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# Use of Viral Entry Assays and Molecular Docking Analysis for the Identification of Antiviral Candidates against Coxsackievirus A16

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| **Corresponding Author:** | Liang-Tzung Lin, Ph.D.<br>Taipei Medical University<br>Taipei, Taipei TAIWAN |
| **Corresponding Author's Institution:** | Taipei Medical University |
| **Corresponding Author E-Mail:** | ltlin@tmu.edu.tw |
| **Order of Authors:** | Jonathan Y. Wang |
| | Chien-Ju Lin |
| | Ching-Hsuan Liu |
| | Liang-Tzung Lin, Ph.D. |
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We thank you and the reviewers for their thoughtful reviews and the helpful suggestions in improving our manuscript.

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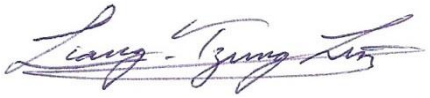

Liang-Tzung Lin, PhD.
Associate Professor
Department of Microbiology and Immunology
Taipei Medical University

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**TITLE:**

# Use of Viral Entry Assays and Molecular Docking Analysis for the Identification of Antiviral Candidates against Coxsackievirus A16

**AUTHORS AND AFFILIATIONS:**

Jonathan Y. Wang[1], Chien-Ju Lin[2], Ching-Hsuan Liu[3,4], Liang-Tzung Lin[3,5]

[1] Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX, USA
[2] School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan
[3] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei Taiwan
[4] Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada
[5] Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

Jonathan Y. Wang (wang.ying.jonathan@utexas.edu)
Chien-Ju Lin (mistylin@kmu.edu.tw)
Ching-Hsuan Liu (julia.chliu@gmail.com)

**CORRESPONDING AUTHOR:**

Liang-Tzung Lin
ltlin@tmu.edu.tw

**KEYWORDS:**

Antivirals, drug development, entry inhibitors, viral entry, binding analysis, molecular docking, Autodock, PyMol, UCSF Chimera.

**SUMMARY:**

The goal of the protocol is to illustrate the different assays relating to viral entry that can be used to identify candidate viral entry inhibitors.

**ABSTRACT:**

Antiviral assays that mechanistically examine viral entry are pertinent to discern at which step the evaluated agents are most effective, and allow for the identification of candidate viral entry inhibitors. Here, we present the experimental approaches for the identification of small molecules capable of blocking infection by the non-enveloped coxsackievirus A16 (CVA16) through targeting the virus particles or specific steps in early viral entry. Assays include the time-of-drug-addition analysis, flow cytometry-based viral binding assay, and viral inactivation assay. We also present a molecular docking protocol utilizing virus capsid proteins to predict potential residues targeted by the antiviral compounds. These assays should help in the identification of candidate antiviral agents that act on viral entry. Future directions can explore these possible inhibitors for further drug development.

## INTRODUCTION:

Hand, foot, and mouth disease (HFMD) is a disease most commonly caused by coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) in young children. Recently across the Asia-Pacific region, there has been a significant uptick in CVA16–induced HFMD. While symptoms can be mild, severe complications can occur that affect the brain and the heart, with potential fatality[1,2]. At present, there are no licensed antiviral therapies or vaccinations available for CVA16, and thus there is a pressing need to develop antiviral strategies to curb future outbreaks and the associated complications.

CVA16 is a non-enveloped virus which has an icosahedral capsid assembled from pentamers that each contain 4 structural proteins namely VP1, VP2, VP3, and VP4. Encircling each five-fold axis in the pentamer is a 'canyon' region that shows as a depression and is noted for its role in receptor binding[3]. At the bottom of this canyon lies a hydrophobic pocket in the VP1 region that contains a natural fatty ligand named sphingosine (SPH). Cellular receptors, such as human P selectin glycoprotein ligand 1 (PSGL-1) and scavenger receptor class B member 2 (SCARB2), have been suggested to play a role in viral binding by displacing this ligand which results in conformational changes to the capsid and the subsequent ejection of viral genome into the host cell[4-6]. Identifying possible inhibitors that block the successive events in the viral entry process could provide potential therapeutic strategies against CVA16 infection.

The steps in the virus life cycle can be dissected through experimental approaches as targets to help identify mode-specific antiviral agents. A time-of-drug-addition analysis examines the drug treatment effect at different times during the viral infection, including pre-entry (added prior to the virus infection), entry (added concurrent to the virus infection), and post-entry (added following the virus infection)[7]. The impact can be assessed using a standard plaque assay by quantitating the number of viral plaques formed in each of the treatment conditions. The flow cytometry-based viral binding assay determines if the drug prevents viral attachment to host cells. This is achieved by shifting the temperature from 37 °C, at which the majority of human virus infections occur, to 4 °C, where the virions are able to bind to the host cell surface but are unable to enter the cells[7]. The cell membrane-bound virus particles are then quantified through immunostaining against viral antigens and assessed by flow cytometry. The viral inactivation assay on the other hand helps to assess potential physical interactions of the drug with free virus particles, either shielding or neutralizing the virions, or causing aggregations or conformational changes that render them inactive for subsequent interactions with the host cell surface during the infection[8,9]. In this experiment, the viral inoculum is allowed to first incubate with the drug before being diluted to titrate out the drug prior to infecting the host cell monolayer and performing a standard plaque assay[8]. Finally, molecular docking is a powerful tool to predict potential drug interaction sites on the virion surface, including the viral glycoproteins from enveloped viruses and the viral capsid proteins from non-enveloped viruses, by using computational algorithms. This helps to mechanistically pinpoint targets of the drug's mode of action and provide useful information that can be further validated by downstream assays.

We recently employed the above described methods to identify antiviral compounds that

efficiently blocked infection by the non-enveloped CVA16[9]. Herein, the detailed protocols that were used are described and discussed.

**PROTOCOL:**

NOTE: All cell culture and virus infections must be conducted in certified biosafety hoods that are appropriate for the biosafety level of the samples being handled. The two tannin-class of small molecules chebulagic acid (CHLA) and punicalagin (PUG), that were observed to efficiently block CVA16 infection[9], are used as examples of candidate inhibitory agents. For basic principles in virology techniques, virus propagation, determination of virus titer, and concepts of plaque forming units (PFU) or multiplicity of infection (MOI), the reader is referred to reference[10].

## 1. Cell culture, virus preparation, compound preparation, and compound cytotoxicity

1.1) Human rhabdomyosarcoma (RD) cells are host cells permissive to CVA16 infection[11]. Grow the RD cells in 10 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 U/mL penicillin G, 200 µg/mL streptomycin, and 0.5 µg/mL amphotericin B in T-75 flasks at 37 °C in a 5% $CO_2$ incubator.

