Stenoparib, an inhibitor of cellular poly (ADP-ribose) polymerases (PARPs), blocks *in vitro* replication of SARS-CoV-2 variants

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Running Head: Stenoparib inhibits SARS-CoV-2 variants *in vitro*.

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Abstract

We recently published a preliminary assessment of the activity of a poly (ADP-ribose) polymerase (PARP) inhibitor, stenoparib, also known as 2X-121, which inhibits viral replication by affecting pathways of the host. Stenoparib is an inhibitor of mammalian poly (ADP-ribose) polymerases (PARPs). Here we show that stenoparib effectively inhibits additional SARS-CoV-2 variants, including an additional wild-type strain (Germany/BavPat1/2020), and the variants *alpha* (B.1.1.7), *beta* (B.1.351) and *gamma* (P.1) *in vitro*, with 50% effective concentration (EC₅₀) estimates of 4.1 μ M, 8.5 μ M, 24.2 μ M and 13.6 μ M, respectively. A second study focusing on a combination of 10 μ M stenoparib and 0.5 μ M remdesivir resulted in over 90% inhibition of the *alpha* (B.1.1.7) variant, which is substantially greater than what was achieved with stenoparib or remdesivir alone at these concentrations.

Importance

The coronavirus disease (COVID-19) pandemic, caused by SARS-CoV-2, has caused over 247 million infections and over 5 million deaths (1). Although protective vaccines are available, the pandemic continues and both old and new SARS-CoV-2 variants may exhibit degrees of resistance to vaccination. To date, only two antiviral drugs, remdesivir and molnupiravir, or treatment with monoclonal antibodies, have been approved by the United States Food and Drug Administration as COVID-19 therapies in certain situations. Additional effective therapeutics are urgently needed. Here we describe the activity of a small molecule, stenoparib, that effectively inhibits replication of SARS-CoV-2 wild-type and variant strains *in vitro*. Stenoparib is an inhibitor of mammalian poly (ADP-ribose) polymerases (PARPs). A host-targeting therapeutic like stenoparib could be a significant benefit for COVID-19 patients as a standalone therapy, or especially as part of a combinatorial COVID-19 treatment strategy with an antiviral drug such as remdesivir or molnupiravir.

Observation

Since the beginning of the COVID-19 pandemic, SARS-CoV-2 has mutated, which has resulted in different variants of the virus. Only two antiviral drugs - remdesivir and molnupiravir, or treatment with monoclonal antibodies, have been approved by the United States Food and Drug Administration as COVID-19 therapies (2, 3). Remdesivir and molnupiravir are nucleoside analogs that affect the activity of the RNA-dependent RNA polymerase (RdRp). After incorporation into viral RNA, remdesivir stalls the RdRp during elongation (4), while molnupiravir results in the incorporation of mutations that can be functionally deleterious (5). In contrast to inhibitors that target viral replication, we recently published a study on the activity of a poly (ADP-ribose) polymerase (PARP) inhibitor, stenoparib, also known as 2X-121, which inhibits viral replication by affecting pathways of the host (6). ADP-ribosylation (ADPR) pathways may have either anti- or pro-viral properties, and their importance in host-virus interactions is becoming increasingly recognized (7). Unlike remdesivir, which inhibits viral replication downstream of entry into the cell, stenoparib inhibits virus entry and post-entry processes (6). Stenoparib inhibited the SARS-CoV-2 USA-WA1/2020 virus and the HCoV-NL63 human seasonal respiratory coronavirus *in vitro*, exhibiting dose-dependent suppression of virus multiplication and cell-cell spread in cell culture (6). Stenoparib exhibits a unique dual activity against the PARP1, 2 and PARP5a, 5b (tankyrase 1, 2) enzymes, which are important intermediates in the Wnt/ β -catenin immune checkpoint (8, 9).

