The Integrin Binding Peptide, ATN-161, as a Novel Therapy for SARS-CoV-2 Infection

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Abstract

Many efforts to design and screen therapeutics for the current severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic have focused on inhibiting viral host cell entry by disrupting ACE2 binding with the SARS-CoV-2 spike protein. This work focuses on the potential to inhibit SARS-CoV-2 entry through a hypothesized α5β1integrin-based mechanism, and indicates that inhibiting α5β1 integrin interaction with ACE2 and the spike protein using a novel molecule ATN-161 represents a promising approach to treat COVID-19.

Main

As of June 14, 2020, there have been 431,192 deaths out of a total 7,805,148 confirmed COVID-19 cases, for an estimated fatality rate of 5.5%[1]. This viral outbreak began in China in late 2019[2], with a likely origin in bats, with selection resulting in efficient human-to-human transmission occurring before or after transfer to the human host[3]. This follows the same epizoontic transmission events seen in other severe viral infections, including SARS-CoV[4] and Ebola[5], and was predicted prior to this outbreak[6]. Interaction between the SARS-CoV-2 spike protein and the angiotensin-converting enzyme II (ACE2) receptor has been implicated in SARS-CoV-2 entry and replication[7]. Many therapeutic efforts spurred by the current pandemic have focused on disrupting an aspect of the viral replication process[8,9], including host entry[10], often focusing on inhibition of ACE2/spike protein binding[11].

Integrin binding has also been implicated in the SARS-COV-2 cell entry mechanism, as the spike protein contains an integrin binding motif (RGD)[12–16]. Integrins are extracellular matrix receptors expressed throughout the body, including in the respiratory tract (e.g. epithelial cells[17]) and vasculature (e.g. endothelial cells[18]), and the β1 family of integrins are closely associated (in proximity and functional regulation) with ACE2[19,20]. A non-RGD peptide derived from the extracellular matrix component fibronectin, referred to herein as ATN-161, can bind to and inhibit the activity of certain integrins, including α5β1[21,22], and has been previously used to study viral replication[23]. ATN-161 binds outside the RGD-binding pocket, thus acting as a non-competitive inhibitor of integrin binding, especially for α5β1[24]. Likewise, ACE2 binds to α5β1 in an RGD-independent fashion, although it possesses an RGD motif in a region inaccessible for protein-protein interaction[18]. We hypothesize that SARS-CoV-2 entry is facilitated by binding to ACE2-associated α5β1 integrin via its spike protein, and that ATN-161 treatment will inhibit infection by blocking this binding event and by disrupting the initial ACE2 and α5β1 interaction (Fig. 1a).

In this study, we explored the binding of the SARS-CoV-2 spike protein with ACE2 and α5β1, utilizing ELISA-based methods. To determine the spike protein's ability to bind α5β1, plates were coated with α5β1 and incubated with a mixture of ATN-161 and a trimeric version of the spike protein. The SARS-CoV-2 spike protein binds to α5β1 with an affinity that is roughly equivalent to α5β1's native ligand, fibronectin[25], and inhibits binding in a dose-dependent manner, with maximum effect at 100nM (Fig. 1b). To our knowledge, this is the first report of

SARS-CoV-2 spike protein interaction with integrins, and specifically α5β1. We performed similar assays to investigate ACE2 binding to α5β1, using a mixture of ATN-161 and human ACE2 protein (hACE2). Clear inhibition of ACE2/α5β1 binding by ATN-161 was apparent and dose-dependent (Fig. 1c). Application of ATN-161 also did not reduce binding of trimeric spike protein to hACE2, but did reduce binding of monomeric spike (Figure S1).

The *in vitro* assessment of ATN-161 and therapeutic potential was performed using a once-passaged Vero (E6) African green monkey (*Chlorocebus atheiops*) kidney cell line utilizing competent SARS-CoV-2. ATN-161 was effective at reducing viral loads after infection (Fig. 2a), with an estimated IC_{50} of 3.16 μM. The EC_{50} value for ATN-161 approximates the value for remdesivir [8].