1.2) Prepare CVA16 by propagating the virus in RD cells and determine viral titer in PFU/mL. For optimized protocol, please refer to reference[11].

1.3) Prepare the test compounds and controls using their respective solvents: for example, dissolve CHLA and PUG in dimethyl sulfoxide (DMSO). For all infection steps, the basal medium consisted of DMEM plus 2% FBS and antibiotics.

NOTE: The final concentration of DMSO in the test compound treatments is equal to or below 0.25% in the experiments; 0.25% DMSO is included as a negative control treatment in the assays for comparison.

1.4) Perform cytotoxicity assay of the test compounds on the RD cells using a cell viability determining reagent such as XTT ((2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-phenylamino)-carbonyl]-2H-tetrazolium hydroxide). For detailed protocol, please refer to reference[12]. Determine the cytotoxicity concentrations of the test compounds using an analytical software such as GraphPad Prism according to manufacturer's protocol. Drug concentrations that do not significantly influence the cell viability (≥ 95% viable cells) are used for the remainder of the study.

## 2. Time-of-drug-addition assay

2.1) To evaluate the influence of drugs on host cells prior to viral infection (pretreatment)

2.1.1) Seed RD cells in 12-well plates at a seeding density of 2 x $10^5$ cells/well. Incubate overnight at 37 °C in a 5% $CO_2$ incubator to obtain a monolayer.

2.1.2) Treat RD cells with test compounds at non-cytotoxic concentrations (determined from step 1.4) in 1 mL of basal media volume for 1 h or 4 h.

2.1.3) Wash cells with 1 mL of PBS before adding 50 PFU/well of virus in basal medium (final volume of the inoculum is 300 µL) for 1 h. Rock the plate every 15 min.

2.1.4) Following the infection, wash the monolayers again using PBS, then overlay with 1 mL of basal media containing 0.8% methylcellulose for further incubation at 37 °C in a 5% $CO_2$ incubator.

2.1.5) After 72 h of incubation, remove the overlay media and wash the wells using 2 mL of PBS.

2.1.6) Fix the wells using 0.5 mL of 37% formaldehyde for 15 min.

2.1.7) Remove the supernatant and wash again using PBS.

2.1.8) Stain the wells using 0.5 mL of 0.5% crystal violet solution. Then remove the stain solution within 2 min and wash the wells with a gentle stream of water before air drying.

2.1.9) Count the viral plaques by placing the plate on a white-light box. Calculate the percent (%) CVA16 infection as follows: (Mean # of plaque $_{\text{virus+drug}}$ / Mean # of plaque $_{\text{virus+DMSO control}}$) × 100%.

2.2) To evaluate the effect of adding the drugs and the virus concurrently (co-addition)

2.2.1) Seed RD cells in 12-well plates at a seeding density of 2 x $10^5$ cells/well. Incubate overnight at 37 °C in a 5% $CO_2$ incubator to obtain a monolayer.

2.2.2) Treat RD cells with the test compounds at the appropriate concentrations and 50 PFU/well of CVA16 (final volume of the inoculum is 300 µL) simultaneously for 1 h. Rock the plate every 15 min.

2.2.3) Wash cells with 1 mL of PBS and then overlay with 1 mL of basal media containing 0.8% methylcellulose for further incubation at 37 °C in a 5% $CO_2$ incubator.

2.2.4) Stain viral plaques with crystal violet after 72 h post infection and determine the% CVA16 infection as described above.

2.3) To evaluate drug treatment effect after viral entry (post-infection)

2.3.1) Seed RD cells in 12-well plates at a seeding density of 2 x $10^5$ cells/well. Incubate overnight at 37 °C in a 5% $CO_2$ incubator to obtain a monolayer.

2.3.2) Inoculate the RD cells with 50 PFU/well of CVA16 (final volume of the inoculum is 300 µL) for 1 h. Rock the plate every 15 min.

2.3.3) Wash the wells with 1 mL of PBS and overlay the cells with basal media containing 0.8% methylcellulose and the appropriate concentrations of test compounds.

2.3.4) Stain viral plaques with crystal violet and count after 72 h post infection and determine the% CVA16 infection incubations described above.

NOTE: Perform all PBS washes gently to avoid lifting the cells.

**3. Flow cytometry-based binding assay**

3.1. Seed RD cells in 12-well plates at a seeding density of 2 x $10^5$ cells/well. Incubate overnight at 37 °C in a 5% $CO_2$ incubator to achieve a monolayer.

3.2) Pre-chill the cell monolayer at 4 °C for 1 h.

3.3) Infect RD cells with CVA16 (MOI = 100) in the presence and absence of the test compounds for 3 h at 4 °C.

NOTE: Perform the viral inoculation on ice and the ensuing incubation in a 4 °C refrigerator to maintain the temperature at 4 °C, which permits viral binding but not entry.

3.4) Remove virus inoculum and wash once with 1 mL of ice-cold PBS.

3.5) Lift cells by adding 1 mL of ice-cold dissociation buffer to the wells on ice for 3 min, before collecting the cells and resuspending them in ice-cold flow cytometry buffer (1x PBS plus 2% FBS).

3.6) Wash cells twice using the ice-cold flow cytometry buffer and fix the cells with 0.5 mL of 4% paraformaldehyde for 20 min on ice.

3.7) Wash the cells using PBS to remove any unbound or weakly bound viruses, and then stain the cells with 1 mL of anti-VP1 antibody (1:2000; diluted in PBS containing 3% BSA) on ice for 1 h, followed by incubation with a secondary Alexa 488-conjugated anti-mouse IgG (1:250; diluted in PBS containing 3% BSA) on ice for 1 h. Perform PBS washes (3 times) following each antibody treatment.

3.8) Resuspend the cells in 0.5 mL of the ice-cold flow cytometry buffer and perform flow cytometry analysis on a flow cytometer using standard procedures. Present data in histograms using the associated software and quantitate for bar graph representation.

**4. Viral inactivation assay**

4.1) Perform the viral inactivation assay as previously described[12] using the following conditions:

- A starting concentration of $10^6$ PFU/mL of CVA16.
- RD cell monolayers in 12-well plates from a seeding density of 2 x $10^5$ cells/well.
- 50-fold dilution for titrating out the drug compounds resulting in a final virus concentration of 50 PFU/well.
- Wash steps using 1 mL of PBS.
- Overlay media containing 0.8% methylcellulose.

4.2) Perform final readout of viral infection using the crystal violet staining of viral plaques procedure as detailed above.

