**B. Studies and Results**

**In Vitro Antiviral Assay Throughput**

The Antiviral Efficacy and Resistance Core (AER) is tasked with the development and standardization of antiviral assays specific to the needs of all projects in the ASAP consortium. Increasing the throughput of the primary antiviral screening assays was a primary goal in the first year of the core’s operation. These live-virus antiviral assays are one of the primary drivers of the SAR through all ASAP projects, and therefore the speed and quality of these data sets are key to the success of this core and the consortium overall. As described and funded in our initial proposal, we have designed, purchased, and installed a liquid handling robotics system for full automation of live-virus antiviral assays under BSL2 conditions, and partial automation for BSL3 and BSL3e assays. This automated system is centered around a Biomek i7 Hybrid liquid handler and a Tecan D300e Digital Drug Dispenser.

We have established end-to-end processing of our live-virus antiviral assays under BSL2 conditions, including EV-D68, EV-A71, DENV-2, ZIKV, and the attenuated rSARS-CoV-2 Δ3a/Δ7b.We have obtained the appropriate permissions from the Mount Sinai IBC and NIH to work with the attenuated rSARS-CoV-2 Δ3a/Δ7b under BSL2 conditions. Our MERS-CoV assay is partially automated for the work that can be performed outside of the BSL3e environment. This end-to-end automated processing includes: Compound addition in 8-point dose-response to 96-well plates, viral infection in 96-well format, plate washing and immunofluorescence staining for viral antigen, automated plate reading and data formatting using the onboard Cytation 1 plate cytometer to quantify infected cell percentage, and finally uploading of data to the ASAP CDD vault for automated data processing, analysis, and storage.

The end-to-end automation in 96-well format has allowed the Antiviral Core to boost antiviral efficacy throughput from ~20-30 compound batches up to 60 compound batches. We plan further improvements in our antiviral assay throughput as we miniaturize down to 384-well format, which now can be accomplished with end-to-end automation complete.

**In Vitro Live-Virus Antiviral Screening**

The Antiviral Core has focused its second-year effort in the development and establishment of standardized *in vitro* live-virus antiviral assays to meet the needs of all projects in the ASAP consortium. These assays have been established and run by hand while our automated liquid handling system was under construction.

**MERS-CoV/SARS-CoV-2 Mpro Protease Program**

The first major lead optimization campaign undertaken by the ASAP consortium is the development of a novel coronavirus Mpro inhibitor with *in vivo* efficacy against MERS-CoV and SARS-CoV-2. Broad-spectrum activity against MERS-CoV is poor in the current clinically approved Mpro inhibitors developed against SARS-CoV-2. Therefore, this program will provide another therapeutic to combat the ongoing SARS-CoV-2 pandemic and add what will be the first effective therapeutic against MERS-like coronaviruses to the world arsenal. Furthermore, a therapeutic with cross-MERS-CoV/SARS-CoV-2 activity will have a greater chance of broad-spectrum anti-coronavirus activity.A graph with black dots

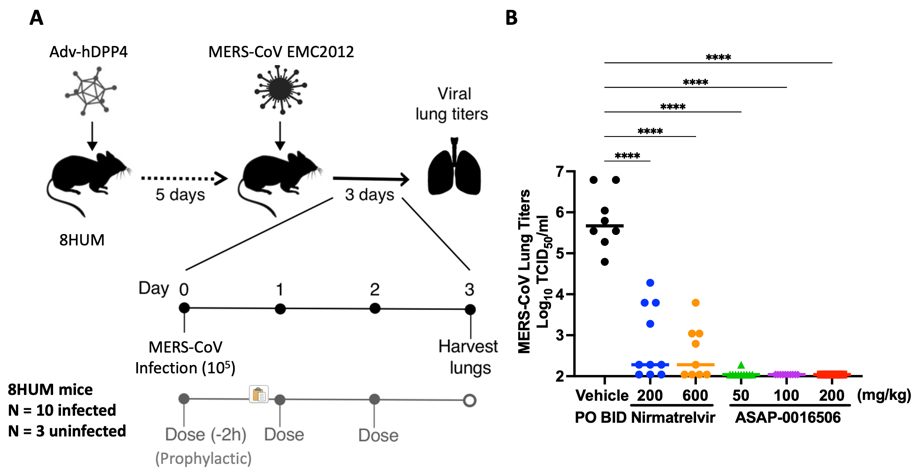
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Although a BSL3e maintenance shut-down lasting 4 months significantly delayed MERS-CoV assays, the Antiviral Core has screened through 364 compounds in full dose-response (EMC2012, Vero-TMPRSS2, +P-gp inhibitor, plate cytometry quantifying infected cell percentage) over the last 6 months. To manage this 4-month disruption, the Antiviral Core has engaged backup capacity at both NIH Preclinical Services (EMC2012, Vero 76, no P-gp inhibitor, 36 compounds screened by PCS to date) and the MAVDA AViDD center (EMC2012, Vero-TMPRSS2, +P-gp inhibitor, 424 compounds screened to date) as two secondary MERS-CoV screening sites to provide bridging capacity during the 4-month shutdown and to supplement Antiviral Core MERS-CoV screening capacity since it has been reinstated. Comparing the 82 compounds which have duplicates screened at both the Antiviral Core and MAVDA, we see a strong correlation (R2=0.82) of EC50s from the two data sets **(Fig. 1)**. The EC50s had an average of 2.22-fold difference across the entire data sets. This strong correlation between data sets generated in two similar, but not identical, assays performed in separate laboratories yielded significant progress in this SAR campaign. 

