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Development of a nano-luciferase based assay to measure the binding of SARS-CoV-2 spike receptor binding domain to ACE-2

Marcelo A. Lima a, Mark Skidmore a, Farhat Khanim b, Alan Richardson c, *.

a Molecular & Structural Bioscience, School of Life Sciences, Keele University Staffordshire, ST5 5BG, United Kingdom
b School of Biomedical Sciences, Institute of Clinical Sciences Medical School University of Birmingham, Birmingham, B15 2TT, United Kingdom
c School of Pharmacy and Bioengineering, Keele University Staffordshire, ST5 5BG, United Kingdom

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ABSTRACT

To identify drugs that could potentially be used to treat infection with SARS-CoV-2, a high throughput 384-well assay was developed to measure the binding of the receptor binding domain (RBD) of the viral S1 protein to its main receptor, angiotensin converting enzyme 2 (ACE2). The RBD was fused to both a HiBIT tag and an IL6 secretion signal to enable facile collection from the cell culture media. The addition of culture media containing this protein, termed HiBIT-RBD, to cells expressing ACE2 led to binding that was specific to ACE2 and both time and concentration dependant. Binding could be inhibited by both RBD expressed in E. coli and by a full length S1 - Fc fusion protein (Fc-fused S1) expressed in eukaryotic cells. The mutation of residues that are known to play a role in the interaction of RBD with ACE2 also reduced binding. This assay may be used to identify drugs which inhibit the viral uptake into cells mediated by binding to ACE2.

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1. Introduction

Zoonotic viruses make up the vast majority of emerging, human diseases owing to their ability to cross-spread between species from animals to humans [1]. SARS-CoV-2, the seventh coronavirus known to infect humans [2], hit the world rapidly and uncontrollably, resulting in the death of over 1 million people as of October 2020, alongside devastating social-economical burdens. To-date, no widely effective treatment or vaccines are available and a significantly limited range of therapies that ameliorate the significant health and socio-economic burdens caused by SARS-CoV-2.

The viral tropism is determined by the interactions that occur between viral surface proteins and host cell receptors, and as such, these interactions are vital for effective cross-species and intra-species transmission [3]. Coronaviruses (CoVs) express a surface glycoprotein, termed spike, that binds to cell-surface receptors and mediate the entry of the virus into cells to initiate an infection [4]. Many host receptors for coronaviruses have been identified [4,5]; in the case of SARS-CoV-2, the viral surface glycoprotein S1 contains a receptor binding domain (RBD) that interacts with angiotensin converting enzyme 2 (ACE2) and this is thought to mediate viral uptake into cells [6]. Thus, blocking this interaction could potentially limit viral infections. More recently, it has also been suggested that neuropilin can also mediate SARS-COV-2 uptake [7].

The current state of the pandemic demands a rapid response. Drug repurposing screens are a convenient and attractive route to the identification of drugs that could address unmet medical needs. This is particularly attractive in the case of SARS-COV-2 because drugs that are already licensed for the treatment of other diseases could be rapidly redeployed to treat the current pandemic [8]. Many marketed drugs are readily available in large quantities and their safety profiles are well understood. When compared to traditional drug discovery paradigms, drug repurposing significantly reduces the time required for the identification of drug candidates that may treat emerging viral infections. Nevertheless, suitable assays are required for the rapid identification of candidate SARS-COV-2 therapeutic drugs from the vast list of approved medicinal drugs. Computational screens provide an alternative strategy, although they lack a full consideration of the complex physiological environment [9] that dictates how molecules interact in vivo and, in any case, still require biochemical assays to validate the in silico results.
To develop an assay to measure the binding of SARS-CoV-2 spike protein to ACE2, here the HiBIT system has been used. HiBIT is an 11 amino acid peptide that binds with high affinity to LgBIT, a fragment of an engineered form of luciferase termed nanoluc [10]. The binding of HiBIT to LgBIT renders the complex catalytically competent, creating a luminescence signal when an appropriate substrate is provided. The binding of HiBIT to LgBIT is of sufficient high affinity that additional proteins are not required to colocalize the two proteins and create a bioactive complex. Thus, LgBIT can be utilized for the detection of a HiBIT-tagged protein. In this study, SARS-CoV-2 RBD was fused inframe with a sequence encoding HiBIT to create a reagent that was anticipated to bind ACE2 and which could be detected by addition of LgBIT and the nanoluc substrate. An IL6 secretion signal was also included to allow HiBIT-tagged RBD secretion into cell culture medium of transfected cells. The resultant culture medium, containing recombinantly expressed HiBIT-tagged RBD, was subsequently added to COS cells ectopically expressing ACE2 to measure RBD-ACE2 binding. This was used in a proof-of-concept screen of a small library of marketed drugs.