Measuring cellular viability and underlying cytotoxicity is another metric for antiviral therapeutic potential that we explored with ATN-161[26]. After 24 hours infection at a MOI of 0.01, cells were lysed with CellTiterGlo and luminescence values were taken to measure ATP production in each treatment. Pretreatment with ATN-161 increased ATP production in infected cells, indicating increased viability, and was consistent with viral PCR data at concentrations as low as 1μM ATN-161 (Fig. 2b). Addition of 10 μM ATN-161 resulted in a decreased cytopathic effect (i.e. fewer apparent rounded, bright cells) when cells were visualized by phase contrast microscopy (Fig. 2c).

In summary, we show that SARS-CoV-2 spike protein binds to both α5β1 and α5β1/hACE2, and that this binding can be effectively inhibited by ATN-161, which also disrupts SARS-CoV-2 infection *in vitro*. Prophylatic treatment of ATN-161 increased cell viability in the presence of SARS-CoV-2 and decreased cytopathic effects associated with viral infection. Taken together, and in light of ATN-161's previously demonstrated *in vivo* therapeutic efficacy against a closely related beta-coronavirus (porcine hemagglutinating encephalomyelitis virus[23]) and its successful use in human cancer clinical trials [27], these results support the performance of *in vivo* studies to assess the potential efficacy of ATN-161 as an experimental therapeutic agent for COVID-19.

Methods

Cells and Virus

VeroE6 cells (ATCC# CRL-1586) were cultured in complete DMEM containing 10% fetal bovine serum (FBS). SARS-CoV-2 stock from viral seed (SARS-CoV-2; 2019-nCoV/USA-WA1/2020 (BEI# NR-52281) was obtained by infecting nearly confluent monolayers of VeroE6 cells for one hour with a minimal amount of liquid in serum free DMEM. Once adsorption was complete, complete DMEM containing 2% FBS was added to the cells and the virus was allowed to propagate at 37°C in 5% CO_2. Upon the presence of CPE in the majority of the monolayer, the virus was harvested by clearing the supernatant at 1,000 xg for 15 minutes, aliquoting and freezing at -80°C. Sequencing confirmed consensus sequence was unchanged from the original isolate.

ELISA Analysis of ATN-161 Inhibition of SARS-CoV-2 Spike Protein Binding to ACE2 and Integrin

Enzyme-Linked Immunosorbent Assay (ELISA) was utilized to determine the ability of ATN-161 to disrupt binding events essential to entry of SARS-CoV-2 into a host cell. For determination of inhibition of ACE2/ α5β1 integrin binding by ATN-161, α5β1 was coated on 96-well plates at 1 µg/mL for 2 hours at room temperature and blocked overnight with 2.5% BSA. Addition of 0.5 µg/mL of hACE2-Fc (Sino Biological, Cat# 10108-H02H) in differing concentrations of ATN-161 followed, incubating for 1 hour at 37°C. Incubation with an HRP labeled goat anti-human Fc secondary antibody at 1:5000 for 30 minutes at 37°C was followed by detection by TMB substrate.

In order to assess disruption of binding of α5β1 to SARS-CoV-2 Spike protein, 96-well plates were coated as before, but incubation with ATN-161 was performed in conjunction with 1µg/mL spike (produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Spike Glycoprotein Receptor Binding Domain (RBD) from SARS-Related Coronavirus 2, Wuhan-Hu-1, Recombinant from HEK293 Cells, NR-52306) in the presence of 1mM $MnCl_2$, followed by detection with an anti-spike antibody. The rest of the procedure was consistent with the previous.

***In vitro* assessment of ATN-161 Inhibition of SARS-CoV-2 Infection**

In order to determine the ability of ATN-161 to reduce the infection capability of SARS-CoV-2 *in vitro*, a cell-based assay was utilized. VeroE6 cells were plated at a density of 1.25 x 10^4 cells/well in a 96-well plate and incubated overnight at 37°C in 5% CO_2. The next day, cells were treated with dilutions of ATN-161 in complete DMEM with 2% FBS for one hour at 37°C in 5% CO_2, followed by viral infection at an MOI of 0.1. After 48 hours, virus and cells were lysed via Trizol LS and RNA was extracted using a Zymo Direct-zol 96 RNA Kit (#R2056) according to manufacturer's instructions. Experiments were performed under Biosafety Level 3 conditions in accordance with institutional guidelines.