|  | MERS-CoV Vero-TMPRSS2 | | SARS-CoV-2 HeLa-ACE2 | |
| --- | --- | --- | --- | --- |
| Molecule Name | **EC50 (µM)** | **CC50 (µM)** | **EC50 (µM)** | **CC50 (µM)** |
| ASAP-0017238 | 0.0154 | > 5.00 | 0.00913 | > 10.0 |
| ASAP-0016506 | 0.0161 | > 5.00 | 0.0145 | > 10.0 |
| ASAP-0017239 | 0.0169 | > 5.00 | 0.0221 | > 10.0 |
| ASAP-0017449 | 0.0183 | > 5.00 | 0.0545 | > 10.0 |
| ASAP-0014786 | 0.0188 | > 5.00 | 0.0235 | > 20.0 |
| ASAP-0017446 | 0.0189 | > 5.00 | 0.0686 | > 10.0 |
| ASAP-0017445 | 0.0192 | > 5.00 | 0.0142 | > 10.0 |
| ASAP-0014925 | 0.0228 | > 5.00 | 0.0393 | > 10.0 |
| ASAP-0017448 | 0.0245 | > 5.00 | 0.0126 | > 10.0 |
| ASAP-0016509 | 0.0285 | > 5.00 | 0.11 | > 10.0 |
| ASAP-0017370 | 0.0346 | > 5.00 | 0.0524 | > 10.0 |
| Nirmatrelvir | 0.0349 | > 5.00 | 0.112 | > 10.0 |
| ASAP-0014750 | 0.0402 | > 5.00 | 0.217 | > 20.0 |
| ASAP-0015657 | 0.0508 | > 5.00 | 0.0546 | > 10.0 |
| ASAP-0016845 | 0.0512 | > 5.00 | 0.0951 | > 10.0 |
| ASAP-0014886 | 0.0517 | > 5.00 | 0.0883 | > 10.0 |
| ASAP-0016846 | 0.0524 | > 5.00 | 0.285 | > 10.0 |
| ASAP-0017339 | 0.0539 | > 5.00 | 0.0397 | > 10.0 |
| ASAP-0012337 | 0.0556 | > 5.00 | 0.869 | > 20.0 |
| ASAP-0013299 | 0.0558 | > 4.88 | 0.0584 | > 20.0 |

In the process of establishing the Antiviral Core MERS-CoV antiviral assay in Vero-TMPRSS2 cells and discussions with the MAVDA AViDD center, we identified that this cell line has an extremely high expression level of P-glycoprotein (P-gp). Therefore, we have elected to run all antiviral assays in the Vero-TMPRSS2 cell line with 2 µM Elacridar to inhibit P-gp to aim to provide better predictive value of *in vivo* activity. The Antiviral Core also runs Elacridar-free antiviral assays as a metric to assess the status of lead compounds as P-gp substrates as part of our ADMET analysis. The AER also continues to counter screen against SARS-CoV-2/WA1 in HeLa-ACE2 without P-gp inhibitor (Table 1) to ensure broad-spectrum anti-coronavirus activity is maintained in the lead series. Over the last year the AER has screened a total of 939 compounds in the SARS-CoV-2 antiviral assay. 

