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

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VISUAL ABSTRACT

HIGHLIGHTS

- SARS-CoV-2 spike protein binds to α5β1 integrin protein and human ACE2/α5β1, facilitating entry into host cells.
- ATN-161, an integrin binding peptide, inhibits the interaction between the SARS-CoV-2 spike protein and its host binding partners, with 3 sites of binding identified via molecular modeling.
- ATN-161 inhibits infection in vitro and demonstrates increases in cell viability when administered prophylactically.
- ATN-161 is well-studied, with the potential for more rapid introduction into clinical trials than many other compounds currently undergoing preclinical evaluation.

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ABBREVIATIONS AND ACRONYMS

ACE2 = angiotensin-converting enzyme 2
CO2 = carbon dioxide
COVID-19 = coronavirus disease-2019
DMEM = Dulbecco’s modified eagle media
ELISA = enzyme-linked immunosorbent assay
hACE2 = human angiotensin-converting enzyme 2
IC50 = half-maximal inhibitory concentration
quPCR = quantitative polymerase chain reaction
RBD = receptor binding domain
RGD = arginine-glycine-aspartate
SARS-CoV-2 = severe acute respiratory syndrome-coronavirus-2

SUMMARY

Many efforts to design and screen therapeutics for the current severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) pandemic have focused on inhibiting viral host cell entry by disrupting angiotensin-converting enzyme-2 (ACE2) binding with the SARS-CoV-2 spike protein. This work focuses on the potential to inhibit SARS-CoV-2 entry through a hypothesized α5β1 integrin-based mechanism and indicates that inhibiting the spike-protein interaction with α5β1 integrin (+/− ACE2) and the interaction between α5β1 integrin and ACE2 using a novel molecule (ATN-161) represents a promising approach to treat coronavirus disease-19. (J Am Coll Cardiol Basic Trans Science 2021;6:1-8) © 2021 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

As of September 28, 2020, there were 995,836 deaths of a total 32,968,853 confirmed coronavirus disease-2019 (COVID-19) cases (1), for an estimated fatality rate of approximately 3.1% (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 that occurred before or after transfer to the human host (3). This follows the same epizootic transmission events seen in other severe viral infections, including severe acute respiratory syndrome-coronavirus (SARS-CoV) (4) and Ebola (5), and was predicted before this outbreak (6). Interaction between the SARS-CoV-2 spike protein and the angiotensin-converting enzyme 2 (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 because the spike protein contains an integrin binding motif (arginine-glycine-aspartate [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 noncompetitive 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.

METHODS

CELLS AND VIRUS. VeroE6 cells (ATCC® CRL-1586) were cultured in complete Dulbecco’s modified eagle media (DMEM) containing 10% fetal bovine serum. 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 1 h with a minimal amount of liquid...
in serum-free DMEM. Once adsorption was complete, complete DMEM containing 2% fetal bovine serum was added to the cells, and the virus was allowed to propagate at 37°C in 5% CO₂. At the presence of the cytopathic effect in most of the monolayer, the virus was harvested by clearing the supernatant at 1,000×g for 15 min, aliquoting and freezing it at −80°C. Sequencing confirmed that the 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 used 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/αSβ1 integrin binding by ATN-161, αSβ1 was coated on 96-well plates at 1 μg/ml for 2 h at room temperature and blocked overnight with 2.5% bovine serum albumin. Addition of 0.5 μg/ml of human ACE2 (hACE2)-Fc (Sino Biological, Cat# 10108-H02H) in differing concentrations of ATN-161 followed, incubating for 1 h at 37°C. Incubation with a horse radish peroxidase–labeled goat anti-human Fc secondary antibody at 1:5,000 for 30 min at 37°C was followed by detection by 3,3′,5,5′-tetramethylbenzidine substrate.