## 5. Molecular docking analysis

5.1) Download 3D molecules of test compounds from PubChem (https://pubchem.ncbi.nlm.nih.gov/). If molecules do not have a 3D structure uploaded, download the 2D structures or use the SMILES string sequence and transform into 3D molecules via a molecular program (e.g. CORINA).

5.2) Download viral biological assembly unit from RCSB Protein Data Bank (https://www.rcsb.org/) and prepare the viral structure model using a biocomputing program (e.g. UCSF Chimera). For example, in the case of CVA16 mature virion crystal structure (PDB: 5C4W)[3], delete solvents from the PDB file, replace incomplete side chains using data from the Dunbrach 2010 Rotamer Library, and add hydrogens and charges to the structure as previously reported[13]. Docking targets can be any relevant viral proteins for the intended analysis with a biological assembly information (Protein Data Bank).

5.3) Dock test compounds onto the prepared virus unit using for example UCSF Chimera, and analyze the output files with a visualization software (e.g., Autodock Vina, PyMol):

5.3.1) Upload the test compound file into UCSF Chimera as the 'ligand' and perform blind docking by selecting the whole prepared viral protein as the 'receptor'. Use the computer mouse or trackpad to resize the search volume to the entire 'receptor'. In 'Advanced options', allow the number of binding modes to be at maximum. Docking frames will be automatically ranked from highest to lowest binding energy.

5.3.2) (Optional) Further to blind docking, confine the docking site onto the viral protein in regions of interest derived from the blind docking results using the mouse or trackpad again to reduce the search volume (e.g., 100 Å x 100 Å x 100 Å). This step helps confirm the blind docking results and increases specificity.

5.3.3) Use a molecular graphics system (e.g., PyMol) to analyze the binding modes' positions by uploading the docking file. Find polar contacts from the compound to the viral protein by selecting the 'ligand' and identifying polar contacts with the option 'to any atoms'; examine the results.

**REPRESENTATIVE RESULTS:**

The time-of-drug-addition assay is indicated in **Figure 1** and shows the influence from treatment using the small molecules CHLA and PUG on CVA16 infection either pre-viral entry (pretreatment), during viral entry (co-addition), or post-viral entry (post-infection). Both small molecules only produced marginal impact against CVA16 infectivity whether in the pretreatment of the host cells prior to viral infection (**Figure 1A**) or in the post-infection treatment (**Figure 1C**). In contrast, CHLA and PUG efficiently abrogated the CVA16 infection by >80% in the co-addition treatment (**Figure 1B**). These observations therefore suggest that the two compounds are most effective when they are concurrently present with the virus particles on the host cell surface during the infection.

In **Figure 2,** the flow cytometry-based binding analysis (schematically illustrated in **Figure 2A**) confirms that the two tannins prevent CVA16 entry by preventing the viral particle binding to the host cells. The quantification data in **Figure 2B** shows that the amount of virus detected on the RD cell surface in the presence of the two drugs, is less than 10%, similar to the heparin positive control which is known to prevent CVA16 attachment[14]. **Figures 2C**, **2D**, and **2E** depict the associated flow cytometry histograms where the band shift due to detection of CVA16 on the RD cell surface is significantly reduced when CHLA and PUG are present.

**Figure 3A** depict how the viral inactivation experiment was performed. The drug compound was either mixed with the CVA16 virus particles and incubated for 1 h (long-term) prior to the dilution step, or mixed and immediately diluted (short-term) prior to the infection. As shown in **Figure 3B**, a pre-incubation of the CV16 particles with the test agents for 1 h led to a near complete protection of the RD cells against the viral infection compared to short-term incubation and the DMSO control. The results therefore suggest that both CHLA and PUG interact with the CVA16 particles and are able to render them inactive in the subsequent infection.

Since our data indicate that the drug compounds can directly inactivate CVA16 particles, and hence identifying the virion itself as a plausible target of their antiviral activity, we used molecular docking to predict the potential interaction(s) between these agents and the CVA16 capsid pentamer. **Figure 4A** shows a surface projection of the CVA16 pentamer which makes up the icosahedral capsid of the CVA16 virion. Molecular docking of the tannins CHLA (**Figure 4B**; green) and PUG (**Figure 4C**; blue) indicate that they both are predicted to bind in the canyon region of the CVA16 pentamer. Specifically, both small molecules bound just above the pocket entrance (**Figures 4B** and **4C**, zoomed panels), which holds the pocket factor and plays an important role for mediating CVA16 binding and entry into the host cell. Both CHLA and PUG therefore appear to mask the pocket entrance region, which theoretically would obstruct interactions between the virus particles and the host cell receptors. **Figures 4D** and **4E** indicate the unique residues predicted from the polar contacts of CHLA and PUG, respectively, around the pocket entrance, with most of these interactions occurring with VP1 for both compounds and the 3 amino acids $Asn^{85}$, $Lys^{257}$, and $Asn^{417}$ being in common between the two tannins.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Time-of-drug-addition effect of CHLA and PUG against CVA16 infectivity.** RD cells were treated with CHLA (20 μM) or PUG (25 μM) at different times of CVA16 inoculation (50 PFU/well). DMSO (0.25%) treatment was included as negative control and all assays were analyzed by plaque assay using crystal violet staining 72 h after incubation. (**A**) For pretreatment, cells were incubated with the test compounds for 1 h or 4 h and then were washed before CVA16 infection. (**B**) For co-addition assays, cells were administered with drugs and virus simultaneously for 1 h and then washed. (**C**) In post-infection, cells were infected with CVA16 for 1 h, washed, and then treated with test compounds. Data shown are the means ± standard deviation (SD) from three independent experiments. *$p < 0.05$ compared to the respective 'virus only' group. Statistical analysis was performed using one-way analysis of variance. This figure has been adapted from reference[9].

**Figure 2: CHLA and PUG abolish CVA16 binding to the host cell.** (**A**) Schematic of the flow cytometry- based binding assay. (**B**) RD cells ($2 \times 10^5$ cells/well) were infected with CVA16 (MOI = 100) in the presence or absence of CHLA (20 μM), PUG (25 μM), soluble heparin (500 μg/mL, positive control), or DMSO (0.25%, negative control) for 3 h at 4 °C. Inocula from wells were collected into tubes, washed with PBS twice, fixed, and stained with anti-VP1 antibody followed by Alexa 488-conjugated secondary antibody for flow cytometry detection of surface-bound viruses. Quantified data from the detected fluorescence signals were plotted as the means ± SD from three independent experiments in bar graph as 'Virus binding (%)'. *$p < 0.05$ compared to the 'DMSO' control treatment. Statistical analysis was performed using one-way analysis of variance. The representative flow cytometry histograms of CHLA (**C**), PUG (**D**), and heparin (**E**) treatments are shown. This figure has been adapted from reference[9].