**The top 20 compounds, ranked by MERS-CoV EC50, of that SAR campaign can be found in Table 1. Through close collaboration between Project 5 (Lead opt), the Biochemical Core, and the AER, we have removed inefficiencies and improved assay cycle times within the ASAP center. This has led to rapid and significant progression in the potency of our lead chemical scaffolds targeting both the MERS-CoV and SARS-CoV-2 Mpro active sites simultaneously. We have identified 5 analogs with EC50s below 23.5 nM against both MERS-CoV and SARS-CoV-2, significantly outperforming nirmatrelvir in our live-virus assays.** Furthermore, these compounds have been significantly optimized for ADMET properties. The report for Project 5 (Lead opt) can be referenced for the pharmacokinetic analysis of ASAP-0016506, which is well tolerated in mice at dosages up to 450 mg/kg PO BID and shows significant plasma stability that indicates coverage of 5x EC50 is obtainable with a significantly lower dosage. We are now finalizing conditions for a MERS-CoV animal efficacy experiment within the next month testing multiple dosage levels of ASAP-0016506.

The Antiviral Core is also tasked with providing animal efficacy models for all programs pursued by the ASAP consortium. The MERS-CoV/SARS-CoV-2 Mpro program is the first of ASAP to reach proof-of-concept *in vivo* animal efficacy studies. ASAP-0016506 is highly stable in both mouse and human (8HUM mouse), with the 8HUM model yielding approximately 2-fold better plasma exposure levels. Therefore, we moved forward with the 8HUM model, which has a humanized cytochrome P450 systems that accurately mimics human PK of well-established FDA-approved drugs. The Antiviral Core has established MERS-CoV EMC2012 infection of 8HUM mice transduced with an adenovirus vector expressing the viral receptor, human DPP4 (hDPP4). The MERS-CoV infection is limited to the lung in this model, which is the only site that should express the hDPP4. Therefore, this model does not show outward viral pathogenesis, and we will rely on viral lung titers and lung histopathology scoring as our major readouts of efficacy. The Antiviral Core has performed a MERS-CoV proof-of-concept efficacy study for ASAP-0016506 at 50, 100, and 200 mg/kg (PO BID) in hDPP4-transduced 8HUM mice (**Fig.**). **ASAP-0016506 was successful at near complete inhibition of MERS-CoV replication in the lung at 3 days post infection at all dosage levels tested (Fig.)**. ASAP-0016506 significantly outperformed the nirmatrelvir controls, which were run at much higher dosages (200 and 600 mg/kg). Histopathology analysis of inflammation and damage is underway for these samples, as well as PK analysis of plasma samples taken during the experiment. **This proof-of-principle experiment validates the ASAP AI-guide fragment-based approach to drug discovery, delivering a potential clinical candidate within the first 2 years of funding.**



**SARS-CoV-2 Nucleocapsid Program**

|  | SARS-CoV-2 HeLa-ACE2 | | SARS-CoV-2 VLP  293T | |
| --- | --- | --- | --- | --- |
| Molecule Name | **EC50 (µM)** | **CC50 (µM)** | **EC50 (µM)** | **CC50 (µM)** |
| Nirmatrelvir | 0.112 | > 10.0 |  |  |
| ASAP-0010877 | 0.404 | > 5.43 | 0.773 | > 49.9 |
| ASAP-0011004 | 1.18 | > 50.0 |  | 48.7 |
| ASAP-0010848 | 4.31 | > 50.0 |  | > 49.9 |
| ASAP-0010978 | 7.83 | > 50.0 |  | 12.2 |
| ASAP-0010965 | 8.17 | > 50.0 | 5.338 | > 49.9 |
| ASAP-0010837 | 12 | > 50.0 |  | 6.78 |
| ASAP-0011009 | 17.5 | > 50.0 |  | 30.5 |
| ASAP-0010806 | 18.7 | > 50.0 |  | > 49.9 |
| ASAP-0011082 | 20.2 | 22.4 |  |  |
| ASAP-0010801 | 34.8 | > 50.0 |  | 18.7 |