RT-qPCR

Viral load was quantified using a Reverse Transcriptase qPCR targeting the SARS-CoV-2 nucleocapsid gene. RNA isolated from cell cultures was plated in duplicate and analyzed in an Applied Biosystems 7300 using TaqPath supermix with the following program: i)50°C for 15 min., ii) 95°C for 2 min. and iii) 45 cycles of 95°C for 3s and 55°C for 30s. The primers and probes were as follows: 2019-nCoV_N1 Forward :5'-GAC CCC AAA ATC AGC GAA AT-3', 2019-nCoV_N1 Reverse: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3', and 2019-nCoV_N1 Probe: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'. Standard curves were generated for each run using a plasmid containing SARS-CoV-2 nucleocapsid gene (Integrated DNA Technologies, USA).

Cell Imaging

The day before infection, Nunc LabTek II chamber slides (Thermo, USA) were seeded with 2.5 x 10^4 cells per chamber. On the day of infection, chambers were treated with dilutions of ATN-161 in complete DMEM with 2% FBS for one hour prior to infecting with SARS-CoV-2 at an MOI of 0.01. Slides were placed in a 37°C 5% CO_2 incubator for 24 hours prior to imaging via phase contrast using an EVOS XL inverted microscope (Thermo, USA).

Cell Viability Assay

Ability of ATN-161 to increase cell viability was performed with CellTiterGlo (Promega, USA). Cell supernatant was removed 24 hours post infection and cells were lysed via pre-mixed CellTiterGlo reagent. Cells were incubated for 15 minutes and allowed to shake briefly before ATP was quantified via luminescence readout on the GloMax Explorer multimode plate reader (Promega).

Statistics

Differences between groups was determined via the one-way ANOVA using Dunnett's multiple comparisons test. Experiments are represented as weighted mean and standard deviation of a total of three replicates. For IC_{50} estimation, the data points directly bounding the IC_{50} value were used and calculation was made in GraphPad Prism.

References

1. Coronavirus disease (COVID-19) pandemic. *World Health Organization* https://www.who.int/emergencies/diseases/novel-coronavirus-2019 (2020).
2. Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270–273 (2020).
3. Andersen, K. G., Rambaut, A., Lipkin, W. I., Holmes, E. C. & Garry, R. F. The proximal origin of SARS-CoV-2. *Nat. Med.* **26**, 450–452 (2020).
4. Wang, L.-F. *et al.* Review of Bats and SARS. *Emerg. Infect. Dis.* **12**, 1834–1840 (2006).
5. Gire, S. K. *et al.* Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. *Science (80-.).* **345**, 1369–1372 (2014).
6. Menachery, V. D. *et al.* A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat. Med.* **21**, 1508–1513 (2015).
7. Lan, J. *et al.* Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* **581**, 215–220 (2020).
8. Wang, M. *et al.* Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* **30**, 269–271 (2020).
9. Caly, L., Druce, J. D., Catton, M. G., Jans, D. A. & Wagstaff, K. M. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. *Antiviral Res.* **178**,

104787 (2020).

10. Xia, S. *et al.* Inhibition of SARS-CoV-2 (previously 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting its spike protein that harbors a high capacity to mediate membrane fusion. *Cell Res.* **30**, 343–355 (2020).

11. Wang, C. *et al.* A human monoclonal antibody blocking SARS-CoV-2 infection. *Nat. Commun.* **11**, 2251 (2020).

12. Yan, S., Sun, H., Bu, X. & Wan, G. New Strategy for COVID-19: An Evolutionary Role for RGD Motif in SARS-CoV-2 and Potential Inhibitors for Virus Infection. *Front. Pharmacol.* **11**, (2020).

13. Tresoldi, I., Sangiuolo, C. F., Manzari, V. & Modesti, A. SARS-COV-2 and infectivity: Possible increase in infectivity associated to integrin motif expression. *J. Med. Virol.* (2020) doi:10.1002/jmv.25831.

14. Aguirre, C., Meca-Lallana, V., Barrios-Blandino, A., del Río, B. & Vivancos, J. Covid-19 in a patient with multiple sclerosis treated with natalizumab: May the blockade of integrins have a protective role? *Mult. Scler. Relat. Disord.* **44**, 102250 (2020).