**Figure 3: CHLA and PUG inactivate cell-free CVA16 virus particles.** (**A**) Schematic of the viral inactivation assay. (**B**) CVA16 ($10^6$ PFU/well) was treated with CHLA (20 μM) or PUG (25 μM) and mixed immediately for short-term inactivation or incubated for 1 h at 37 °C for long-term inactivation before being diluted 50-fold to a non-effective concentration of test compounds before inoculating on RD cells (final virus concentration = 50 PFU/well). DMSO (0.25%) was used as a negative control. Experiments were analyzed by plaque assay using crystal violet staining 72 h post-infection. Data shown are the means ± SD from three independent experiments. *$p < 0.05$ compared to the respective 'virus only' group. Statistical analysis was performed using one-way analysis of variance. This figure has been adapted from reference[9].

**Figure 4: CHLA and PUG target the CVA16 capsid near the pocket entrance.** Surface projection of the CVA16 virion particle with the monomeric structural pentamer delineated by red lines (**A**). Additional pentamers on the virion are shown in cyan, magenta, indigo, bronze, and green. Molecular docking analysis of CHLA (**B**, green) and PUG (**C**, blue) on the CVA16 pentamer (PDB: 5C4W); zoomed-in panels are demarcated in yellow. VP1 = orange, VP2 = gray, VP3 = white; polar contacts are shown as black dashes. Residues that make-up pocket entrance are colored red ($Ile^{94}$, $Asp^{95}$, $Gln^{207}$, $Met^{212}$, $Met^{213}$, $Lys^{257}$, $Thr^{258}$). **D, E.** Close-up side view into the canyon where the pocket entrance is located and where CHLA (**D**) and PUG (**E**) bind to. Unique residues that are polar contacts from the compounds' polar contacts on the pentamer are labeled in yellow (VP1),

white (VP2), and in black (VP3) fonts. The white dashed line indicates the pocket entrance region. This figure has been adapted from reference[9].

**DISCUSSION:**

In this report, we described the protocols that are useful for the identification of antiviral candidates that target viral entry, in particular against the non-enveloped CVA16. The assays are designed in ways to dissect the early events during viral entry, which is helpful to clarify the mechanism(s) of action and potential target(s) of the test agents' antiviral activity. The 'time-of-drug-addition assay' permits to broadly determine the potential target of the test compounds, for instance the uninfected host cells (pretreatment analysis), the virus particles or its interactions with the host cell surface (co-addition analysis), or the virus-infected host cell during the viral replicative phase (post-infection analysis). This assay alone can determine the method of interaction from the compounds (e.g., co-addition) that leads to the subsequent assays described in this protocol (e.g., viral inactivation assay and binding analysis). Wash steps are critical to ensure that the treatment method examined is specific to the one analyzed. The use of the 'flow cytometry-based binding assay' helps to assess the influence of the compounds specifically on virus binding to the host cell. Maintaining the temperature of the experiment at 4 °C is important to the final detection of the virions on the cell surface, as this temperature permits viral binding but not entry. The 'viral inactivation assay' can aid to determine potential physical interaction of the drug compounds with the cell-free virus particles. The critical step is the dilution for titrating out the drug compounds following incubation with the viral inoculum, as this is necessary to prevent any meaningful interaction of the drugs with the host cell surface in the subsequent infection step[12].

Since viral entry is a multi-step event, a viral entry inhibitor class of antiviral agents could possibly exert several types of mechanisms, including: (1) modulating cell surface entry factors/receptors or its associated signaling pathways; (2) affecting cell membrane fluidity or integrity; (3) targeting electrostatic or van der Waals interactions between the virus particles and the host cell surface; (4) inducing physical changes to the virions such as particle breakage or aggregation; (5) binding to viral glycoproteins or capsid proteins and preventing their functions or conformational changes; (6) blocking fusion associated mechanisms; and (7) prevent release of viral genome inside the host cell. The analyses described in this report can therefore help point to the above-listed potential modes of action that can be further validated by additional experiments. Lastly, the 'molecular docking analysis' described here is instrumental to predict potential interaction regions between the drug compounds and the virus particles, and as such can help identify candidate viral capsid or glycoprotein binders and the targeted residues on the virus particles. However, these predictions are dependent on the docking software, and the resolution and accuracy of the viral protein crystal structures. It is important to note that the optional confined docking method in step 5.3.2 was added because oftentimes when using viral structural proteins as the 'receptor' molecule, the ligand can possibly bind to regions normally not accessible or exposed on the surface (e.g., under surface of the virion capsid facing the inside of the virion, transmembrane regions of envelope glycoproteins, etc.). Confining the search box allows only accessible regions of the viral protein to be targeted and rules out any unrealistic interactions. Molecular docking is dependent on crystallized structures, but recent advances in homology

modeling have enabled analysis of non-crystallized structures by fitting its amino acid sequence onto a closely related crystallized structure[15]. This has allowed more structures to be analyzed and the information acquired can be useful for further studies including mutational analyses that can help validate the predicted interactions.

In conclusion, the assays and protocols described in this report are specifically catered to identify candidate antiviral agents that target viral entry, and provide information on which step of the viral entry process the test agent targets to, whether they interact with free virus particles, and predicting possible drug interaction sites on the virions. These types of assays can be repeated on other non-enveloped viruses or adapted to enveloped viruses as a method of screening antiviral drug compounds for possible inhibitors of viral entry. Using such mechanism-driven approach to identify antiviral candidates could help expedite the drug development process and expand the scope of antiviral therapeutics.

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**DISCLOSURES:**

The authors declare that they have no conflict of interest.

**REFERENCES:**

1 Legay, F. et al. Fatal coxsackievirus A-16 pneumonitis in adult. *Emerging Infectious Diseases.* **13**, 1084-1086 (2007).

2 Wang, C. Y., Li Lu, F., Wu, M. H., Lee, C. Y., Huang, L. M. Fatal coxsackievirus A16 infection. *Pediatric Infectious Disease Journal.* **23**, 275-276 (2004).

3 Ren, J. et al. Structures of coxsackievirus A16 capsids with native antigenicity: implications for particle expansion, receptor binding, and immunogenicity. *Journal of Virology.* **89**, 10500-10511 (2015).

4 Nishimura, Y. et al. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. *Nature Medicine.* **15**, 794-797, doi:10.1038/nm.1961 (2009).