2. Methods

Materials. The plasmid pCDNA3 encoding ACE2 was obtained from Genescrip (OHu 20260); the plasmid encoding prolactin was obtained from Sino biological (HG10275-CV). pRSETa encoding the SARS-CoV-2 S1 protein was obtained from GeneArt Gene Synthesis. OptiMem and lipofectamine 2000 were obtained from Thermo- fisher. HiBIT detection reagent was obtained from Promega. The flexicloning transfer system (C8820), pFN39K and HiBIT detection reagent were obtained from Promega. The Q5 mutagenesis kit was obtained from New England Biolabs. SARS-CoV-2 spike S1 protein was obtained from the Centre for AIDS Reagents, NIBSC, UK: Recombinant SARS-CoV-2 spike S1 protein, consisting of a combination of the FC fusion (25 kDa) and the SARS-CoV-2 S1 domain (70 kDa) was obtained from Prof. Ian M Jones.

2.1. Molecular biology

RBD constructs were prepared using the flexicloning transfer system. The region encoding amino acids 328–536 of the SARS-CoV-2 spike S1 were amplified using Pfu polymerase and pRSETa SARS-CoV-2 S1 as the template. Forward (CCCGCCGATCCGCATGGTTTACATTTCCACCAATGC) and reverse (GTCGGTAAACCTCATCCGTGCAGACATTTCTCCT) primers introduced 5gF1 and Pme1 restriction sites flanking the RBD. The product was gel purified, digested with flexiblend and ligated into pF4ACMV. After restriction analysis, selected clones were verified by sequencing. For site directed mutagenesis, mutations were introduced into the resulting pF4ACMV-RBD using a Q5 mutagenesis kit according to the manufacturer’s instructions. The desired clones were identified by sequencing. The wild-type and mutated pF4ACMV-RBD clones and pFN39K, which includes a HiBIT tag and an IL6 secretion signal, were subsequently digested with flexiblend, heat inactivated and ligated. pFN39K clones containing the RBD were selected using kanamycin and identified by restriction digest. Plasmids for transfection were prepared using a Qiagen plasmid purification kit.

2.2. Recombinant SARS-CoV2 spike S1 and SARS-CoV2 S1 receptor binding domain (RBD)

Residues 319–597 of the SARS-CoV-2 spike S1 RBDS (GenBank: MN908947) were cloned upstream of an N-terminal 6XHisTag in the pPCSET A expression vector and transformed into BL21(DE3) pLysS Competent Cells (Novagen, UK). Protein expression was carried out in MagicMedia™ E. coli Expression Media (Invitrogen, UK) at 37 °C for 24 h, 250 rpm. The bacterial pellet was suspended in 5 mL of lysis buffer (BugBuster Protein Extraction Reagent, Merck Millipore, UK; containing DNase) and incubated at room temperature for 30 min. The protein was purified from inclusion bodies using IMAC chromatography under denaturing conditions [11]. Fractions were pooled and buffer-exchanged to phosphate-buffered saline (PBS; 140 mM NaCl, 5 mM NaH2PO4, 5 mM Na2HPO4, pH 7.4; Lonza, UK) using a Sephadex G-25 column (GE Healthcare, UK). Recombinant protein was stored at −20 °C until required.

2.3. Binding assay

COS cells were grown in DMEM supplemented with 10% (v/v) fetal calf serum and penicillin-streptomycin (50 U/ml). For each well of a 384-well plate, a transfection mix was prepared containing plasmids encoding ACE2 or prolactin (0.025 µg) in 6.25 µl optiMem and mixed with an equal volume of 0.8% (v/v) lipofectamine 2000 in optiMem. COS cells were collected, cleared by centrifugation at 37 °C for 24 h, 250 rpm. The bacterial pellet was suspended in 5 mL of lysis buffer (BugBuster Protein Extraction Reagent, Merck Millipore, UK; containing DNase) and incubated at room temperature for 30 min. The protein was purified from inclusion bodies using IMAC chromatography under denaturing conditions [11]. Fractions were pooled and buffer-exchanged to phosphate-buffered saline (PBS; 140 mM NaCl, 5 mM NaH2PO4, 5 mM Na2HPO4, pH 7.4; Lonza, UK) using a Sephadex G-25 column (GE Healthcare, UK). Recombinant protein was stored at −20 °C until required.