15. Sigrist, C. J., Bridge, A. & Le Mercier, P. A potential role for integrins in host cell entry by SARS-CoV-2. *Antiviral Res.* **177**, 104759 (2020).

16. Wang, Y., Liu, M. & Gao, J. Enhanced receptor binding of SARS-CoV-2 through networks of hydrogen-bonding and hydrophobic interactions. *Proc. Natl. Acad. Sci.* 202008209 (2020) doi:10.1073/pnas.2008209117.

17. Ishibashi, Y., Relman, D. A. & Nishikawa, A. Invasion of human respiratory epithelial cells by Bordetella pertussis: Possible role for a filamentous hemagglutinin Arg-Gly-Asp sequence and α5β1 integrin. *Microb. Pathog.* **30**, 279–288 (2001).

18. Napione, L., Cascone, I., Mitola, S., Serini, G. & Bussolino, F. Integrins: A flexible platform for endothelial vascular tyrosine kinase receptors. *Autoimmun. Rev.* **7**, 18–22 (2007).

19. Clarke, N. E., Fisher, M. J., Porter, K. E., Lambert, D. W. & Turner, A. J. Angiotensin Converting Enzyme (ACE) and ACE2 Bind Integrins and ACE2 Regulates Integrin Signalling. *PLoS One* **7**, e34747 (2012).

20. Lin, Q., Keller, R. S., Weaver, B. & Zisman, L. S. Interaction of ACE2 and integrin β1 in failing human heart. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1689**, 175–178 (2004).

21. Donate, F. *et al.* Pharmacology of the Novel Antiangiogenic Peptide ATN-161 (Ac-PHSCN-NH2): Observation of a U-Shaped Dose-Response Curve in Several Preclinical Models of Angiogenesis and Tumor Growth. *Clin. Cancer Res.* **14**, 2137–2144 (2008).

22. Edwards, D. N. *et al.* Integrin α5β1 inhibition by ATN-161 reduces neuroinflammation and is neuroprotective in ischemic stroke. *J. Cereb. Blood Flow Metab.* 0271678X1988016 (2019) doi:10.1177/0271678X19880161.

23. Lv, X. *et al.* ATN-161 reduces virus proliferation in PHEV-infected mice by inhibiting the

integrin α5β1-FAK signaling pathway. *Vet. Microbiol.* **233**, 147–153 (2019).

24. Khalili, P. A non-RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo. *Mol. Cancer Ther.* **5**, 2271–2280 (2006).

25. Aota, S., Nomizu, M. & Yamada, K. M. The Short Amino Acid Sequence Pro-His-Ser-Arg-Asn in Human Fibronectin Enhances Cell-Adhesive Function. *J. Biol. Chem.* **269**, 24756–61 (1994).

26. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280.e8 (2020).

27. Cianfrocca, M. E. *et al.* Phase 1 trial of the antiangiogenic peptide ATN-161 (Ac-PHSCN-NH2), a beta integrin antagonist, in patients with solid tumours. *Br. J. Cancer* **94**, 1621–1626 (2006).

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Author contributions

G.B. conceived the study. B.B. conducted all live SARS-CoV-2 studies. N.I. and W.Z. conducted all ELISA's. B.B., N.I. and W.Z. collected data and performed computational analysis. B.B., C.R., T.H., J.K. and G.B. interpreted data and wrote the manuscript with input from all of the authors.

Competing interests

G.B. is the inventor on a filed provisional patent with the USPTO related to this work. The remaining authors declare no competing interests.