To assess the disruption of binding of αSβ1 to the SARS-CoV-2 spike protein, 96-well plates were coated as previously described, but incubation with ATN-161 was performed in conjunction with a 1 μg/ml spike (produced under HHSN272201400008C and obtained through BEI Resources, National Institutes of Allergy and Infectious Disease, National Institutes of Health: spike glycoprotein receptor binding domain [RBD] from SARS-related Coronavirus 2, Wuhan-Hu-1, Recombinant from HEK293 Cells, NR-52306) in the presence of 1 mM manganese chloride, followed by detection with an antispike antibody. The rest of the procedure was consistent with previously described procedures.

**IN VITRO ASSESSMENT OF ATN-161 INHIBITION OF SARS-CoV-2 INFECTION.** To determine the ability of ATN-161 to reduce the infection capability of SARS-CoV-2 in vitro, a cell-based assay was used with VeroE6 cells due to their permissive nature for infection by this virus, as well as common use in neutralization studies (25,26). Furthermore, the use of a kidney cell line for in vitro SARS-CoV-2 studies was supported by the fact that kidney injury due to direct SARS-CoV-2 infection and multiple indirect mechanisms commonly occurs in seriously affected patients with COVID-19 (27). VeroE6 cells were plated at a density of 1.25 × 10⁴ cells/well in a 96-well plate and incubated overnight at 37°C in 5% CO₂. The next day, cells were treated with dilutions of ATN-161 in complete DMEM with 2% fetal bovine serum for 1 h at 37°C in 5% CO₂, followed by viral infection at a multiplicity of infection of 0.1. After 48 h, virus and cells were lysed via Trizol LS, and RNA was extracted using a Zymo Direct-zol 96 RNA Kit (#R2056, Zymo Research, Irvine, California) according to manufacturer’s instructions. Experiments were performed under Biosafety Level 3 conditions in accordance with institutional guidelines.

**REVERSE TRANSCRIPTASE QUANTITATIVE POLYMERASE CHAIN REACTION.** Viral load was quantified using a reverse transcriptase quantitative polymerase chain reaction (qPCR) that targeted the SARS-CoV-2 nucleocapsid gene. RNA isolated from cell cultures was plated in duplicate and analyzed in an Applied Biosystems 7300 (Thermo Scientific, Waltham, Massachusetts) using a TaqPath supermix with the following program: 1) 50°C for 15 min; 2) 95°C for 2 min; and 3) 45 cycles of 95°C for 3 s and 55°C for 30 s. 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 GAC CCC AAA ATC AGC GAA AT-3′, 2019-nCoV_N1 Probe: 5′-FAM-ACC CCG CAT TAC GTG TGG ACC-BHQ1-3′. Standard curves were generated for each run using a plasmid containing the SARS-CoV-2 nucleocapsid gene (Integrated DNA Technologies, Coralville, Iowa).

**CELL IMAGING.** The day before infection, Nunc Lab-Tek II chamber slides (Thermo Scientific, Waltham, Massachusetts) were seeded with 2.5 × 10⁴ cells/chamber. On the day of infection, chambers were treated with dilutions of ATN-161 in complete DMEM with 2% fetal bovine serum for 1 h before infecting with SARS-CoV-2 at a multiplicity of infection of 0.01, which was chosen for ease of visibility of viable cells versus cytopathic cells under microscopy. Slides were placed in a 37°C 5% CO₂ incubator for 24 h before imaging via phase contrast using an EVOS XL inverted microscope (Thermo Scientific).

**CELL VIABILITY ASSAY.** Ability of ATN-161 to increase cell viability was performed with CellTiterGlo (Promega, Madison, Wisconsin). Cell supernatant was removed 24 h post-infection, and cells were lysed via pre-mixed CellTiterGlo reagent. Cells were incubated for 15 min and allowed to shake briefly before adenosine triphosphate was quantified via luminescence readout on the GloMax Explorer multimode plate reader (Promega).

