CoV inhibitors, was determined by testing an anti-ACE-2 antibody specific for the SARS-CoV receptor in the screening assays. The antibody showed a selective and potent inhibition of SARS-CoV at non-cytotoxic concentrations (Fig. 4 and Table 4).

The antiviral EC_{50} value was 1.43 μM when measuring virus inhibition based on EGFP signal (Table 4) and 1.56 μM when using CPE as read-out (data not shown). This further shows the close correlation between EGFP expression and viral cytopathic activity. Together, the validation experiments showed that the screening system was reproducible and specific for the detection of agents active against SARS-CoV.

3.3. Screening of antiviral agents and chemical library compounds

The SARS-CoV screening system was initially used to test a selection of pharmacological agents. The compounds included agents targeting different viral factors as well as inhibitors of cellular p38 kinase, TGF-β, and ACE (Table 1). One of the 21 ACE inhibitors (Table 4) showed a selective antiviral activity against SARS-CoV using a cytotoxicity/antiviral activity SI criterion of 3. However, the LLC-PK1-EGFP cell line appeared to be more sensitive to the cytotoxic effects of the compound than the VeroE6-EGFP cell line, indicating that the ACE inhibitor may exhibit cellular effects not observed in the SARS-CoV/VeroE6-EGFP assay system. The other tested agents showed no antiviral activity below the cytotoxic concentration (Table 4 and data not shown).

To test the possible use of the assay system in a high-throughput setting, a total of 3388 small molecule compounds were tested for selective antiviral activity against SARS-CoV. Sixty-four compounds were active in the SARS-CoV assay and 18 of these compounds exhibited a cytotoxicity/antiviral activity SI of at least 3 (listed as compounds A–R in Table 4). The EC_{50} values ranged between 0.83 and 9.57 μM. Out of the 18 anti-SARS-CoV active and non-cytotoxic compounds, 2 showed a TGEV/SARS-CoV SI of at least 3 in the absence of cytotoxic effects on any of the two indicator cell lines (compounds N and P in Table 4). Together, these results show that the combination of assays used in this screening system has the potential to provide an efficient basis for drug discovery.

4. Discussion

An important aspect of drug screening for new viral diseases is the choice of assay system. A drug candidate has to fulfill a number of requirements regarding potency, target specificity and cytotoxicity. Thus, a screening system was designed with three parallel types of assays with similar set-up and read-out to evaluate all these parameters concurrently at the initial screening phase. Susceptible cell lines were equipped with EGFP reporter genes and used for quantification of cytotoxicity, SARS-CoV and TGEV activity. This assay system can be readily applied to high-throughput screening and has many advantages over assays based on

Table 3
Intra- and inter-experimental reproducibility

		Experiment #1	Experiment #2	Experiment #3	Inter-experiment
SARS-CoV infected cells	Mean[a]	146328	176300	405419	242682
	S.D.[a]	6329	31787	55651	126894
	CV (%)	4.3	18	13.7	52.3
Uninfected cells	Mean[a]	2594210	2515356	1580705	2230090
	S.D.[a]	89300	222138	112550	505836
	CV (%)	3.4	8.8	7.1	22.7
	Ratio[b]	19.8	14.7	4.1	14.7

[a] Mean and S.D. values are given as EGFP signal (arbitrary units of fluorescence/well).
[b] EGFP signal ratio for uninfected control cells versus virus-infected cells.

laborious and subjective scoring of CPE or plaque formation. The assay system described in this study requires minimal manipulation after assay set-up, and no staining, washing, fixation or manual inspection of the tissue culture plates which is especially advantageous when working with hazardous viruses such as SARS-CoV.

The strategy to use parallel counter-screening of chemical compounds on a porcine-specific member of the coronavirus family was employed to select drug candidates with high specificity for the human SARS-CoV. Highly virus-specific compounds are more likely to exhibit a well-defined single viral target and binding site. This greatly facilitates structure-activity analysis and optimization of active compounds identified in an initial screening. Thus, the platform of three parallel screening assays described here obtains more data from the potentially useful compounds at the initial screening step than simple infectivity assays, and therefore speeds up downstream drug development. It is, of course, conceivable that potent antiviral drug candidates can be developed from compounds targeting multiple coronaviruses: compounds active against other coronaviruses may be selected from the data obtained in this screening system and analyzed separately.

