Identification of a pharmacological approach to reduce ACE2 expression and development of an *in vitro* COVID-19 viral entry model

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Abstract

Because of rapid emergence and circulation of the SARS-CoV-2 variants, especially Omicron which shows increased transmissibility and resistant to antibodies, there is an urgent need to develop novel therapeutic drugs to treat COVID-19. In this study we developed an *in vitro* cellular model to explore the regulation of ACE2 expression and its correlation with ACE2-mediated viral entry. We examined ACE2 expression in a variety of human cell lines, some of which are commonly used to study SARS-CoV-2. Using the developed model, we identified a number of inhibitors which reduced ACE2 protein expression. The greatest reduction of ACE2 expression was observed when CK869, an inhibitor of actin-related protein 2/3 (ARP2/3) complex, was combined with 5-(N-ethyl-N-isopropyl)-Amiloride (EIPA), an inhibitor of sodium-hydrogen exchangers (NHEs), after treatment for 24 hours. Using pseudotyped lentivirus expressing SARS-CoV-2 full-length spike protein, we found that ACE2-dependent viral entry was inhibited in CK869 + EIPA-treated Calu-3 and MDA-MB-468 cells. This study provides an *in vitro* model that can be used for screening of novel therapeutic candidates that may be warranted for further pre-clinical and clinical studies on COVID-19 countermeasures.

Introduction

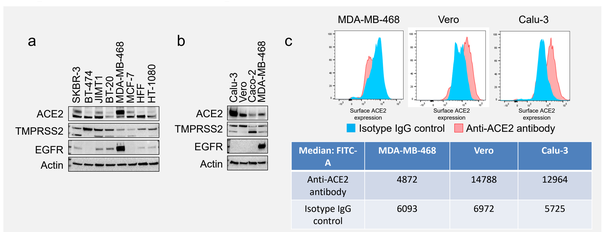
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged as a global pandemic at the end of 2019. Since the Pfizer-BioNTech COVID-19 vaccine was authorized for emergency use by the FDA on Dec. 11, 2020, a total of three COVID-19 vaccines have been authorized or approved for use in the U.S.A. However, subsequent emergence of SARS-CoV-2 variants, e.g., Alpha, Beta, Gamma, Delta, and Omicron, gave rise to additional public health concerns. Furthermore, it has been reported that waning efficacy of COVID-19 vaccines was most notable against the Omicron variant. Thus, there is still an urgent need to develop novel and effective therapeutic drugs to treat COVID-19.

Angiotensin-converting enzyme 2 (ACE2) is a dimeric, type 1 membrane protein expressed in a wide variety of human tissues, including lungs, heart, kidneys, and intestines. The SARS-CoV-2 spike protein binds to ACE2 to facilitate viral entry. The expression pattern of ACE2 suggests that in addition to playing important roles in the regulation of the biological functions in those tissues and organs, ACE2 also serves as the receptor for SARS-CoV-2 to infect other tissues and organs apart from the lungs. A correlation has been shown between a high level of ACE2 expression and increased SARS-CoV-2 infection, and downregulating ACE2 expression may reduce SARS-CoV-2 infection.

In this study, in order to establish an in vitro model of SARS-CoV-2 cellular entry, we first examined ACE2 expression in a variety of mammalian cell lines, some of which are commonly used to study SARS-CoV-2. Based on ACE2 expression levels, Calu-3, Vero, and MDA-MB-468 cells were selected for further study to develop the *in vitro* model. As the second step, a variety of compounds or inhibitors were screened based on ACE2 expression changes, and a treatment combination of CK869 plus EIPA was selected because this combination efficiently decreased ACE2 protein expression in the cell lines we tested. Next, we used pseudotyped lentiviruses expressing SARS-CoV-2 full-length spike protein from the Wuhan-Hu-1 strain, Delta and Omicron variants to study viral entry. Finally, we confirmed that the combination of CK869 plus EIPA inhibited viral entry in MDA-MB-468 and Calu-3 cells.