SARS-CoV-2 variants may carry mutations throughout the genome, particularly in the sequence coding for the spike (S) protein. As a host-targeting therapeutic, we hypothesized that the antiviral activity of stenoparib should be able to inhibit all variant strains. We explored the activity of stenoparib against four additional SARS-CoV-2 variants of concern; SARS-CoV-2 Germany/BavPat1/2020 wild-type strain (wt) and variant strains (B.1.1.7 [*alpha*], B.1.351 [*beta*], and P1 [*gamma*] – hereafter referred to using the Greek nomenclature). SARS-CoV-2 virus variants were mixed with serial dilutions of compound and transferred to Vero E6 *Cercopithecus*

aethiops kidney cells, which were fixed and stained 20 hours (h) later with a SARS-CoV/SARS-CoV-2 Nucleocapsid monoclonal antibody (Sino Biological) followed by a horseradish peroxidase (HRP)-labeled Goat anti-Mouse IgG (Thermo Scientific) and TrueBlue substrate. The 2 controls included on all assay plates were 1) infected and untreated cells and 2) uninfected and untreated cells. The experiment was conducted in quadruplicate and the results were calculated as the average fraction of wells that showed positive staining for the virus using ViroSpot assay (10). Inhibition of virus replication by stenoparib was dose-dependent, with EC₅₀ ranging between 4.1 μ M to 24.2 μ M across the 4 variants tested (Fig. 1A). Interestingly, the EC₅₀ for the *beta* variant (24.2 μ M) was 1.8- to 5.8-fold higher than for the wt, *alpha*, and *gamma* variants (4.1, 8.5, and 13.6 μ M, respectively). A similar phenomenon was noted for remdesivir (Fig. 1B), where the EC₅₀ for the *beta* variant (9.9 μ M) was 2.0- to 2.6-fold higher than for the wt, *alpha*, and *gamma* variants (3.8, 5.0, and 4.3 μ M, respectively). According to a recent report, the *beta* variant appears to exhibit a significantly reduced eclipse period and more rapid replication *in vitro* (11), which would likely affect its dose response toward stenoparib and remdesivir. The *beta* variant carries numerous changes to the S protein, several of which affect the receptor binding domain and are also common to the *alpha* and *gamma* variants. In addition to these changes, the *beta* variant carries mutations in the N protein and in the Nsp3 polyprotein (ORF1a), which may be involved in ADPR following infection (12). Differential susceptibility to ADPR may contribute to the altered replication kinetics of the *beta* variant, although this awaits experimental validation.

In an additional set of experiments, we focused on the SARS-CoV-2 *alpha* variant. Vero E6 cells were infected with the *alpha* variant using a multiplicity of infection of 0.1, and the activity of stenoparib was assessed using the plaque reduction assay. Plaques are areas of dead or destroyed cells and appear as small, clear regions in an infected cell monolayer after fixation and staining with crystal violet. Following infection and treatment with an inhibitor, cells were overlaid with low melting point agarose, fixed at 120 h post infection, stained with crystal

violet, and plaques were manually counted. The 3 control types were 1) infected and untreated cells, 2) uninfected and untreated cells, and 3) infected cells treated with a combination of camostat mesylate and E64d (CE), which are protease inhibitors that prevent spike protein cleavage and virus entry into the cell (13). For the analysis shown in Fig. 2A and 2B, we combined lower doses of stenoparib (2.5, 5.0, and 10 μ M) with the previously reported 50% effective concentration (EC_{50}) of remdesivir (0.5 μ M) (6). In Fig. 2A and 2B, neither 10 μ M stenoparib nor 0.5 μ M remdesivir achieved greater than a 50% reduction in plaquing efficiency compared to the infected, untreated cells. When combined, however, synergy occurred as plaque inhibition increased to over 90% - superior to what was achievable with either drug alone ($P < 0.01$) at these doses. Notably, this was accomplished without significant cytotoxicity (Fig. 2C). Combinations of two or more drugs may lead to synergism through different mechanisms of action, which has the potential benefit of reducing individual doses of each drug and minimizing undesirable side effects.