**MOLECULAR MODELING.** The structure of the ACE2-spike protein RBD complex (7) was obtained from the protein data bank (ID 6m0j). To obtain the orientation of the SARS-CoV-2 spike protein trimer relative to ACE2, the
RBD was aligned with the sprung out RBD of the prefusion conformation of the spike protein trimer (protein data bank ID 6vsb) (28). Similarly, the integrin α5β1 ectodomain structure (29) was obtained from the protein data bank (ID 3vi3), with the calf1 and calf2 domains of α5 added from the protein data bank (ID 6naj) (30). ATN-161 (Ac-PHSCN-NH2) was prepared for docking with AutoDock vina (31). ATN-161 was docked to the α5β1 complex, ACE2, and ACE2-spike RBD complex. The ZDock 3.0.2 (32) server was used for protein/C0 protein docking to generate the α5β1 complexed with ACE2 and with the spike RBD. The structures were rendered using PyMol 2.3.0 (33).

**STATISTICAL METHODS.** Data are presented using the mean ± SD. Differences between groups were determined via the 1-way analysis of variance using Dunnett’s post hoc multiple pairwise comparisons test. Experiments are represented as the mean ± SD of a total of 3 replicates. For half-maximal inhibitory concentration (IC50) estimation, the data points directly bounding the IC50 value were used and calculation was made in GraphPad Prism (GraphPad, La Jolla, California). Viral load studies were performed 3 separate times, ELISA studies 2 times, and the cell viability assay was performed a single time, with each condition done in triplicate for each study. A p value <0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

In this study, we explored the binding of the SARS-CoV-2 spike protein with ACE2 and α5β1, using 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 was bound to α5β1 with an affinity that was roughly equivalent to α5β1’s native ligand, fibronectin (34), and inhibited binding with a U-shaped, dose-dependent manner, with maximum effect at 100 nM (Figure 1A) (21). This U-shaped response was not surprising because it was consistent with previous in vitro and in vivo preclinical studies on ATN-161 in the context of blocking angiogenesis and solid tumor growth (21). This might be due to the
ability of ATN-161, at saturating concentrations, to affect the expression of α5β1 integrin, which we demonstrated to occur in experimental stroke (22). To our knowledge, this was 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 hACE2. Clear inhibition of ACE2/α5β1 binding by ATN-161 was apparent and dose-dependent (Figure 1B). Furthermore, application of ATN-161 reduced binding of the trimeric spike protein to hACE2, either alone or in combination with α5β1, the latter of which tended to support greater spike binding than to hACE2 alone (Figure 1C). Application of ATN-161 also reduced binding of the monomeric spike to hACE2 (Supplemental Figure S1).

The in vitro assessment of ATN-161 and its therapeutic potential was performed using a once-passaged VeroE6 African green monkey (Chlorocebus aethiops) kidney cell line that used competent SARS-CoV-2. ATN-161 was effective at reducing viral loads after infection (Figure 2A), with an estimated IC50 (Concentration which inhibits 50% of viral replication) of 3.16 μM. The EC50 (concentration which inhibits 50% of viral replication) value for ATN-161 approximated the value for remdesivir (8). Importantly, VeroE6 was previously shown to express α5β1 integrin (35).

Measuring cellular viability and underlying cytotoxicity was another metric for the antiviral therapeutic potential that we explored with ATN-161 (36). After 24 h infection at a multiplicity of infection of 0.01, cells were lysed with CellTiterGlo, and luminescence values were taken to measure adenosine triphosphate production in each treatment. Pretreatment with ATN-161 increased adenosine triphosphate production in infected cells, which indicated increased viability, and was consistent with viral PCR data at concentrations as low as 1 μM ATN-161 (Figure 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

![FIGURE 2 ATN-161 Effects on SARS-CoV-2 Infection In Vitro](image)

(A) Viral load of SARS-CoV-2 with and without ATN-161 treatment. VeroE6 cells were incubated with indicated ATN-161 concentrations for 1 h before 48 h infection with SARS-CoV-2 at a multiplicity of infection of 0.1. (B) Cell viability via luminescence-based CellTiterGlo 24 h post-infection. (C) Representative phase contrast microscope images of VeroE6 cells 24 h post-infection with and without 10 μM ATN-161 treatment. Solid arrows indicate some of the visible viral cytopathic effect (rounded, phase bright cells). Scale bar is 10 μM. (D) Number of cells taking on a rounded, cytopathic appearance per 4× field under varying treatments. Data represent mean ± SD (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001. Abbreviations as in Figure 1.
phase contrast microscopy (Figure 2C), with increased ATN-161 administration resulting in fewer cells appearing rounded from viral damage (Figure 2D).