5 Yamayoshi, S. et al. Scavenger receptor B2 is a cellular receptor for enterovirus 71. *Nature Medicine.* **15**, 798-801, doi:10.1038/nm.1992 (2009).

6 Yamayoshi, S. et al. Human SCARB2-dependent infection by coxsackievirus A7, A14, and A16 and enterovirus 71. *Journal of Virology.* **86**, 5686-5696, doi:10.1128/JVI.00020-12 (2012).

7 Lin, L. T. et al. Hydrolyzable tannins (chebulagic acid and punicalagin) target viral glycoprotein-glycosaminoglycan interactions to inhibit herpes simplex virus 1 entry and cell-to-cell spread. *Journal of Virology.* **85**, 4386-4398 (2011).

8 Lin, L. T. et al. Broad-spectrum antiviral activity of chebulagic acid and punicalagin against viruses that use glycosaminoglycans for entry. *BMC Microbiology.* **13**, 187 (2013).

9 Lin, C. J. et al. Small molecules targeting coxsackievirus A16 capsid inactivate viral particles and prevent viral binding. *Emerging Microbes & Infections.* **7**, 162, doi:10.1038/s41426-018-

0165-3 (2018).
10 Flint, S. J., Enquist, L. W., Racaniello, V. R., Skalka, A. M. *Principles of Virology*. 3rd edn, (ASM Press, 2008).
11 Velu, A. B. et al. BPR-3P0128 inhibits RNA-dependent RNA polymerase elongation and VPg uridylylation activities of Enterovirus 71. *Antiviral Research.* **112**, 18-25 (2014).
12 Tai, C. J., Li, C. L., Tai, C. J., Wang, C. K., Lin, L. T. Early Viral Entry Assays for the Identification and Evaluation of Antiviral Compounds. *Journal of Visualized Experiments*. e53124 (2015).
13 Lang, P. T. et al. DOCK 6: combining techniques to model RNA-small molecule complexes. *RNA.* **15**, 1219-1230, doi:10.1261/rna.1563609 (2009).
14 Zhang, X. et al. Coxsackievirus A16 utilizes cell surface heparan sulfate glycosaminoglycans as its attachment receptor. *Emerging Microbes & Infections.* **6**, e65, doi:10.1038/emi.2017.55 (2017).
15 Waterhouse, A. et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research.* **46**, W296-W303, doi:10.1093/nar/gky427 (2018).

Fig1

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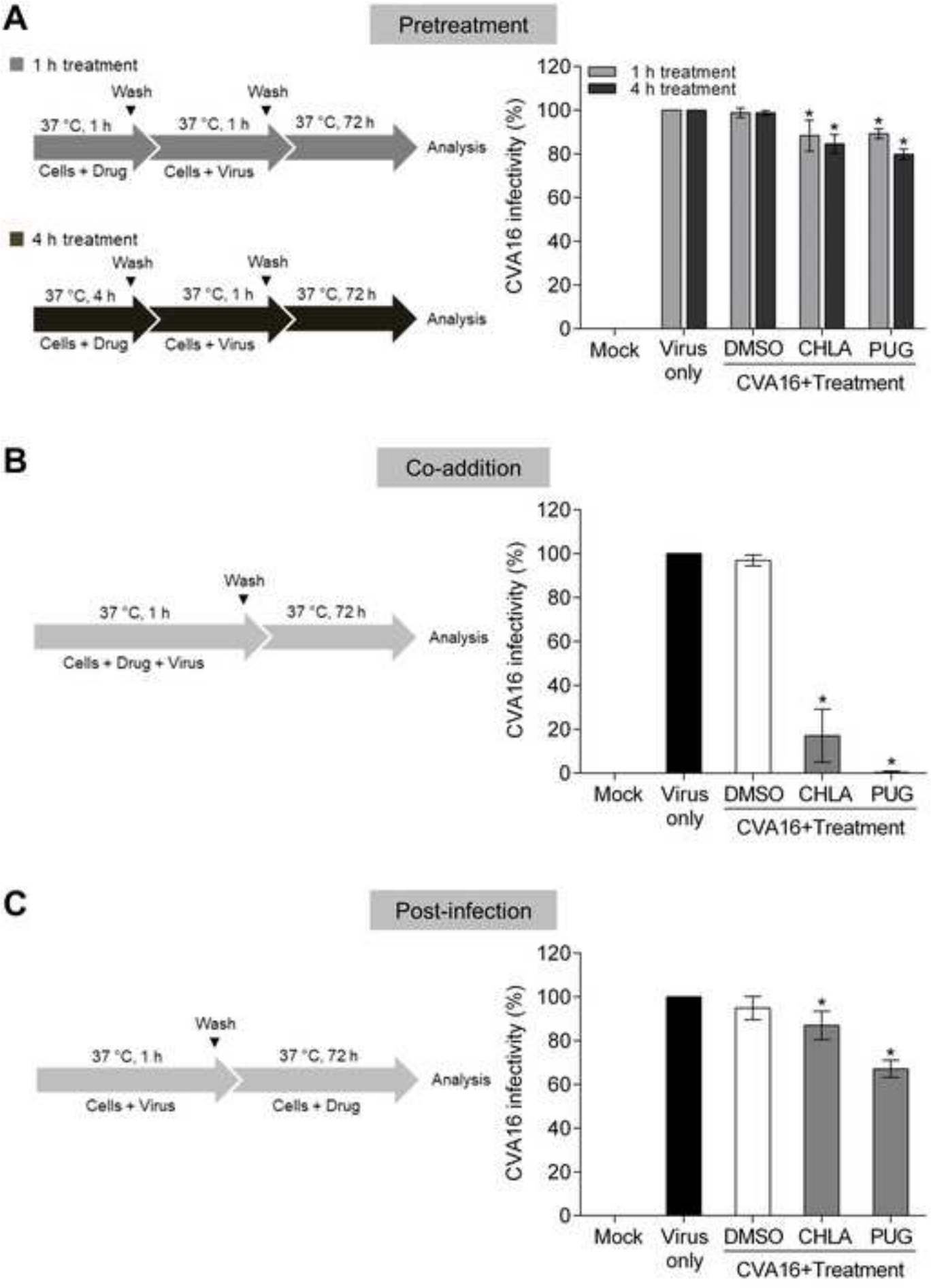