The ASAP consortium has a focus on unique structural targets that polymerize, such as capsid and nucleoproteins. Such targets offer dominant targeting effects that make them potentially resistance refractory (see Project 1 for detailed discussion). The nucleoprotein target (N protein) is a structural protein and does not have an known enzymatic process for the establishment of a biochemical assay, which led us to pause our discovery program while searching for a viable alternative assay with sufficient throughput. Recently, ASAP has collaborated with the laboratory of Jennifer Doudna to screen our top fragment-based hits in the SARS-CoV-2 lentivirus VLP assays she has established. After observing potent hits in the VLP assay, we began screening in live-virus SARS-CoV-2/WA1 assays in the Antiviral Core. In **Table 2** you can see the top 10 hits from 40 total compounds tested to date in an ongoing screening campaign. These initial 8 early hits in this campaign have clear antiviral activity, with 2 compounds (including ASAP-0010877, with sub-micromolar activity) corroborated with the orthogonal VLP assay, give us high confidence that we could elect to pursue this nucleoprotein target with Project 3 (Fragment-to-lead) should ASAP elect to restart this program.

**SARS-CoV-2 nsp3 Mac1 Macrodomain Program**

ASAP launched a SARS-CoV-2 nsp3 Mac1 macrodomain targeted program toward the end of the consortium’s first year. While deletion or ablation of Mac1 activity has a profound effect *in vivo*, the involvement of innate immune response meant that it would likely be challenging to identify a suitable cellular model for which *in vitro* cellular antiviral activity could drive the lead optimization phase of the discovery program. Project 3 (fragment-to-lead) produced multiple potent molecules that demonstrated target engagement in X-ray structures, in vitro biochemical assays, and cell-based CETSA assays. It is known that Mac1 inhibition shows clear *in vivo* reductions in viral replication, but impacts on cell models are still unclear. We believed the primary-like iPSC-derived pneumocytes with intact innate immune systems (see Developmental Award – Zwaka) could A graph of different types of cells

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|  | EV-D68  RD Cells | |
| --- | --- | --- |
| Molecule Name | **EC50 (µM)** | **CC50 (µM)** |
| Rupintrivir | 0.007 | > 5.00 |
| ASAP-0017288 | 0.063 | > 5.00 |
| ASAP-0017291 | 0.16 | > 5.00 |
| ASAP-0017289 | 0.208 | > 5.00 |
| ASAP-0017285 | 0.364 | > 5.00 |
| ASAP-0017292 | 0.478 | > 5.00 |
| ASAP-0017286 | 0.52 | > 5.00 |
| ASAP-0017294 | 0.574 | > 5.00 |
| ASAP-0017295 | 0.861 | > 5.00 |
| ASAP-0017287 | 1.37 | > 5.00 |
| ASAP-0017290 | 1.51 | > 5.00 |
| ASAP-0017284 | 5.15 | > 5.00 |
| ASAP-0017293 | 18.5 | > 5.00 |
| ASAP-0017296 | > 50.0 | > 5.00 |
| ASAP-0017297 | > 50.0 | > 5.00 |
| ASAP-0017298 | > 50.0 | > 5.00 |

potentially offer a viable cell model to observe Mac1 inhibition. Our cellular assays in HeLa-ACE2 cells and iPSC-derived type II pneumocytes initially identified multiple sub-micromolar hits in our live-virus SARS-CoV-2 antiviral screening assays, initially giving hope that we could drive the program with these assays. Since then, we have screened 81 macrodomain targeted compounds, and have noted that the observed antiviral activity does not correlate well with the biochemical data (see Biochemical Core report), suggesting the antiviral activity may be from off-target effects. We have therefore paused this program while we explore the observed target engagement and the impact on SARS-CoV-2 infected cells. To further explore the impact of the of inhibiting macrodomain function during viral infection, we have observed global mono-ADP-ribosylation expression by IF, gated between infected and uninfected cell populations within the same well. We have compared the mono-ADP-ribosylation states of uninfected and infected cells in the presence increasing amounts of 10 ASAP macrodomain compounds with differing profiles. In **Figure 2**, we show an example of two differing compounds, ASAP-0010715 and ASAP-0011184. ASAP-0010715 has weak biochemical macrodomain activity, but potent antiviral activity in our live-virus assay. ASAP-0011184 has potent biochemical macrodomain activity, but no detectable antiviral activity in our live-virus assay. We found significant increases in ADP-ribosylation mean fluorescence intensity can be detected with increasing concentrations of ASAP-0011184, but not ASAP-0010715. This would be expected for a macrodomain inhibitor, which functions to remove ADP-ribosylation modifications from proteins. This correlates well with the CETSA target engagement for these compounds and indicates that the observed antiviral activity of ASAP-0010715 is most likely off-target. This program will remain paused until a high-throughput cell-based assay can be established to drive SAR in lead optimization. We aim to disseminate the results in a forthcoming publication. All compound structures, biochemical activity data, cellular antiviral data, and ADMET data has already been released publicly.