Analyzing a panel of reported antiviral compounds with different mechanisms of activity tested the screening system. The finding that an ACE inhibitor showed selective activity against SARS-CoV is in line with the reported usage of ACE-2 as a receptor for this virus (Li et al., 2003). Ribavarin, although active against a wide range of viruses and used as SARS therapy (So et al., 2003), was shown in this study to be inactive against SARS-CoV in vitro at non-cytotoxic concentration, which is in line with previous reports (Tan et al., 2004; Cinatl et al., 2003; Wu et al., 2004; Yi et al., 2004). The variation in results obtained in this study from novaron, glycirrihizin and *S*-nitroso-*N*-acetylpenicillamine as compared to previously published results may be due to assay conditions including virus strain, multiplicity of infection, detection method and compound concentration and handling (Cinatl et al., 2003; Keyaerts et al., 2004). Notably, other independent groups have also observed lack of anti-SARS-CoV activity of glycyrrhizin (Hertzig et al., 2004; Yi et al., 2004). The

Table 4
Screening activities for validation-, general antiviral- and chemical library compounds.

Compound	SARS-CoV EC_{50} (μM)	VeroE6-EGFP CC_{50} (μM)	TGEV EC_{50} (μM)	LLC-PK1-EGFP CC_{50} (μM)	SI SARS CC_{50}/EC_{50}	SI TGEV EC_{50}/SARS EC_{50}
Validation compound						
ACE-2 antibody	1.43[a]	>125[a]	>125[a]	>125[a]	>87.4	>87.4
General antiviral compound						
Ribavirin	>100	>100	>20.2[b]	20.2	–[c]	–[c]
AZT	>100	>100	>100	>100	–[c]	–[c]
Lopinavir/ritonavir	>100	>100	>100	>100	–[c]	–[c]
ATA	>9.19[b]	9.19	>56.9[b]	56.9	<1.00	–[c]
DS5000	>100	>100	>100	>100	–[c]	–[c]
Novaron	>12.0[b,d]	12.0[d]	>13.1[b,d]	13.1[b,d]	<1.00	–[c]
Glycirrhizin	>100	>100	>100	>100	–[c]	–[c]
S-Nitroso-*N*-acetylpenicillamine	>530[b]	530	>312[b]	312	<1.00	–[c]
ACE inhibitor #1	4.83	15.1	>5.80[b]	5.80	3.13	>1.20
Chemical library compounds						
A	0.83	5.05	1.41	1.85	6.08	1.70
B	2.76	15.0	1.28	6.54	5.43	0.464
C	3.11	22.0	5.83	13.8	7.07	1.87
D	9.43	32.0	6.41	16.1	3.39	0.680
E	1.47	7.04	>2.28[b]	2.28	4.79	>1.55
F	1.64	8.27	>3.99[b]	3.99	5.04	>2.43
G	1.83	7.70	2.67	6.31	4.21	1.46
H	1.89	7.49	3.16	3.15	3.96	1.67
I	2.18	7.51	2.40	5.91	3.44	1.10
J	2.69	20.3	1.34	7.97	7.55	0.498
K	2.90	11.1	2.27	7.66	3.83	0.783
L	3.79	>15.0	>0.06[b]	0.06	>3.96	>0.016
M	3.93	13.2	>4.67[b]	4.67	3.36	>1.19
N	4.32	>32.0	>32.0	>32.0	>7.41	>7.41
O	4.83	>15.0	>9.17[b]	9.17	>3.11	>1.90
P	5.00	>15.0	>15.0	>15.0	>3.00	>3.00
Q	9.53	>32.0	13.9	>32.0	>3.36	1.46
R	9.57	>32.0	5.04	15.5	>3.34	0.527

[a] Antibody concentrations represent μg/ml.
[b] No inhibition was detected below the cytotoxic concentration.
[c] Undefined.
[d] Compound concentrations represent mg/ml.