Besides the known roles for the PARP1 and PARP2 enzymes in DNA repair (14, 15), members of the PARP family have numerous additional functions (16). The 18 known human PARPs appear to differentially affect viral replication; some exhibit proviral activity and others exhibit antiviral roles (7, 17-21). In coronavirus, the N protein is mono (ADP) ribosylated (MARylated) during infection. Stability of the N protein is critical for viral genome replication, translation, packaging, and modulation of the host cell cycle (22). The N protein is the only ADPR target definitively identified in coronavirus thus far (17), and the prevalence of this modification across multiple coronavirus families suggests an important role in virus stability (17). Despite this, the role of N protein MARylation, and of ADPR in general with regards to coronavirus stability, is not well characterized. Possibly ADPR has a role in regulating virus genome structure, similar to ADPR of histones or the adenovirus core proteins (23, 24). While remdesivir and molnupiravir inhibit the viral replicon, stenoparib likely acts through multiple

121 targets. A combination of stenoparib and remdesivir or molnupiravir may be highly potent at
122 inhibiting SARS-family coronaviruses including SARS-CoV-2.

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Conflict of Interest Statement

SK is employed by and holds a financial interest in Allarity Therapeutics, which stands to potentially benefit from these results.

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Figures

Figure 1. Dose-response curves for stenoparib and remdesivir on SARS-CoV-2 variants. **A.** stenoparib. **B.** remdesivir. Vero E6 cells (ATCC CRL-1586) were infected for 20 hours with SARS-CoV-2 wild type (wt) or the indicated variants in the presence or absence of stenoparib or remdesivir as indicated. Results are the average fraction of quadruplicate wells that exhibited positive staining for SARS-CoV-2 (y-axis) vs. the concentration of the inhibitors (x-axis). These data were used to estimate the EC₅₀ of stenoparib and remdesivir. The EC₅₀ values were approximated with the aid of the online calculator from AAT Bioquest ("Quest Graph™ EC₅₀ Calculator." AAT Bioquest, Inc, 26 Oct. 2020, <https://www.aatbio.com/tools/ec50-calculator>). Viruses; wild-type (wt): BetaCoV/Munich/BavPat1/2020, European Virus Archive Global. *Alpha*: USA/CA_CDC_5574/2020, BEI Resources, Cat# NR-54011. *Beta*: hCoV-19/South Africa/KRISP-K005325/2020, BEI Resources. *Gamma*: hCoV-19/Japan/TY7-503/2021 (Brazil P.1), BEI Resources.

Figure 2. Activity of stenoparib and remdesivir on the *alpha* (B.1.1.7) variant. **A.** Virus Plaque assays. Vero E6 cells (ATCC CRL-1586) were infected for 2 hours with SARS-CoV-2 *alpha* (USA/CA_CDC_5574/2020, BEI Resources, Cat# NR-54011), washed, and overlaid with low-melting temp agarose/MEM with or without stenoparib and/or remdesivir at the concentrations indicated. The cell monolayers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet prior to manual counting of clear plaques. The experiment was performed using technical triplicates, and error bars represent the standard deviation of three independent experiments. Statistical analysis was performed using the Student's unpaired t-test. * $P < 0.05$, ** $P < 0.01$ applies where indicated. **B.** Photographs of plate wells after crystal violet staining. Labels indicate the concentrations of stenoparib or remdesivir. Abbreviations: S = stenoparib, R = remdesivir at the concentrations indicated (e.g. S2.5 / R0.5 = stenoparib 2.5 μ M, remdesivir 0.5 μ M, etc.). **C.** Cytotoxicity measurements performed by the lactate dehydrogenase release assay (Promega Cytotox 96) 48 hours after infection. Inhibitors stenoparib or remdesivir were used at the indicated concentrations (x-axis). The assays were performed according to the manufacturer's recommendations. Results from drug treatments were normalized to the fraction of maximum cytotoxicity (percent cytotoxicity; y-axis) achieved by detergent lysis of cells. The experiment was performed using technical triplicates, and error bars represent the standard deviation of three independent experiments.

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