Molecular docking of ATN-161 with ACE2 or ACE2-spike RBD complex revealed 3 potential binding sites (Figure 3A). One of these was at the interface between the ACE2 and the spike RBD. This might have affected the binding of RBD with ACE2. ATN-161 was also found to bind the integrin α5β1 ectodomain complex near the RGD motif binding site located at the interface between the α5 and β1 chain (29), which potentially affected the binding of α5β1 with proteins containing the RGD motif. Although ACE2 contains the RGD sequence, it is inaccessible for binding under physiological conditions. Therefore, it is believed that another sequence, lysine-glycine-aspartate (residues 353, 354, 355), which closely resembles the sequence RGD, may bind α5β1 via the RGD-binding site (37).
**Figure 3B** shows the ACE2-α5β1 complex obtained from protein–protein docking using Zdock with the ACE2 residues around the lysine-glycine-aspartate and the α5β1 residues around the RGD-binding site selected as preferred binding partners. This docking resulted in a complex with the desired orientations of the integrin chains (38) and ACE2 relative to the plasma membrane (**Figure 3A**). As shown in **Figure 3B**, the binding of the α5β1 to ACE2 at this site masked the binding site for the spike RDB, potentially inhibiting SARS-CoV-2 entry (37). The binding of ATN-161 in the interface may disrupt the α5β1-ACE2 complex.

Separately, we performed docking of α5β1 to the spike protein RBD, which contained the RGD sequence that was accessible for binding. This resulted in a complex of the spike RDB and α5β1 (**Figure 3C**). For this binding to occur, the RGD-binding interface of integrin needs to be oriented differently than the binding with ACE2, which is consistent with the active conformation of integrin (7). ATN-161 binding near the RGD motif binding site of integrin might inhibit the α5β1-spike RBD complex formation. We hypothesized that SARS-CoV-2 entry was facilitated by binding to the ACE2-associated α5β1 integrin via its spike protein, and that ATN-161 treatment would inhibit infection by blocking this binding event and by disrupting the initial ACE2 and α5β1 interaction (**Figure 3D**). One potential limitation of our study was that ATN-161, although primarily characterized as an inhibitor of α5β1 integrin, could also bind to and inhibit αvβ3 integrin, a receptor that is present in VeroE6 cells and implicated as a viral coreceptor (23,35). However, although this possible mechanism of action should be investigated in future studies, α5β1 integrin’s known association with ACE2, which has not been demonstrated for αvβ3 integrin, makes this possibility less likely.

**CONCLUSIONS**

In summary, we showed that the SARS-CoV-2 spike protein binds to both α5β1 and α5β1/hACE2, and that this binding could be effectively inhibited by ATN-161, which also disrupted SARS-CoV-2 infection in vitro. Prophylactic 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 betacoronavirus [porcine hemagglutinating encephalomyelitis virus (23)] and its successful use in human cancer clinical trials (39), 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.

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**COMPETENCIES IN MEDICAL KNOWLEDGE:** Entry into host cells is 1 of the most essential functions of a virion, with a large amount of variation in approaches among species that are capable of infecting humans. Increasing knowledge of how these entries occur is crucial to understanding and applying future therapeutics targeted against these mechanisms.

**TRANSLATIONAL OUTLOOK:** Use of host proteins that are not typically considered targets for therapeutics that block viral entry can expand the pool of novel antivirals. Future work directed at both in vivo efficacy of ATN-161 as well as other integrin-binding molecules is warranted. In addition, more work to elucidate integrin binding and entry in SARS-CoV-2 would facilitate the increase in potential antivirals.
ATN-161, a Novel Therapy for SARS-CoV-2 Infection

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