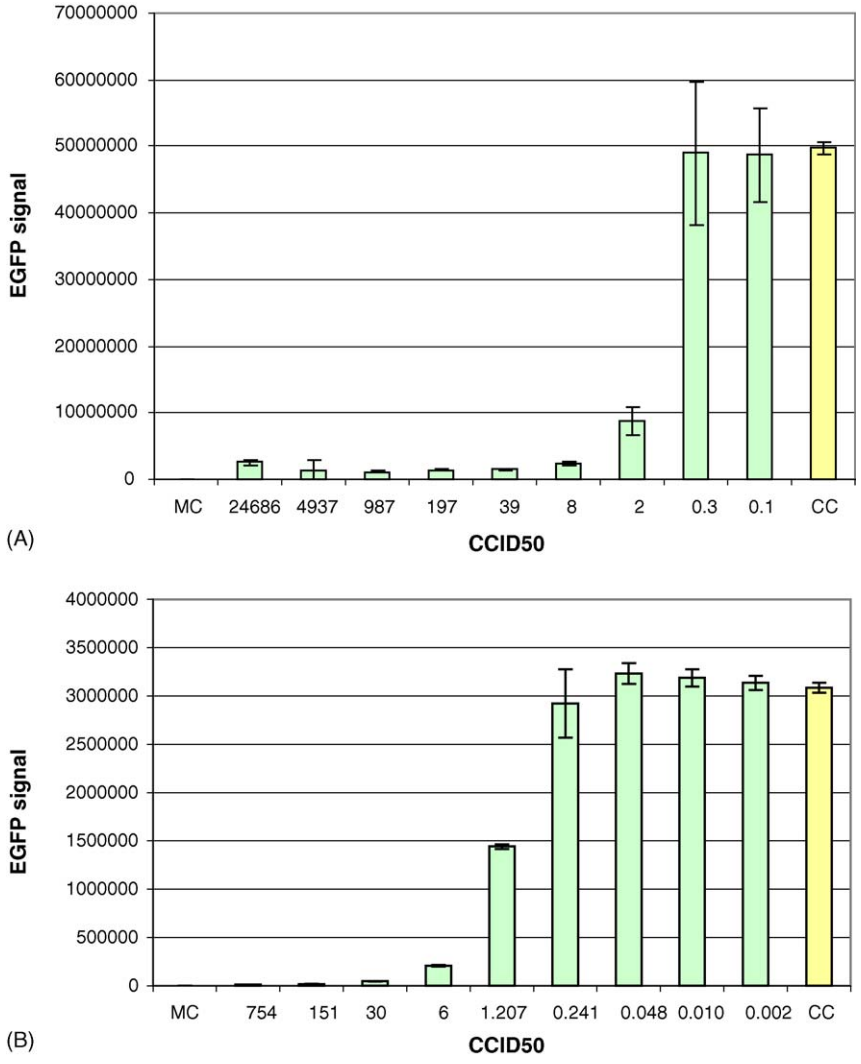

Fig. 3. Sensitivity of SARS-CoV and TGEV assays. The assays showed significant reduction in EGFP signal for virus titers of $\geq$2.0 $CCID_{50}$ of SARS-CoV (A) and $\geq$1.2 $CCID_{50}$ of TGEV (B). Data points and error bars represent mean and S.D. obtained in one single experiment using eight replica wells. MC, medium control and CC, uninfected cell control.

assay system was also evaluated in the setting of a chemical library screen. Compounds that were selectively active against SARS-CoV at non-cytotoxic compound concentrations were identified in the library screen. These compounds show a potential for further optimization into potent inhibitors of SARS-CoV and merit detailed characterization regarding mechanism of action.