**EV-D68/EV-A71 Programs**

There are currently no approved treatments for enterovirus infection. ASAP has multiple enterovirus programs which are progressing from Project 3 (Fragment-to-lead) toward Project 5 (Lead opt), which now require cell-based antiviral assay support to continue towards project transition. The Antiviral Core has established live-virus cell-based for EV-D68 (US/MO/14-18949) and EV-A71 (Strain H). These assay uses an 8-point dose-response of ASAP compounds added to RD cells 2 hours prior to infection with EV-D68/EV-A71 under BSL2 conditions. 24 hours later, the infected cells are fixed and stained with EV-VP1-specific monoclonal antibodies, and infected cells are quantified over total cells (DAPI) using our Cytation 1 plate cytometer. A cytotoxicity assay (MTT) is run in parallel in uninfected RD cells for all compounds. The assay was then standardized by generating EC50s for known EV inhibitors and comparing with published results for consistent potency values. The Antiviral Core has also engaged with the laboratory of Johan Neyts at KU Leuven to provide the planned back-up screening capacity for these two viruses to further confirm the accuracy of our results. The Neyts lab is providing CPE-based assays for both viruses in HeLa cells.

**EV-D68/EV-A71 Capsid Program**

The ASAP consortium has a focus on unique structural targets that polymerize, such as capsid and nucleoproteins. Such targets offer dominant effects that make them potentially resistance refractory (See Project 1 for detailed discussion). While this program has just entered into cell-based assays, we have already identified multiple hits with sub-micromolar EC50s against EV-D68 **(Table 3)**, with our top hit ASAP-0017288 having a 63 nM EC50. The rupintrivir control had a 7 nM EC50, which is comparable to the literature reported EC50. We will continue to screen against EV-D68 and are now collecting our initial live-virus data for EV-A71, with the goal of broad-spectrum anti-enterovirus activity.

**EV-D68/EV-A71 3CL Protease Program**

Viral proteases are classical targets for antiviral drug development with a proven track record, most recently with Paxlovid for SARS-CoV-2. Therefore, ASAP is bringing our fragment-based approach to the 3CLpro of enterovirus. This program is just entering the cell-based assays of the Antiviral Core, with an initial 12 compounds screened against EV-D68 and EV-A71 and no activity below 50uM to date. We expect this program to increase throughput in the Antiviral Core in the near future.

**DENV/ZIKV NS2B/3 Protease Program**

|  | DENV-2 Huh7 | | DENV-2  VeroE6 | |
| --- | --- | --- | --- | --- |
| Molecule Name | **EC50 (µM)** | **CC50 (µM)** | **EC50 (µM)** | **CC50 (µM)** |
| Mosnodenvir | < 0.01 | 5 |  |  |
| ASAP-0016305 | < 0.01 | 16 | 2.54 | 4.99 |
| ASAP-0016436 | < 0.01 | 19 | 1.82 | > 10.0 |
| ASAP-0016843 | > 1.90 | 1.9 | 2.75 | 8.95 |
| ASAP-0013916 | > 1.90 | 1.9 | 0.677 | < 0.08 |
| ASAP-0016818 | > 3.10 | 3.1 | 8.64 | 6.62 |
| ASAP-0015919 | 5.3 | 15 | 3.46 | 7.73 |
| ASAP-0016815 | > 5.50 | 5.5 | 1.31 | 4.32 |
| ASAP-0016554 | > 6.10 | 6.1 | > 10.0 |  |
| ASAP-0014782 | 6.2 | > 32.0 | > 10.0 | > 10.0 |