Figure

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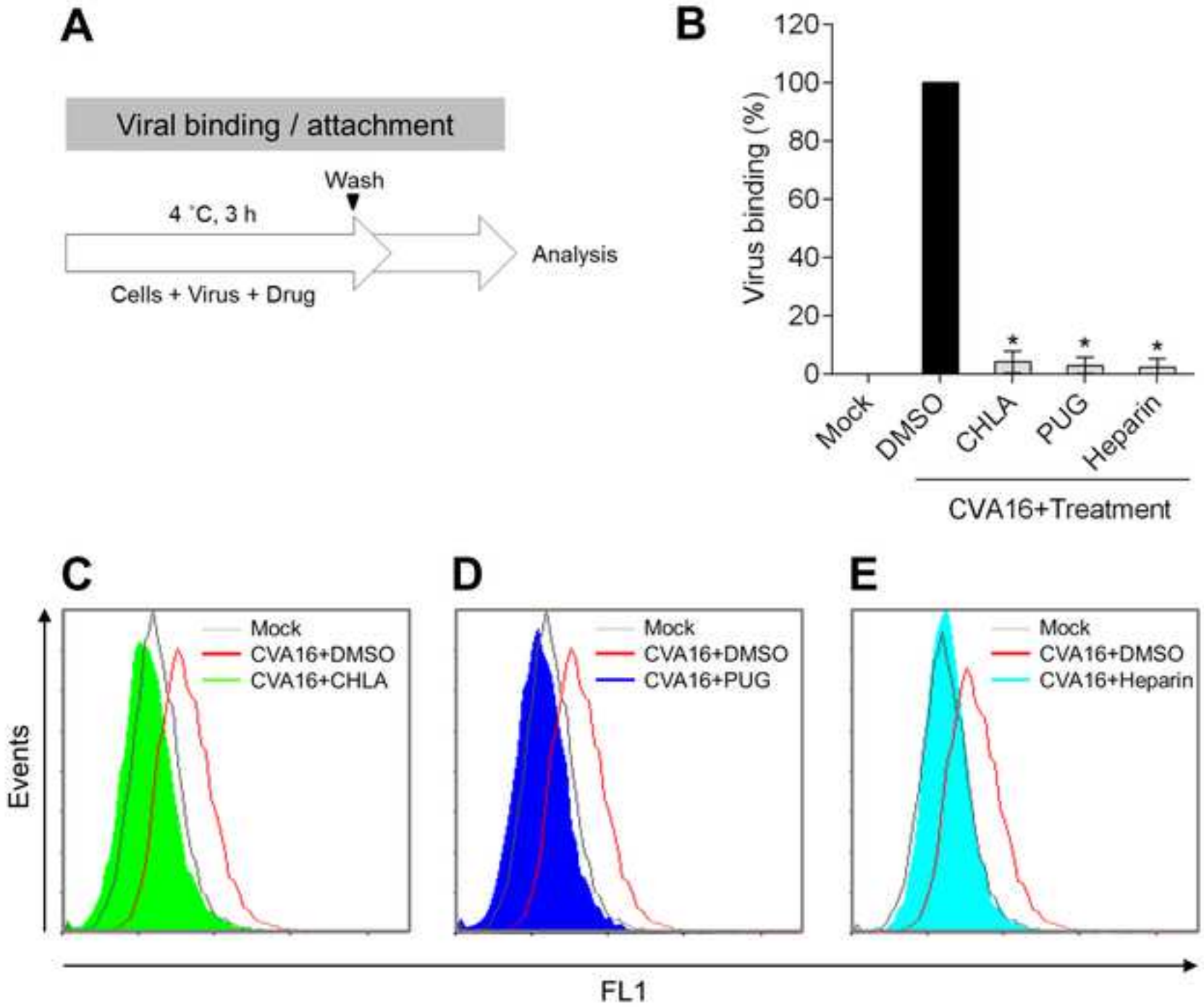

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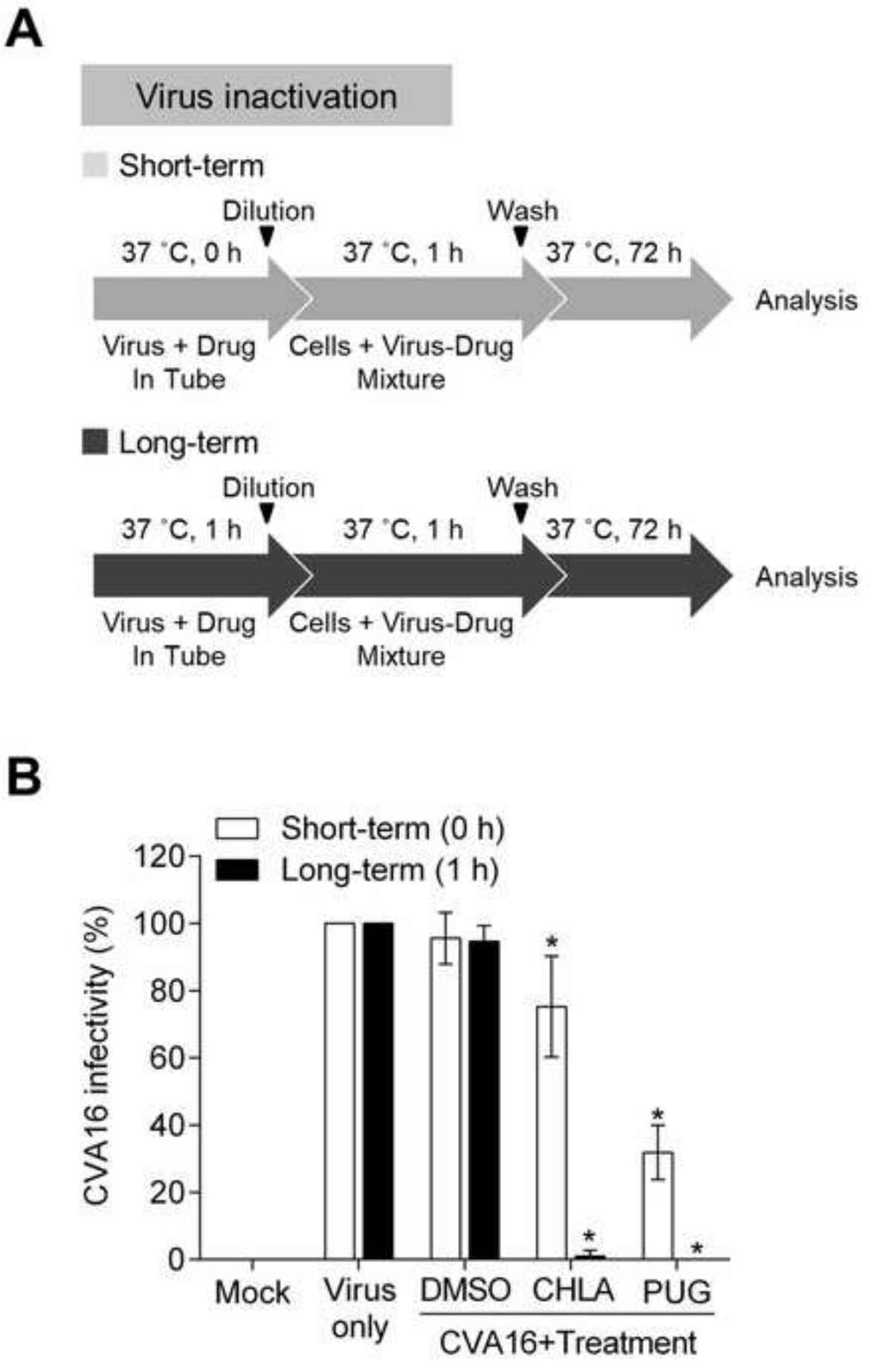