In conclusion, this report describes a robust screening system for identification of drug candidates selectively active against SARS-CoV. This screening system meets the requirements of a successful high-throughput assay such as minimal manipulation after assay set-up, low sample volume and automated detection of end-point. Furthermore, this system offers considerable benefits from a first round of screening regard-

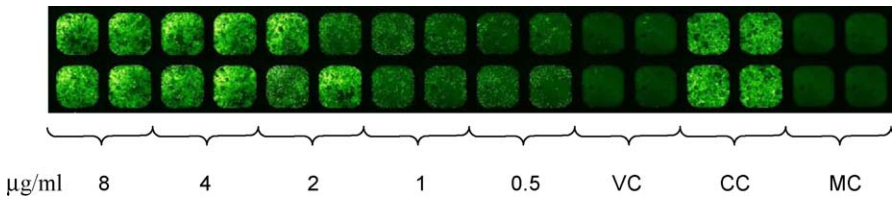

Fig. 4. Titration of the ACE-2 antibody on VeroE6 cells. The ACE-2 antibody showed a concentration-dependent protection of the EGFP signal after the SARS-CoV challenge. Quadruple wells of infected and treated VeroE6-EGFP cells are shown and the numbers indicate the tested antibody concentration in µg/ml. VC, virus control, CC, cell control, and MC, medium control.

ing selectivity, specificity and toxicity of the compound, resulting in an improved data set from an initial screen in comparison with existing assays based on simpler observation such as plaque formation or analysis of CPE. This system, with its ease of handling and good level of containment is well suited to drug discovery against the SARS-CoV, and should be applicable to other emerging high-risk pathogenic viruses.

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References

Balasubramanian, A., Ganju, R.K., Groopman, J.E., 2003. Hepatitis C virus and HIV envelope proteins collaboratively mediate interleukin-8 secretion through activation of p38 MAP kinase and SHP2 in hepatocytes. J. Biol. Chem. 278, 35755–35766.

Chu, C.M., Cheng, V.C.C., Hung, I.F.N., Wong, M.M.L., Chan, K.H., Chan, K.S., Kao, R.Y.T., Poon, L.L.M., Wong, C.L.P., Guan, Y., Peiris, J.S.M., Yuen, K.Y., HKU/UCH SARS Study Group, 2004. Role of lopinavir/ritonavir in the treatment of SARS: initial virological and clinical findings. Thorax 59, 252–256.

Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H., Doerr, H.W., 2003. Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. Lancet 361, 2045–2046.

Cushman, M., Sherman, P., 1992. Inhibition of HIV-1 integration protein by aurintricarboxylic acid monomers, monomer analogs, and polymer fractions. Biochem. Biophys. Res. Commun. 185, 85–90.

De Haan, C.A.M., Volders, H., Koetzner, C.A., Masters, P.M., Rottier, P.J.M., 2002. Coronaviruses maintain viability despite dramatic rearrangements of the strictly conserved genome organization. J. Virol. 76, 12491–12502.

Furman, P.A., Fyfe, J.A., St Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Lehrman, S.N., Bolognesi, D.P., Broder, S., Mitsuya, H., Barry, D.W., 1986. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Hertzig, T., Scandella, E., Schelle, B., Ziebuhr, J., Siddell, S.G., Ludewig, B., Thiel, V., 2004. Repid identification of coronavirus replicase inhibitors using a selectable replicon RNA. J. Gen. Virol. 85, 1717–1725.

Keyaerts, E., Vijgen, L., Chen, L., Maes, P., Hedenstierna, G., Van Ranst, M., 2004. Inhibition of SARS-coronavirus infection in vitro by *S*-nitroso-*N*-acetylpenicillamine, a nitric oxide donor compound. Int. J. Infect. Dis. 8, 223–226.

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.E., Humphrey, C.D., Shieh, W.J., Guarner, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J., Anderson, L.J., SARS Working Group, 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348, 1953–1966.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H.-Y., Farzan, M., 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426, 450–454.

Liu, S., Xiao, G., Chen, Y., He, Y., Niu, J., Escalante, C.R., Xiong, H., Farmar, J., Debnath, A.K., Tien, P., Jiang, S., 2004. Interaction between heptad repeat one and two regions in spike protein of SARS-associated coronavirus: implications for its fusogenic mechanism and identification of fusion inhibitors. Lancet 363, 938–947.

Mangum, E.M., Graham, K.K., 2001. Lopinavir-Ritonavir: a new protease inhibitor. Pharmacotherapy 21, 1352–1363.