Another classical protease target that ASAP is pursuing with a fragment-to-lead campaign is the flavivirus NS2/B3 protease. The Antiviral Core has established live-virus cell-based for ZIKV (PRVABC59) and DENV-2 (16681). These assay uses an 8-point dose-response of ASAP compounds added to Vero-TMPRSS2 cells 2 hours prior to infection with ZIKV/DENV-2 under BSL2 conditions. 24 hours (ZIKV) or 6 days (DENV-2) later, the infected cells are fixed and stained with flavivirus E-specific monoclonal antibodies, and infected cells are quantified over total cells (DAPI) using our Cytation 1 plate cytometer. A cytotoxicity assay (MTT) is run in parallel in uninfected Vero-TMPRSS2 cells for all compounds. The assay was then standardized by generating EC50s for known flavivirus inhibitors and comparing with published results for consistent potency values. The Antiviral Core has worked with the laboratory of Johan Neyts at KU Leuven to provide the planned back-up CPE-based screening capacity for DENV-2 in Vero E6 cells to further confirm the accuracy of our results. The Antiviral Core has also engaged with NIH preclinical services (PCS, through the laboratory of Hong Wang at Utah State University) for another CPE based DENV-2 antiviral assay in Huh7 cells.

The flavivirus NS2/B3 protease program has just progressed from biochemical screening to cell-based assays and initial hits have been identified with single-digit micromolar EC50s against DENV-2 **(Table 4)**. The mosnodenvir (JNJ-1802) control had a < 10 nM EC50, which is comparable to the literature reported EC50. There are some discrepancies between the initial NIH PCS and and KU Leuven data sets, repeats will be performed at both sites to bring the data in congruence. Furthermore, these compounds are now being screened in the Antiviral Core live-virus assays for DENV-2 and ZIKV, with the goal of broad-spectrum anti-flavivirus activity.

**Primary Cell Model Development**

The Antiviral Core has also been tasked with the development of high-throughput primary cell models to meet the needs of all ASAP programs. The Antiviral Core now has coronavirus, enterovirus, and flavivirus programs entering our live-virus antiviral assays, and primary cell models will be needed for comprehensive potency analysis of each program as leads approach proof-of-concept animal studies. Furthermore, the Antiviral Core hosts an AViDD Developmental Project Award (led by Thomas Zwaka from Mt Sinai) to develop multiple primary-like iPSC models, representing the many important cell types infected by these target viral families, in 96-well format for medium-throughput antiviral screening (See the Zwaka Developmental Award Report for more details on the generation of these models). The Zwaka laboratory regularly produces iPSC-derived pneumocytes for SARS-CoV-2 antiviral screening in the Antiviral Core, which has been well-established over years of collaboration. Over the first year of the developmental award, the Zwaka laboratory has produce iPSC-derived hepatocytes, macrophages, and neurons in 96-well format for the establishment of IF-based antiviral assays. The Antiviral Core has established IF-based assays for coronavirus **(Fig. 3A)** and enterovirus **(Fig. 3B)** in these 3 iPSC cell types. We generally detected infection of hepatocytes and macrophages ranging from 9-24% infection, which are appropriate for robust medium-throughput assays. EV-D68 was able to infect the iPSC-derived neurons at up to 4%, which is on the borderline of robustness for an effective medium-throughput antiviral assay. SARS-CoV-2 did not show detectable infection of the neurons, which is in line with the 0.1% infection observed in the literature. We are now expanding these observations to flaviviruses to ensure we have assays prepared for all ASAP programs. The Antiviral Core has also established EV-D68 infection of iPSC-derived pneumocytes and validated our rupintrivir control in this assay **(Fig. 3C)**.A collage of graphs and charts

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**C. Significance**

**ASAP has established impressive *in vitro* potency and prophylactic *in vivo* efficacy against MERS-CoV in the 8HUM mouse model, which validates the AI-guide fragment-based approach to antiviral drug discovery. The significance of this cannot be overstated, our academic consortium has delivered a potential broad-spectrum coronavirus clinical candidate within the first 2 years of funding.** This paradigm will be followed for all ongoing ASAP programs, while improving efficiency.

The Antiviral Core has established and standardized antiviral assays that have been critical for driving the SAR for the ASAP enterovirus and flavivirus targeted programs. This has led to the identification of initial hits for both programs with micromolar and sub-micromolar potency. Furthermore, the Antiviral Core has developed primary-like iPSC medium-throughput screening assays for coronavirus, flavivirus, and enterovirus. These iPSC-derived cell lines were differentiated into multiple lineages to establish the screening models in cell types that are relevant to the pathogenesis of each virus. The cell types developed by the Antiviral Core in collaboration with the Zwaka laboratory, through the AViDD Developmental Award, were pneumocytes (coronavirus, enterovirus), hepatocytes (coronavirus, flavivirus, enterovirus), macrophages (coronavirus, flavivirus, enterovirus), and neurons (coronavirus, flavivirus, enterovirus). We believe with these assays in place, the AER can now produce antiviral potency data sets in relevant primary-like cell types to reflect the potency of the compounds most accurately at the relevant site of viral infection for all ASAP targeted viral families. We believe that potency values from these iPSC models will be critical in determining the dosage levels used as we progress into animal models for all ASAP projects.