Fig4

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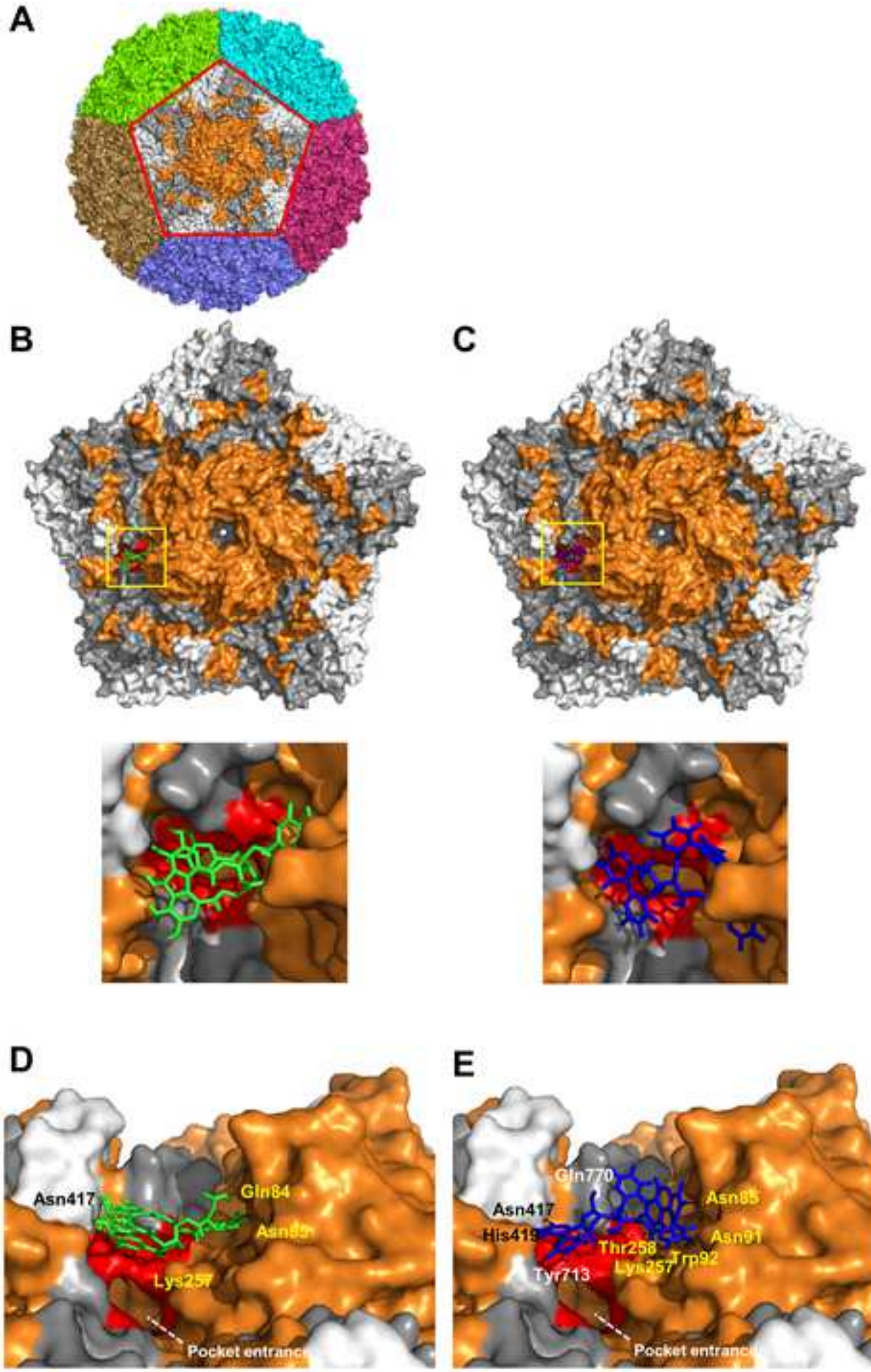

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| 4% Paraformaldehyde | Sigma | AL-158127-500G | |
| Alexa 488-conjugated anti-mouse IgG | Invitrogen | A11029 | |
| Amphotericin B | GIBCO | 15290-018 | |
| Anti-VP1 antibody | Merck-Millipore | MAB979 | Anti-Enterovirus 71 Antibody, cross-reacts with Coxsackie A16, clone 422-8D-4C-4D |
| Beckman Coulter Cytometer | Beckman Coulter | FC500 | |
| Corina | Molecular Networks GmbH | | |
| Crystal violet | Sigma | C3886-100G | |
| DMEM | GIBCO | 11995-040 | |
| DMSO | Sigma | D5879 | |
| FBS | GIBCO | 26140-079 | |
| Formaldehyde | Sigma | F8775 | |
| Graphpad Prism | GraphPad | | |
| Heparin sodium salt | Sigma | H3393 | |
| In vitro toxicology assay kit, XTT-based | Sigma | TOX2 | |
| Methylcellulose | Sigma | M0512-100G | |
| PBS pH 7.4 | GIBCO | 10010023 | |
| Penicillin-Streptomycin | GIBCO | 15070-063 | |
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| | |
|---|---|
| Title of Article: | Use of Viral Entry Assays and Molecular Docking Analysis for the Identification of Antiviral Candidates against Coxsackievirus A16. |
| Author(s): | Jonathan Y. Wang, Chien-Ju Lin, Ching-Hsuan Liu, and Liang-Tzung Lin |

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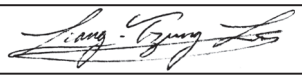
| Signature: |  |
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**Editorial comments:**

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2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Graphpad Prism

Response: The manuscript has been proofread for commercial language, spelling, and grammar issues.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: We have highlighted the filmable content in the manuscript.

2. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. 1.1: Please include more information here (and in other cell culture steps)-volume, cell density, container, etc.

Response: Extra steps for cell culture of RD cells before experimental steps were added in lines 106-107.

2. 2.1.2: What test compounds will you be demonstrating? What are non-toxic concentrations and how do you determine them?

Response: Test compounds demonstrated are mentioned in line 97. Non-toxic concentrations are determined using the XTT-based cytotoxicity analysis of the test compounds on RD cells. More steps and reference can be found in lines 120-125.

3. 3.8: Please include more information on flow cytometry (e.g., gating steps).

Response: The flow cytometry was performed using standard procedures with no gating. We have added further information on data presentation and quantitation in lines 215-216.

4. 5.1, 5.3: Please include step-by-step instructions for software–'click', 'select', etc., for Graphical User Interfaces (GUIs) and/or full commands for command-line programs.

Response: Additional steps to 5.3.1-5.3.3 were added from lines 232-262. Common vocabulary across Graphical User Interfaces were incorporated for easier user access.

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Response: Copyright permission has been obtained and uploaded.

2. Please explain what statistical test was used to produce the p-values in Figures 1-3.

Response: Statistical analysis test explained in lines 313-314, 325-326, and 336-337.

3. Figure 2C-E: Can you include axis labels here?

Response: Axis labels have now been included.

Discussion:
1. Discussion: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) The significance with respect to existing methods

Response: Critical steps in the assays from Steps 2-5 are added in lines 357-372. Troubleshooting step to molecular docking technique is added to lines 356-359. Additional information about the significance of molecular docking, some of the advancements and modifications that can be done have been added in lines 386-396.

References:
1. Please do not abbreviate journal names.

Response: Abbreviated journal names have been replaced with their full names.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: The Table of Materials has been modified to include commercial programs such as GraphPad Prism, PyMol, and UCSF Chimera.

We thank the editor for these comments and hope that the changes are satisfactory.

Reviewer #1 (Comments to the Author):

Manuscript Summary:
In the paper entitled: "Use of viral entry assays and molecular docking analysis for the identification of antiviral candidates against coxsackievirus A16", the authors: Jonathan Y. Wang, Chien-Ju Lin, Ching-Hsuan Liu, and Liang-Tzung Lin, presented a protocol to evaluate the effect of new antiviral compounds in viral entry steps, and the use of molecular docking as a way to suggest a possible mechanism of action. The methods were sufficient and well exposed, and it is noteworthy that in vitro assays are very well explained and showed the knowledge of proper gold standard techniques in virology. The results are interesting and revealed that in silico tools are useful to give insights in the role of new antiviral molecules, and the combination of both in vitro and in silico methodologies could be apply for any other viral model.

Major Concerns:
None

Minor Concerns:
None

Response: We thank Reviewer #1 for the kind comments.

Reviewer #2 (Comments to the Author):

Manuscript Summary:
This paper describes experimental procedures to examine the effect of antiviral drug candidates on the different steps of cell entry of the coxsackievirus A16 (CVA16), one of the causative agents of hand, foot and mouth disease (HFMD) in young children.
The protocols described in the paper are sound and provide good guidelines for establishing conditions to examine drug-mediated inhibition of virus cell entry, as well as whether the drug under consideration has a direct virucidal effect.
Similar protocols, with appropriate modifications, should be applicable to other non-enveloped viruses.

The paper describes also the use of molecular docking to predict sites on the virion surface where antiviral drug candidates bind.

Major Concerns:
None

Minor Concerns:
The section on molecular docking is sound but only relevant to the specific case of CVA16 and therefore it is questionable whether it would be of any interest to any virologist working with a different virus.

Response: We thank the reviewer for raising this issue. In line 243, we have indicated that other viral proteins can be used for molecular docking to broaden the applications of this molecular docking protocol.

The use of FACS to examine virus binding to cell surface should be further discuss and alternatives provided. The assay described in the paper relies in the use of a very high multiplicity of infection (MOI = 100), which could result in virus binding to cell surface molecules that do not play any significant role during the natural course of infection. There are alternative experimental approaches that could be used.

Response: We thank the reviewer for pointing this out. We have added some clarifications in line 207 that states that weakly bound viruses are removed during the PBS wash to prevent any false positive signals.

We thank Reviewer #2 for the helpful suggestions in improving our manuscript.

Reviewer #3 (Comments the Author):

Manuscript Summary:
In this manuscript "Use of viral entry assays and molecular docking analysis for the identification of antiviral candidates against Coxsackievirus A16" by Lin et al., the authors describe experimental approaches for the identification of antiviral drugs targeting different steps in the infection of Coxsackievirus A16 (CVA16). Specifically, the authors describe experimental approaches to determine if antivirals target viral entry and/or binding, or the interaction of the drug with the virus. Moreover, the authors also provide a protocol for molecular docking of the drug to predict residues in the viral capsid targeted by the antiviral compounds.

Overall, the manuscript is well written and clear. There are only some comments that the authors might want to consider to improve the document:

1) It might be useful for those readers not familiar with the virus structure and biology to include a figure showing the schematic representation of the virus and the different steps in the replication cycle of the virus targeted by the assays described in this protocol.

Response: We thank the reviewer for this suggestion. An additional schematic of the virus structure has been included in the manuscript but we have decided not to include the viral life cycle schematic since the assays are only focused on viral entry. We hope to have the reviewer's understanding.

2) The notes sections described in part 2 (Time of drug addiction assay) indicating to perform the washes gently to avoid lifting the cells is repeated 3 times. The authors could include a single note at the end of this section for the three different assays.

Response: All PBS wash notes were consolidated to line 184.

3) Line 262: It is my understanding that Figure 1B shows the co-addition experimental approach, not the post-infection treatment (Figure 1C). Please, revise.

Response: We have revised lines 260 and 263, where the co-addition and post-infection figure references were corrected to 1B and 1C, respectively.

4) Figure 1. The authors should contemplate including an schematic representation of the experimental approach similar to that shown in Figures 2 and 3. Also, please revise the text and Figure for consistency on the description of the different panels.

Response: We have included a schematic representation of the experimental approach for Figure 1 and revised the text and figures for consistency.

We thank Reviewer #3 for the helpful comments. We hope that the revisions made are satisfactory.

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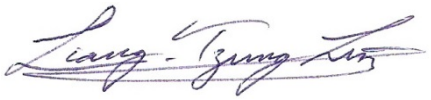

Liang-Tzung Lin, Ph.D.
Associate Professor
Department of Microbiology and Immunology
Taipei Medical University

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