# AT-527, the oral prodrug of a guanosine nucleotide analog, is a potent *in vitro* inhibitor of SARS-CoV-2

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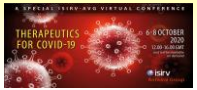



## Background

**In a search for an effective, orally administered treatment for COVID-19, the *in vitro* activity of AT-511, the free base of the antiviral drug candidate AT-527, a modified guanosine nucleotide prodrug known to inhibit the hepatitis C virus NS5B polymerase (1), was evaluated against several coronaviruses, including SARS-CoV-2, the virus responsible for COVID-19. Additionally, the inhibition of replication of SARS-CoV-2 by AT-511 was compared to that by N$^4$-hydroxycytidine, a nucleoside recently reported to be active against SARS-CoV-2 (2).**

## Methods

**Viruses.** Seasonal human coronaviruses (HCoV-229E and HCoV-OC43) were obtained from ATCC (Manassas, VA). MERS-CoV (EMC), SARS-CoV (Urbani) and SARS-CoV-2 (USA-WA1/2020) were supplied by The Centers for Disease Control and Prevention, Atlanta, GA.

**Coronavirus infection and treatment of BHK-21, Huh-7 and RD cells.** The antiviral activity of AT-511 was evaluated against human coronaviruses alpha (229E), beta (OC43), MERS (EMC) and SARS (Urbani) three to seven days post-infection using a neutral red assay (3). The effective concentration of test compound required to prevent virus-induced cytopathic effect (CPE) by 50% ($EC_{50}$) and to cause 50% cell death in the absence of virus ($CC_{50}$) were calculated by regression analysis. Viruses were diluted to achieve multiplicity of infection (MOIs) of 0.003, 0.002, 0.001 and 0.03 $CCID_{50}$ (50% cell culture infectious dose) per cell for 229E, OC43, MERS and SARS, respectively. A virus yield reduction (VYR) assay (4) which calculated virus titer using a standard endpoint dilution $CCID_{50}$ assay and the Reed-Muench equation was used as a second, independent determination of the inhibition of viral replication.

Chloroquine and hydroxychloroquine (Mason-Chem, Palo Alto, CA) were also tested against HCoV-229E in Huh-7 cells. Sofosbuvir (Pharma Sys, Cary, NC; up to 100 µM) was tested against HCoV-229E at a MOI of 0.01 in confluent BHK-21 cells.

**SARS-CoV-2 infection and treatment of HAE cells.** Differentiated normal human airway epithelial (HAE) cells (EpiAirway™ AIR-100 or AIR-112) were prepared by MatTek Corporation (Ashland, MA) from a single donor. The cells form polarized monolayers, the ciliated apical side of which is exposed to air and creates a mucin layer. SARS-CoV-2 virus was diluted in AIR-100-MM medium before infection to yield a MOI when added to cultures of approximately 0.0015 $CCID_{50}$ per cell. Serial dilutions of AT-511 or N$^4$-hydroxycytidine (Oxeltis, Montpellier, France) were applied to the cells (120 µL to the apical side, and 1 mL to the basal side) while virus (120 µL) was applied only to the apical side. As a virus control, some cells were treated with cell culture medium only. After a 2-h infection incubation, the apical medium was removed, and the basal medium was replaced with fresh compound or medium (1 mL). The cells were maintained at the air-liquid interface. On day 5, cytotoxicity was estimated by visual inspection. Virus released into the apical compartment of the HAE cells was harvested by the addition of medium, incubated for 30 min, mixed and plated on Vero 76 cells for VYR titration. Separate wells were used for virus control (no drug) and duplicate wells were used for untreated cell controls (no virus).

**Formation of AT-9010 *in vitro*.** Human bronchial and nasal epithelial cells from single donors were cultured, harvested with DetachKit 2 (PromoCell GmbH) and plated according to the vendor's instructions at 1x10$^6$ cells per well. Once confluent, 10 µM or 100 µM AT-511 or ALS-8112 was added for an 8 h incubation. Cell viability and density, measured using an automatic cell counter after staining cells with acridine orange and propidium iodide, was determined in a subset of samples before plating and at the end of the incubation period along with untreated cells used as vehicle controls. After the 8-h exposure period, media was removed, cells were rinsed with Hepes buffered saline solution (HBSS) and fresh cell culture medium without drug was added. At 0, 15, 24, 48 and 72 h post washout, the media was removed, cells rinsed with HBSS, extracted with internal standards in ice-cold 60% MeOH overnight at -20°C, and analyzed for concentrations of the TP metabolites of AT-511 and ALS-8112 by LC-MS/MS. Untreated cells were collected at each time point as a negative control. The CellTiterGlo® Assay kit was also used to confirm cell viability at the end of the 8-h incubation.