The ASAP consortium and the AER have thoroughly explored the macrodomain target for coronaviruses in multiple biochemical and cell-based assays. Through this work, the crystallographic, biochemical and cellular assays correlating the impact of Mac1 inhibition on viral replication and the ADP-ribosylation states of infected cells will be of great interest to the antiviral drug discovery field. The ASAP consortium is in the process of publishing this work so that our learnings on this target can be widely distributed. Furthermore, the Antiviral Core will continue to explore cell-based models capable of driving the SAR of the macrodomain program, at which time a restart of the program can be determined by the ASAP membership.

The continued implementation and improvement of the automated liquid handling methods will have a large impact on the through-put and consistency of the AER data and therefore will allow the AER to continuing to improve data quality and turn-around for the entire ASAP consortium as more antiviral programs come online.

The establishment of the 8HUM model is critical for addressing one of the major weaknesses of the ASAP animal model pipeline, reliance on murine models and therefore exposure to mouse-specific PK issues. The availability of this model will have a major impact on our ability to navigate the potential for poor mouse-specific PK, which is now being translated to the ASAP MERS-CoV Mpro program.

**D. Plans**

**Selection of a coronavirus Mpro targeted preclinical candidate**

With the successful proof-of-principle MERS-CoV animal efficacy study with the ASAP lead Mpro inhibitor ASAP-0016506. The Antiviral Core will continue work closely with Projects 5 and 6 as we select a final preclinical candidate from a set of compounds with similar properties to ASAP-0016506. The AER plans to run multiple coronavirus animal models in year 3 to determine the therapeutic dosing potential and broad-spectrum activity of the ASAP lead Mpro series.

**Improvements in throughput and cycle times**

The end-to-end automation in 96-well format has allowed the Antiviral Core to boost antiviral efficacy throughput from ~20-30 compound batches up to 60 compound batches. We plan further improvements in our antiviral assay throughput as we miniaturize down to 384-well format, which now can be accomplished with end-to-end automation complete. Over the next year the AER plans to incorporate full automation into all antiviral assays run at the BSL2 level. The Antiviral Core will then establish a fully automated 384-well format antiviral assay for the attenuated rSARS-CoV-2 Δ3a/Δ7b virus. We will work to establish conditions and automated protocols that will yield the necessary z’-factors and increase throughput from 60 compounds up to 200 compounds per run. As more projects progress from Fragment-to-Lead to Lead Optimization, the Antiviral Core will require this throughput to ensure all ASAP projects has cycle times for efficient Lead development.

**Antiviral Resistance Analysis**

The Antiviral Core received direction from the NIH and AViDD program that antiviral resistance analysis for SARS-CoV-2 should be restricted to established attenuated strains of the virus. This guidance caused the Antiviral Core to take our antiviral resistance pipeline using SARS-CoV-2/WA1 offline as we obtained the rSARS-CoV-2 Δ3a/Δ7b virus, obtained permission from the NIH to work with this virus under BSL2 conditions, and established infection and resistance passaging condition for our analysis. This pipeline has been re-established with the rSARS-CoV-2 Δ3a/Δ7b virus, and the Antiviral Core plans to generate resistance profiles for lead ASAP compound series over the next year using this pipeline.

**Antiviral Efficacy Animal Model Development**

The establishment of the 8HUM model is critical for addressing one of the major weaknesses of the ASAP animal model pipeline, reliance on murine models and therefore exposure to mouse-specific PK issues. The availability of this model will have a major impact on our ability to navigate the potential for poor mouse-specific PK. The Antiviral Core will continue to explore the suitability of this mouse model across the enterovirus and flavivirus families. This will be confounded against viruses like DENV-2, ZIKV, EV-A71, and EV-D68, which have previously been established in IFN-deficient mouse models. We will explore options to improve viral infection in this IFN-competent mouse model, such as antibody-mediate pathogenesis observed in DENV-2 mouse models.