To perform the binding assay, the 384-well plate was placed on ice and washed twice (2 × 50 µl) with ice-cold DMEM containing 2% (v/v) FCS and 10 mM HEPES. If drugs were being tested, these were diluted in a 96 well plate in the HiBIT-RBD on ice. After shaking off the medium from the plate, 25 µl of the ice-cold culture medium containing HiBIT-RBD was added per well and the plate incubated on ice for 90 min. The plate was washed four times with 70 µl ice-cold DMEM containing 2% FCS and 10 mM HEPES and before filling wells with 10 µl of the same solution. The HiBIT detection reagent was prepared according to the manufacturer’s instruction and 10 µl added per well. The plate was shaken on ice in the dark for 5 min and then centrifuged briefly (150 × g, 1 min, 0 °C) to remove bubbles. Luminescence was measured on a plate reader at intervals over 45 min returning the plate to ice each time; an integration time of 0.1 s per well was used to minimize plate warming.
2.4. Western blotting

Cells were transfected as described above with pFN39K HiBIT-RBD. Cells in a 12 well plate were lysed with a 250 μL modified RIPA as described [12], separated on a 4–12% SDS-polyacrylamide gel, transferred to PVDF and analysed by immunoblotting with anti ACE2 (ab15348, 1 μg/mL).

3. Results

The receptor binding domain of SARS-CoV-2 was cloned into the pFN39K expression vector in frame with an IL6 secretion signal and the HiBIT tag (Fig. 1a). This allowed the HiBIT-tagged protein (HiBIT-RBD) to be collected from the culture medium of cells transfected with the tag. The expression of the protein was verified by measuring luminescence after adding this culture medium to the HiBIT detection reagent (Fig. 1b). ACE2 was expressed by transient transfection of Ovcar-8 cells and COS cells. Western blotting (Fig. 1c) demonstrated higher levels of protein in COS cells, and subsequent experiments were performed with this cell line. HEK-293 cells were also tested, and although they allowed robust expression, these were considered not to be suitable for establishing a binding assay as the cells readily detached when washed with PBS, a step necessary to remove any unbound HiBIT-RBD. COS cells transiently expressing ACE2 or prolactin (PRL, negative control) were incubated with HiBIT-RBD cell culture supernatant and after washing to remove unbound HiBIT-RBD, specific binding measured. As expected, the specific binding was saturable, consistent with HiBIT-RBD binding to a finite number of receptors.

To provide evidence that the HiBIT-RBD provides a model of the viral spike protein binding to ACE2–2, competition experiments were performed. In these experiments, SARS-CoV-2 S1 RBD and Fc-fused S1 (recombinantly produced in E. coli) were used to give competition. In preliminary experiments, the binding of the SARS-CoV-2 RBD to ACE2 when measured in an ELISA assay [16]. Consequently, mutations encoding L455Y, F486L and Q493N were engineered into the HiBIT-RBD construct. When these were tested in the HiBIT binding assay (Fig. 4), HiBIT-RBDL455Y and HiBIT-RBDF486L exhibited diminished binding to cells expressing ACE2 and, in the case of HiBIT-RBDL455Y, the specific binding

Next, the kinetics of HiBIT-RBD binding to ACE2 were investigated. COS cells expressing ACE2 or PRL were incubated with HiBIT-RBD at 0 °C for different periods and the specific binding, defined as binding to cells expressing ACE2 minus binding to cells expressing PRL, was determined (Fig. 2a). Specific binding reached steady state after approximately 90 min. In all further experiments, an incubation period of 90 min at 0 °C was used. To evaluate the concentration dependency of HiBIT-RBD binding, COS cells expressing ACE2 or PRL were incubated with different dilutions of the HiBIT-RBD cell culture supernatant and after washing to remove unbound HiBIT-RBD, specific binding determined. As expected, the specific binding was saturable, consistent with HiBIT-RBD binding to a finite number of receptors.

To provide evidence that the HiBIT-RBD bound ACE2 in a manner that reflected the virus binding to ACE2, we made use of reports [13–15] that identified crucial amino acids in the RBD that are involved in the RBD-ACE2 interaction. It has also been reported that mutation of L455, F486 and Q493 in the SARS-CoV-2 RBD to the corresponding residues in SARS-CoV-1 substantially reduces the binding of the SARS-CoV-2 RBD to ACE2 when measured in an ELISA assay [16]. Consequently, mutations encoding L455Y, F486L and Q493N were engineered into the HiBIT-RBD construct. When these were tested in the HiBIT binding assay (Fig. 4), HiBIT-RBDL455Y and HiBIT-RBDF486L exhibited diminished binding to cells expressing ACE2 and, in the case of HiBIT-RBDL455Y, the specific binding
in each experiment. The results shown are the combined individual results from 3 separate experiments (each mean binding measured in ACE2 expressing cells minus the binding measured in cells expressing PRL. The results were expressed as a fraction of the maximum specific binding measured in the absence of a competing ligand.

RBDF486. There was no measurable effect of any of the mutations on binding to ACE-2 expressing cells, although not significantly more so than did HiBIT-RBDF486L,Q493N, which contained both F486L and Q493N mutations, also bound weakly to cells expressing ACE2, although not significantly more so than did HiBIT-RBD. There was no measurable effect of any of the mutations on the non-specific binding of the HiBIT-RBDs to control cells expressing prolactin.