Ray, S., Broor, S.L., Vaishnav, Y., Sarkar, C., Girish, R., Dar, L., Seth, P., Broor, S., 2003. Transforming growth factor beta in hepatitis C virus infection in vivo and in vitro findings. J. Gastroenterol. Hepatol. 18, 393–403.

Schols, D., Pauwels, R., Desmyter, J., De Clercq, E., 1990. Dextran sulfate and other polyanionic anti-HIV compounds specifically interact with the viral gp120 glycoprotein expressed by T-cells persistently infected with HIV-1. Virology 175, 556–561.

So, L.K.-Y., Lau, A.C.W., Yam, L.Y.C., Cheung, T.M.T., Poon, E., Yung, R.W.H., Yuen, K.Y., 2003. Development of a standard treatment protocol for severe acute respiratory syndrome. Lancet 361, 1615–1617.

Tan, E.L., Ooi, E.E., Lin, C.Y., Tan, H.C., Ling, A.E., Lim, B., Stanton, L.W., 2004. Inhibition of SARS coronavirus infection in vitro with clinically approved antiviral drugs. Emerg. Infect. Dis. 10, 581–586.

Thiel, V., Ivanov, K.A., Putics, A., Hertzig, T., Schelle, B., Bayer, S., Weissbrich, B., Snijder, E.J., Rabenau, H., Doerr, H.W., Gorbalenya, A.E., Ziebuhr, J., 2003. Mechanisms and enzymes involved in SARS coronavirus genome expression. J. Gen. Virol. 84, 2305–2315.

Toagosei Co. Ltd., 2003. News Release, Prous Daily Essentials, November 17.

Vastag, B., 2003. Old drugs for a new bug: influenza, HIV drugs enlisted to fight SARS. JAMA 290, 1695–1696.

Wu, C.Y., Jan, J.T., Ma, S.H., Kuo, C.J., Juan, H.F., Cheng, Y.S., Hsu, H.H., Huang, H.C., Wu, D., Brik, A., Liang, F.S., Liu, R.S., Fang, J.M., Chen, S.T., Liang, P.H., Wong, C.H., 2004. Small molecules targeting severe acute respiratory syndrome human coronavirus. Proc. Natl. Acad. Sci. USA 101, 10012–10017.

Xu, Y., Liu, Y., Lou, Z., Qin, L., Li, X., Bai, Z., Pang, H., Tien, P., Gao, G.F., Rao, Z., 2004. Structural basis for coronavirus-mediated membrane fusion: crystal structure of MHV spike protein fusion core. J. Biol. Chem. 279, 30514–30522.

Yamamoto, N., Yang, R., Yoshinaka, Y., Amari, S., Nakano, T., Cinatl, J., Rabenau, H., Doerr, H.W., Hunsmann, G., Otaka, A., Tamamura, H., Fujii, N., Yamamoto, N., 2004. HIV protease inhibitor nelfinavir inhibits replication of SARS-associated coronavirus. Biochem. Biophys. Res. Commun. 318, 719–725.

Yi, L., Li, Z., Yuan, K., Qu, X., Chen, J., Wang, G., Zhang, H., Luo, H., Zhu, L., Jiang, P., Chen, L., Shen, Y., Luo, M., Zuo, G., Hu, J., Duan, D., Nie, Y., Shi, X., Wang, W., Han, Y., Li, T., Liu, Y., Ding, M., Deng, H., Xu, X., 2004. Small molecules blocking the entry of severe acute respiratory syndrome coronavirus into host cells. J. Virol. 78, 11334–11339.

Yoshida, K., Tanagawa, M., Atsuta, M., 1999. Characterization and inhibitory effect of antibacterial dental resin composites incorporating silver-supported materials. J. Biomed. Mater. Res. 47, 516–522.

Zhang, J., Balestrieri, E., Grelli, S., Matteucci, C., Pagnini, V., D'Agostini, C., Mastino, A., Macchi, B., 2001. Efficacy of 3′-azido 3′deoxythymidine (AZT) in preventing HTLV-1 transmission to human cord blood mononuclear cells. Virus Res. 78, 67–78.

Zhang, Y., Schols, D., De Clercq, E., 1999. Selective activity of various antiviral compounds against HHV-7 infection. Antiviral Res. 43, 23–35.