Figure legends

Fig. 1

a, Proposed mechanism of ATN-161 inhibition of SARS-CoV-2 infection, where addition of ATN-161 is proposed to inhibit SARS-CoV-2 spike protein binding to host α5β1 integrin, as well as α5β1-ACE2 binding. ELISA data indicates that ATN-161 alters **b**, binding of α5β1 to spike protein-coated plates, when these plates are incubated with α5β1 and concentrations of ATN-161 and **c**, when α5β1-coated plates are incubated with human ACE2 and concentrations of ATN-161. Data was normalized to no-ATN vehicle control. Data represent mean ± SD, n=3, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Fig. 2

a, Viral Load of SARS-CoV-2 with and without ATN-161 treatment. VeroE6 cells were incubated with indicated ATN-161 concentrations for one hour prior to 48 hours infection with SARS-CoV-2 at a MOI of 0.1. **b**, Cell viability via luminescence-based CellTiterGlo 24 hours post-infection. **c**, Representative phase contrast microscope images of VeroE6 cells 24 hours post-infection with and without 10 μM ATN-161 treatment. Black arrows indicate some of the visible viral cytopathic effect (rounded, phase bright cells). Scale bar is 10 μM. Data represent mean ± SD, n=3, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Fig. S1

Inhibition of SARS-CoV-2 spike protein binding to human ACE2 by ATN-161. Plates were pre-coated with **a**, trimeric or **b**, monomeric spike protein and incubated with a mixture of hACE2 and various ATN-161 concentrations, followed by detection of bound hACE2 via HRP-conjugated anti-ACE2 antibody. Data was normalized to a no-ATN vehicle control. Data represent mean ± SD, n=3, * $P<0.05$, ** $P<0.01$.

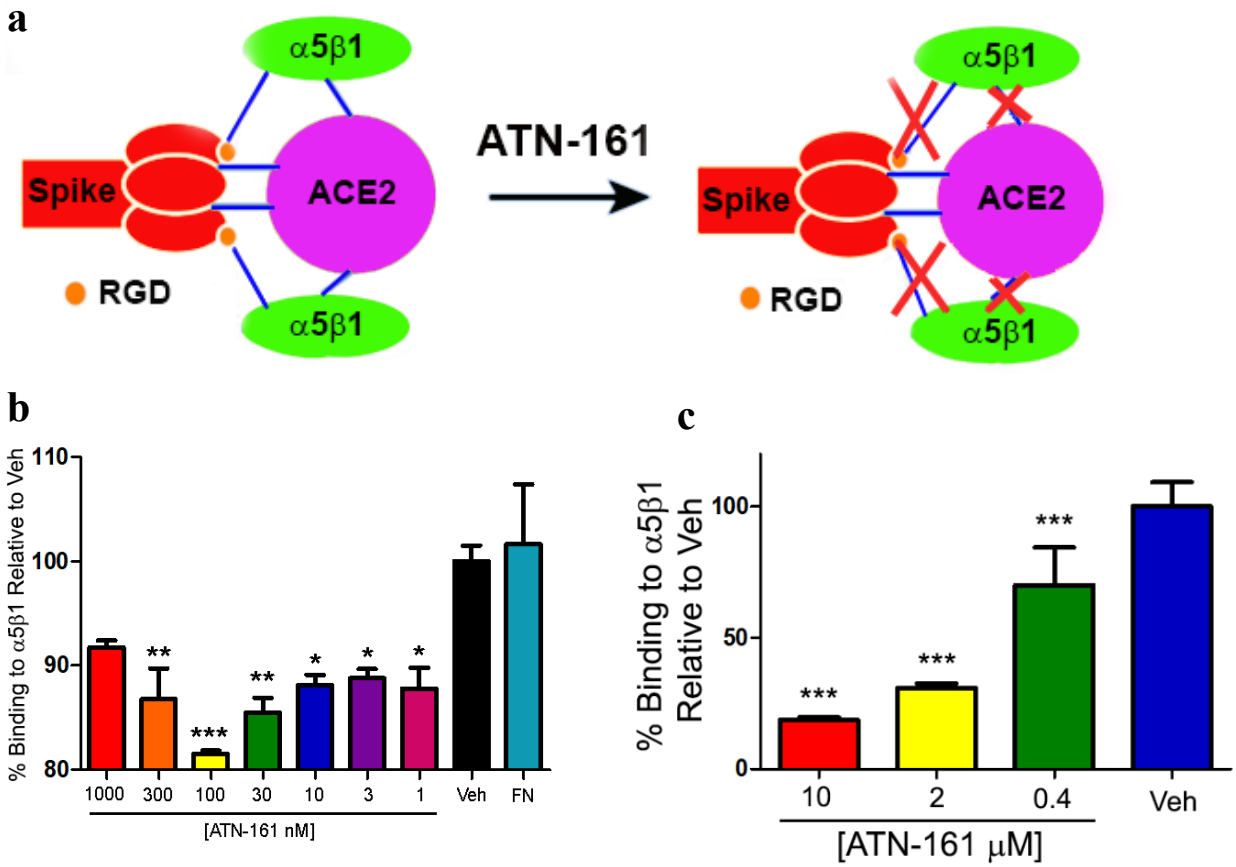
a
α5β1
Spike
ACE2
RGD
α5β1
ATN-161
α5β1
Spike
ACE2
RGD
α5β1
b
% Binding to α5β1 Relative to Veh
110
100
90
80
**
***
**
*
*
*
1000
300
100
30
10
3
1
Veh
FN
[ATN-161 nM]
c
% Binding to α5β1 Relative to Veh
100
50
0
***
***
***
10
2
0.4
Veh
[ATN-161 μM]