Materials and Methods

Cells: Calu-3, Vero, Caco-2, and BT-20 cells were purchased from ATCC and were maintained in MEM media containing 10% FBS. MDA-MB-468 (ATCC) and BT474 cells (ATCC) were maintained in RPMI-1640 containing 10% FBS. SKBR-3 cells (ATCC) were maintained in DMEM/F12 (1:1) containing 10% FBS. JIMT1 (DSMZ), MCF-7 (ATCC), HT1080 (ATCC), and HFF (kindly provided from Susan Yamada, NIH, Bethesda) cells were maintained in DMEM containing 10% FBS. **Ligand, chemical compounds, and inhibitors:** All ligands, chemical compounds, and inhibitors used in this manuscript were cell biological grade and were purchased as follows: EGF (Sigma-Aldrich, cat# E9644), NH_4Cl (Sigma-Aldrich, cat# A0171), NSC23766 (TOCRIS, cat# 2161), Casin (TOCRIS, cat# 5050), ZCL278 (TOCRIS, cat# 4794), ML141 (TOCRIS, cat# 4266), PBP10 (Calbiochem, cat# 529625), Rapamycin (Sigma-Aldrich, cat# S-015), U0126 (Sigma-Aldrich, cat# 19-147), Quercetagetin (Calbiochem, cat# 551590), Afatinib (Selleckchem, cat# S1011), 5-(N,N-Dimethyl)-amiloride hydrochloride (DMA, Sigma-Aldrich, cat# A4562), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA, Cayman chemical company, cat# 14406), Wiskostatin (TOCRIS, cat# 4434), 187-1, N-WASP inhibitor (TOCRIS, cat#2067), CK869 (TOCRIS, cat# 4984), CK666 (TOCRIS, cat# 3950), Cytochalasin D (TOCRIS, cat# 1233), and LY294002 (Sigma-Aldrich, cat# L9908). **Viral entry assay:** Lentiviral particles pseudotyped with SARS-CoV-2 spike protein were produced in 293T cells by transfection of a lentiviral backbone encoding CMV-Luciferase-IRES-ZsGreen as well as lentiviral helper plasmids and Wuhan-Hu-1, Delta (B.1.617.2), and Omicron (BA.1) spike expression plasmid. To measure viral entry, 2.0 x 10^5 Calu-3, Vero and MDA-MB-468 cells were seeded in 96-well plates and incubated overnight at 37°C. The cells were pre-treated with a combination of 50 µM CK869 and 40 µM EIPA or left untreated for 24 hours at 37°C. Prior to addition of lentiviral pseudovirus, the cells were pre-treated with SARS-CoV-2 pseudovirus infection enhance (101Bio, cat# CoV2) at a volume of 1/10 of the cell culture media in each well and incubated for 30 min at 37°C. Lentiviral pseudovirus with a titer of approximately 1 x 10^6 relative luminescence unit (RLU)/mL of luciferase activity was then added, and Calu-3, Vero, and MDA-MB-468 cells were incubated for 72 hours at 37°C. Cell extracts were harvested, lysed, and luciferase levels were assayed using a luciferase-based assay system (Promega, Madison, Wl). The experiment was performed in at least triplicate.



Median: FITC-A	MDA-MB-468	Vero	Calu-3
Anti-ACE2 antibody	4872	14788	12964
Isotype IgG control	6093	6972	5725

Figure 1. ACE2 is highly expressed in Calu-3, Vero and MDA-MB-468 cells. (a) The levels of ACE2, TMPRSS2, and EGFR expression were evaluated by Western blotting in whole cell lysate (WCL) of SKBR-3, BT-474, JIMT1, BT-20, MDA-MB-468, MCF-7, HFF, and HT-1080 cells. (b) The levels of ACE2, TMPRSS2, and EGFR expression were evaluated by Western blotting in WCL of Calu-3, Vero, Caco-2, and MDA-MB-468 cells. (c) The levels of cell surface ACE2 were evaluated using flow cytometry analysis in non-permeabilized MDA-MB-468, Vero, and Calu-3 cells.

Results and Discussion

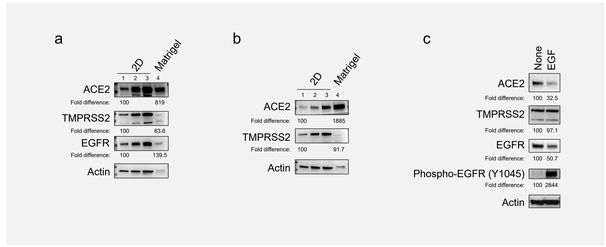


Figure 2. ACE2 expression is increased when MDA-MB-468 and Vero cells grow on a Matrigel matrix and decreased after MDA-MB-468 cells are treated by EGF. (a) The levels of ACE2, TMPRSS2 and EGF expression were evaluated by Western blotting in WCL of MDA-MB-468 cells cultured either on 2D (lanes 1, 2, 3) or grown on Matrigel matrix for 4 days. Lanes 1, 2, and 3 shows different WCLs harvested from three different cell densities of MDA-MB-468 cells on 2D. (b) The level of ACE2 expression was evaluated by Western blotting in WCL of Vero cells cultured either on 2D (WCLs harvested from three different cell densities of Vero cells) or grown on Matrigel matrix for 4 days. Lanes 1, 2, and 3 shows different WCLs harvested from three different cell densities of Vero cells on 2D. (c) The levels of ACE2, TMPRSS2, EGFR, and phosphorylated EGFR (Y1045) were evaluated by Western blotting in WCL of MDA-MB-468 cells in the absence or presence of 100 ng/ml EGF for 2 days.