## Results

### *In Vitro* Potency and Cytotoxicity of AT-511 and Other Oral Drugs Against Several Coronaviruses

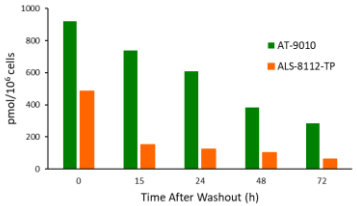
| Virus (genus) | Cell line | Compound | Cytopathic Effect Assay | | Virus Yield Reduction Assay | Selectivity Index |
|---|---|---|---|---|---|---|
| | | | $EC_{50}$ (µM) | $CC_{50}$ (µM)[a] | $EC_{90}$ (µM) | ($CC_{50}/EC_{90}$) |
| HCoV-229E (alpha) | BHK-21 | AT-511 | 1.8 ± 0.3 (2) | >100 | ND | >55[b] |
| | | sofosbuvir | >100 | >100 | | 0 |
| | Huh-7 | AT-511 | 1.7 ± 0.1 (2) | >86 | 1.2 ± 0.1 (2) | >72 |
| | | chloroquine | 7.8 | 28 | <0.050 | 3.6[b] |
| | | hydroxychloroquine | 6.0 | 16 | <0.037 | 2.6[b] |
| HCoV-OC43 (beta) | Huh-7 | AT-511 | ND[c] | >86 | 0.5 / <0.03 | >170 / >3100 |
| | RD | AT-511 | 2.8 | >86 | 2.2 | >38 |
| MERS-CoV (beta) | Huh-7 | AT-511 | 26 ± 15 | >86 | 37 ± 27 | >2.3 |
| SARS-CoV (beta) | Huh-7 | AT-511 | ND[c] | >86 | 0.3 | >250 |
| SARS-CoV-2 (beta) | HAE | AT-511 | ND[c] | >86[d] | 0.5 ± 0.1 (3) | >160 |
| | | N$^4$-hydroxycytidine | | >19[d] | 3.9 | >4.9 |

The activity of AT-511 and other antiviral compounds was measured in cells infected with different coronaviruses, using the neutral red assay and/or the virus yield reduction (VYR) assay as described in the Methods to determine the effective concentration required to achieve 50% inhibition ($EC_{50}$) of the virus-induced cytopathic effect (CPE), the concentration to reduce virus yield by 1 $\log_{10}$ ($EC_{90}$), and the cytotoxic concentration of the drug to cause death to 50% of viable cells without virus ($CC_{50}$). Values represent results from single or multiple [mean ±SD (n)] experiments.

[a]Highest concentration tested
[b]$CC_{50}/EC_{50}$
[c]Not determined because there was no cytopathic effect with this virus in these cells
[d]Cytotoxicity assessed by visual inspection of cell monolayers

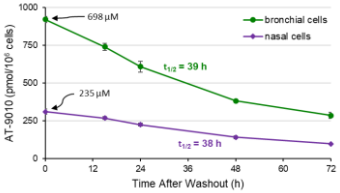
- **AT-511 exhibited a mean $EC_{90}$ value of 0.5 µM against SAR-CoV-2 and similar potencies against HCoV-229E, HCoV-OC43 and SARS-CoV, and no cytotoxicity up to a concentration of 100 µM.**
- **N$^4$-hydroxycytidine inhibited SARS-CoV-2 replication with an $EC_{90}$ = 3.9 µM in the same HAE cell model.**

### Triphosphate Concentrations in Primary Human Bronchial Epithelial (HBE) Cells Incubated with 10 µM AT-511 or ALS-8112




- **Substantial levels of AT-9010, the active triphosphate of AT-511, were attained after 8-h incubation with HBE cells.**
- **Two-fold more AT-9010 was formed in HBE cells from AT-511 as compared to ALS-8112, a drug shown to be clinically effective against respiratory syncytial virus (RSV), with an *in vitro* $EC_{90}$ value of 1.3-2.7 µM in RSV-infected human airway epithelial (HAE) cells. (5)**
- **72 h after drug washout, AT-9010 levels were almost 5-fold more than ALS-8112 TP levels due to the longer half-life of AT-9010 (39 h) as compared to that of the TP of ALS-8112 (6-8 h).**
- **No toxicity was observed in the cells treated with AT-511 up to 100 µM, the highest concentration tested.**

### Intracellular Half-life of AT-9010 in Primary Human Bronchial and Nasal Epithelial Cells after 8-h Exposure to 10 µM AT-511




- **About 3-fold more AT-9010 was formed in human bronchial as compared to nasal epithelial cells.**
- **AT-9010 half-life was similar (38-39 h) in both human cell types**

## Conclusions

- **The substantial formation of the active TP metabolite in incubations with human bronchial and nasal epithelial cells is consistent with the sub-micromolar potency of the free base of AT-511 against SARS-CoV-2 replication in the HAE tissue model.**
- **Considering that these cell types are actual targets of infection in COVID-19 patients, the potency of AT-527, combined with its favorable safety profile previously established in HCV subjects, suggests that AT-527 may be highly efficacious in treating COVID-19.**

## References

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