Figure 1

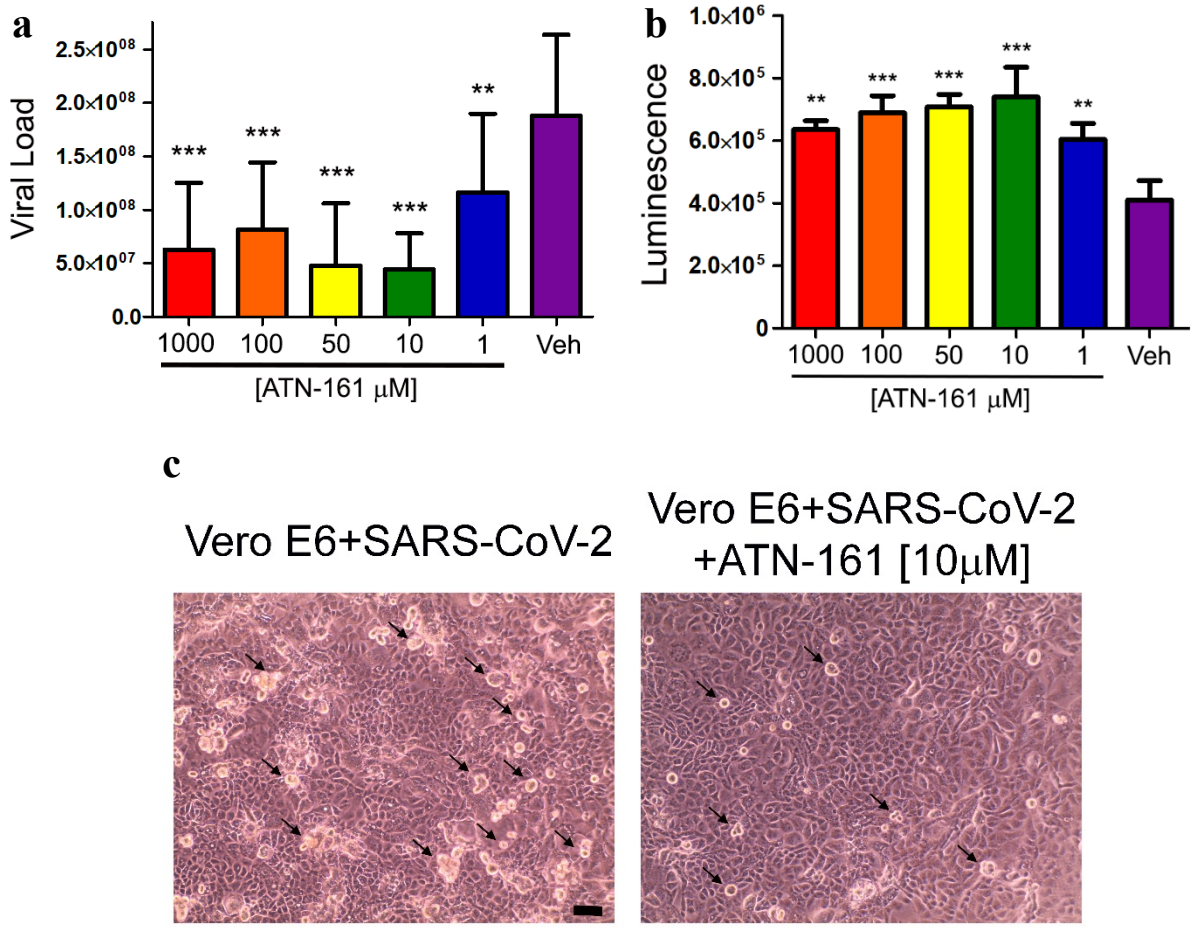
a
Viral Load
2.5×10^08
2.0×10^08
1.5×10^08
1.0×10^08
5.0×10^07
0.0
***
***
***
***
**
1000
100
50
10
1
Veh
[ATN-161 μM]
b
Luminescence
1.0×10^6
8.0×10^5
6.0×10^5
4.0×10^5
2.0×10^5
0
**
***
***
***
**
1000
100
50
10
1
Veh
[ATN-161 μM]
c
Vero E6+SARS-CoV-2
Vero E6+SARS-CoV-2
+ATN-161 [10μM]