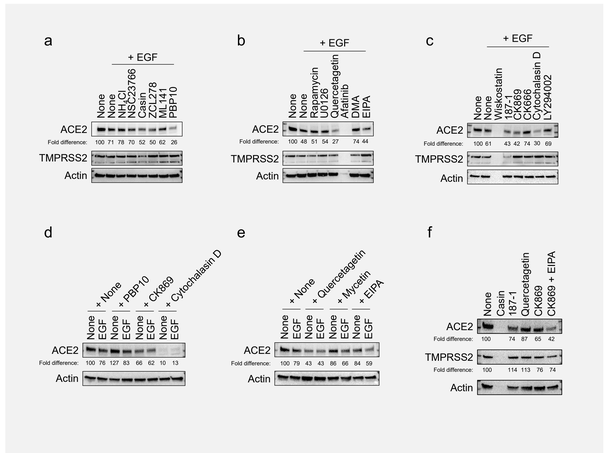


Figure 3. Chemical compounds and inhibitors can downregulate ACE2 expression in MDA-MB-468 and Vero cells. The level of ACE2 expression was evaluated by Western blotting in WCLs of MDA-MB-468 cells (a, b, c) and Vero cells (d, e, f) after the cells were treated with indicated compounds and inhibitors for 24 hours in the absence or presence of EGF.

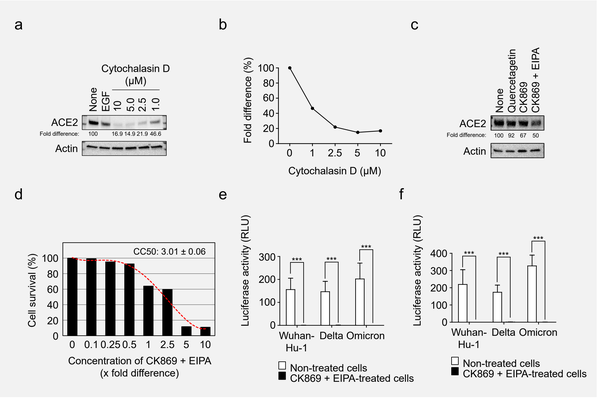


Figure 4. A combination of CK869 and EIPA reduces ACE2 expression in Calu-3 and inhibits ACE2-mediated viral entry in Calu-3 and MDA-MB-468 cells. (a) The levels of ACE2 expression were evaluated by Western blotting in WCLs of Vero cells after the cells were treated with different concentrations of cytochalasin D for 24 hours. (b) Kinetics of ACE2 expression in cytochalasin D-treated Vero cells were obtained from results of Figure 4a. (c) The level of ACE2 expression was evaluated by Western blotting in WCLs of Calu-3 cells after the cells were treated with indicated compounds and inhibitors for 24 hours. (d) Cytotoxicity of CK869 plus EIPA in Calu-3 cells. (e) Luciferase activity is proportional to the number of Calu-3 cells infected with pseudotyped lentivirus particles expressing SARS-CoV-2 full-length spike protein from Wuhan-Hu-1, Delta, and Omicron variants. (f) Luciferase activity is proportional to the number of MDA-MB-468 cells infected with pseudotyped lentivirus particles expressing SARS-CoV-2 full-length spike protein from Wuhan-Hu-1, Delta and Omicron variants. Luciferase expression (RLU) was quantified.

Conclusion

The results from our study provide a proof of concept that the ACE2 protein expression levels can be downregulated by a number of inhibitory compounds that target EGF-mediated signaling and that the downregulation of ACE2 expression reduces ACE2-mediated viral entry in human cells. This study also finds that SARS-CoV-2 may infect human cells via an ACE2-independent pathway. Taken together, our results suggest that further pre-clinical and clinical studies are warranted to develop drugs that could reduce ACE2 protein expression and inhibit ACE2-mediated and ACE2-independent viral entry to treatCOVID-19.

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