4. Discussion

We have developed an assay that specifically measures the binding of SARS-COV-2 to ACE2. The assay was implemented in a 384-well format allowing reasonable throughput. The assay can be conducted using widely available scientific instrumentation (a plate reader capable of measuring luminescence) and reagents, and it is envisaged that this technology could be deployed in many laboratory settings.

For this assay ACE2, the main SARS-COV-2 receptor, was transiently expressed in COS cells because these cells can be transfected with high efficiency. HEK-293 cells were also investigated, but owing to their propensity to become easily dislodged during washing it was deemed unlikely that reproducible data could be obtained with them. COS cells were also preferred over Ovcar-8 cells, which can also be transfected with relatively high efficiency, because of the higher level of expression observed in the former cells. In the future, it may be useful to develop a cell line stably expressing the receptor. However, since we hoped to identify compounds which could inhibit RBD binding to ACE2 as quickly as possible to assist in the SARS-COV-2 pandemic, that option was not pursued here.

Several lines of evidence suggest that the described assay faithfully measures RBD binding to ACE2. As expected, binding of HiBIT-RBD was both time dependant and saturable, consistent with binding to ACE2. Importantly, significantly more binding of HiBIT-RBD was measured in cells expressing ACE2 than to cells transfected with a negative control (PRL). This indicated that the measured binding was specific to ACE2. Furthermore, both RBD and full length S1 proteins obtained from distinct biological sources could compete with HiBIT-RBD for ACE2 binding thereby giving confidence that the binding of HiBIT-RBD reflected binding of the
viral RBD to its receptor in its authentic binding mode. Finally, the mutation of several different residues that have previously been shown to inhibit RBD binding to ACE2 in an ELISA assay [16] also reduced it the binding measured in the HiBiT assay. Taken together, these data strongly suggest that an assay has been developed that replicates and measures the binding of the viral RBD to ACE2 through its authentic binding mode.

The three mutations introduced into the RBD mimicked the corresponding amino acids in SARS-COV-1 and have been previously shown to inhibit SARS-COV-2 binding to ACE2 in an ELISA assay [16]. These residues have also been shown from studies of the structure of ACE-2-RBD complex [13–15] to play a significant role at the interaction interface of the two proteins. In this study, two of the mutations (L455Y and F486L) substantially inhibited HiBiT-RBD binding to ACE2. However, a third mutation (Q493N) had a very modest effect, although it substantially inhibited binding in the ELISA assay [16]. To explore this further, an RBD was generated containing both Q493N and F486L mutations and this also bound more weakly than the wild-type protein and HiBiT-RBD [1486]. The relatively modest effect is perhaps not surprising when considering the conservative nature of the glutamine to asparagine mutation. Furthermore, the crystal structure of ACE2 bound to the RBD shows Q493 in two possible conformations [15], suggesting there is some flexibility in the binding mode.

The assay was employed to screen a library of approximately 100 approved drugs [17]. Unfortunately, no drugs were identified that reproducibly inhibited binding. Some hits appeared to inhibit the binding, but further exploration of these compounds revealed that they also inhibited the luminescence generated by adding HiBiT-RBD to the detection reagent directly, suggesting that the drugs inhibited nanoluc reporter reaction, rather than the HiBiT-RBD-ACE2 binding interaction. This emphasizes the importance of confirming hits identified in drug screens through validation in biochemical assays based on distinct technologies or by using other functional assays such as pseudotyped virus-like particle and/or live virus assays.

One issue with the assay is that the whole assay must be conducted on ice. In preliminary experiments (not shown) binding was not detected when the assay was performed at 37 °C. Consequently, the assay was conducted on ice to prevent endocytosis of RBD bound to ACE2. To avoid the plate warming appreciably during luminescence measurements, a very short integration time per well (0.1s) was used. Even so, some increase in the signal measured in control samples was observed as the plate was read and to control for this, multiple controls were included across the plate. It would be preferable to avoid this problem by using a plate reader that allowed the sample plate to be cooled. Alternatively, it might be possible to conduct the assay at a slightly higher temperature to minimize the effect of warming. It may also be possible to configure the assay to use cell membranes or fixed cells.

The assay developed here is suitable for the identification of drugs that inhibit the binding of SARS-COV-2 to ACE2. It may also be used to validate hits identified by other workers using binding assays based on other technologies. Although the assay has some limitations, these could be ameliorated with further work.

Author contributions

AR conceived the study and performed the experiments with the assay. MAL and MS designed and prepared the RBD expressed in E. Coli and FK designed the drug library. All authors reviewed and contributed to the manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of competing interest

The authors declare no conflict of interest.

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