Figure 2

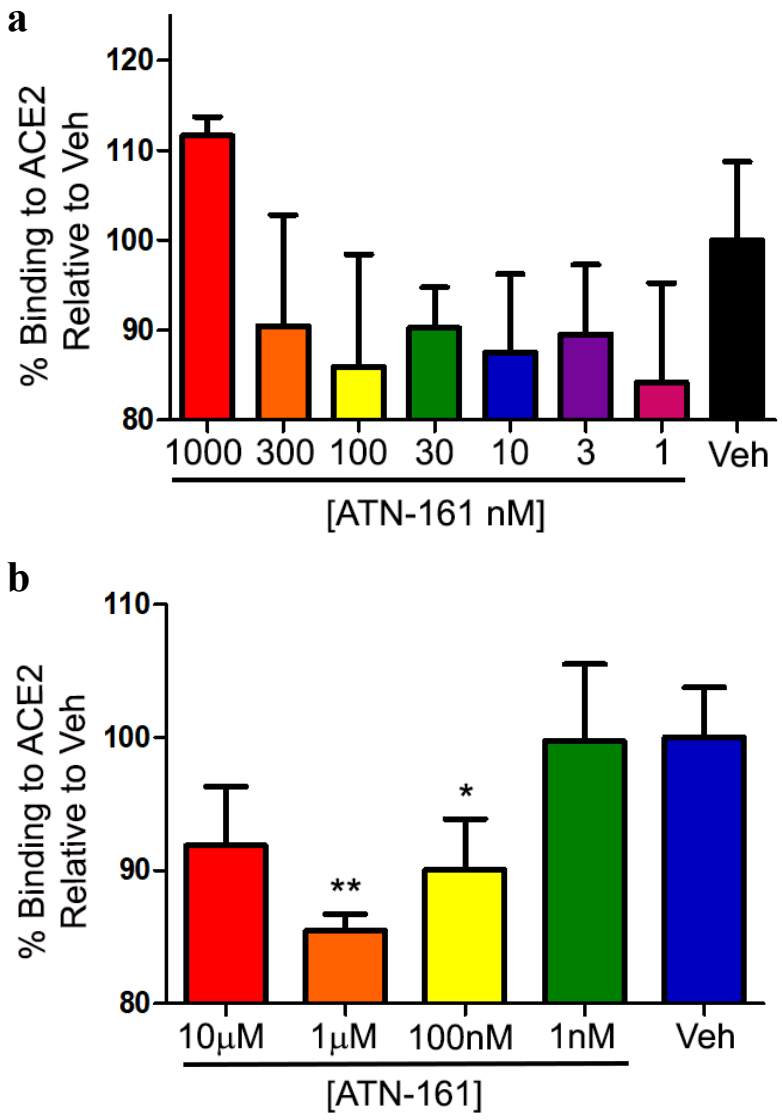
a
% Binding to ACE2
Relative to Veh
120
110
100
90
80
1000
300
100
30
10
3
1
Veh
[ATN-161 nM]
b
% Binding to ACE2
Relative to Veh
110
100
90
80
**
*
10μM
1μM
100nM
1nM
Veh
[ATN-161]

Figure S1