**B. Studies and Results**

**B.1 Publications**

Publications from Project 1 laboratories in Year 2 supported by AViDD funding:

* Doherty JS and Kirkegaard K (2023). Differential inhibition of intra- and inter-molecular protease cleavages by antiviral compounds. J Virol. 21: e0092823. PMID 38047713.
* Bloom JD and Neher RA (2023). Fitness effects of mutations to SARS-CoV-2 proteins. Virus Evolution 9: vead055. PMID: 3772875.
* Hannon WW and Bloom JD (2023). dms-viz: Structure-informed visualizations for deep mutational scanning and other mutation-based datasets. bioRxiv. https://doi.org/10.1101/2023.10.29.564578.
* Flynn JM, Huang QYJ, Zvornicanin SN, Schneider-Nahum G, Shaqra AM, Yilmaz NK, Moquin SZ, Dovala D, Sciffer CA and Bolon DNA (2023) Systematic analyses of the resistance potential of drugs targeting SARS-CoV-2 main protease. ACS Inf. Dis. 9:1372-1386. PMID: 37390404.

**The two genetic approaches by which Project 1 assists the ASAP group** to design antivirals that are robust to the development of drug resistance are:

* To determine the fitness cost of mutations at each residue of target proteins, with the goal of guiding the choice of binding sites for antiviral compounds so that drug-resistant mutations will enact high fitness costs (former Specific Aim 3).
* To choose antiviral targets for which antivirals are most likely to function via sub-stoichiometric inhibition, with the effect that drug-resistant progeny will not be subject to genetic selection during the first round of replication due the presence of drug-susceptible progeny within the same cell. Examples include:  
  1. Viral proteases that target the polyproteins of positive-strand RNA viruses. Failure to make such cleavages can lead to the persistence of dominant inhibitory precursors (former Specific Aim 1)   
  2. Oligomers of viral proteins, because defective subunits can inhibit the functions of non-defective subunits in chimeric complexes (former Specific Aim 2).

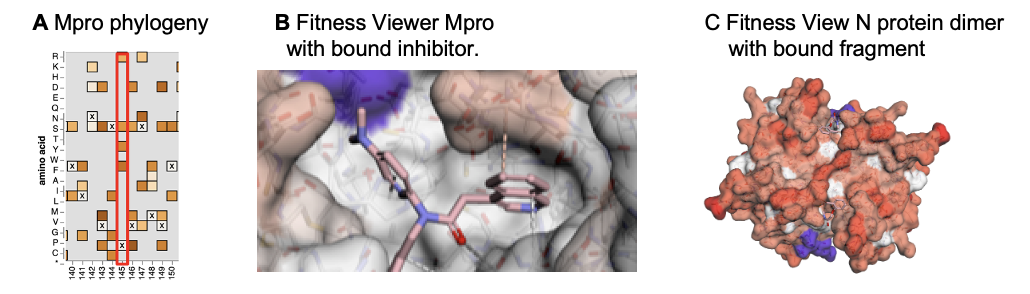
***3CL protease (Mpro) and nsp3 Macrodomain1 (Mac1) of SARS-CoV-2***

*Deep mutational scanning of Mpro in S. cerevisiae.*

Before the commencement of ASAP funding, the laboratory of Dan Bolon had expressed CoV-2 Mpro in *S. cerevisiae*, with a functional readout of its catalytic activity. ASAP funding supported deep mutational scanning experiments in the presence and absence of antiviral compounds (PMID: 37390404). These data were of interest and showed that the mutations that conferred relative resistance to nirmatrelvir and ensitrelvir did not completely overlap. However, we decided as a consortium that the approach of **expressing viral proteases in yeast was not a method stringent enough for our purposes.** Specifically, the expression in yeast made it difficult to achieve potent concentrations of antivirals in early stages of development. In addition, the genetic selection for Mpro function in yeast was only its protease activity, not any of its other functions within the polyprotein.

*Phylogenetic analysis of six million SARS-CoV-2 sequences*.

Evolutionary conservation is not as effective as deep mutational scanning at identifying ALL possible amino acids that can function at any given site in a protein, because evolution does not sample sequences as completely. In addition, evolutionary analyses are prone to over-representing some sequences due to bottleneck effects. These problems could be partially circumvented in the case of SARS-CoV-2 due to the **unprecedented number of individual sequences determined**. Bloom and Neher (PMID: 3772875) mined these sequences for all observed variants and, by quantifying the associated mutations for each new sequence variants, subtracted the effects of bottlenecks on the representation of newly occurring mutations in the population. This analysis has generated 'logo plots' that reflect the relative fitness of each amino acid mutation throughout the SARS-CoV-2 genome for those amino acid changes sampled by evolution; sequences in Mpro surrounding the active-site Cys are shown (Fig. 1A).

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**Figure 1. Phylogenic analysis of SARS-CoV-2 proteins**. A) Visualization of predicted fitness of wild-type sequences (X) and substitutions shown at left from Bloom and Neher (PMID: 3772875). Darker colors show decreasing fitness. Red box highlights Cys145, the active-site Cys. Substitutions observed in the six million sequences are shown. Sequence is more sparsely sampled than in a deep mutational scanning experiment due to the redundancy and organization of the genetic code. Deeper colors show decreasing fitness; substitutions in active-site Cys are higly unift. Those that are observed are likely to be background due to sequencing error or noninfectious RNAs. <https://jbloomlab.github.io/SARS2-mut-fitness/> C) Fitness viewer of dimeric C-terminal oligomerization domain of SARS-Cov-2 nucleocapsid (N) protein bound to one of the chemical fragments in the structural screen. https://a7e363c69a86a9f51702101656b2932994f870c5ddfeed52d1214bcaaacc.s3.amazonaws.com/demo\_for\_jenke/frag\_fitn ess\_view.html.

*Development of Fitness Viewer*

To facilitate interpretation of these data, Dr. Jenke Scheen (Computational Core) has implemented an ingenious interactive approach to render fitness data intelligible in the context of protein structure. Examples are shown in Figure 1, in which a known SARS-CoV-2 Mpro inhibitors (Fig. 1B) and a fragment from the ASAP structural screen (Fig. 1B) are shown as screen shots in the interactive viewer. The Mpro inhibitor constacts primarily mutationally intolerant regions (white). The N-protein -binding fragment, on the other hand, contacts more mutationally tolerant residues (increasing red coloration with increasing number of tolerated residues. The detailed contacts can be seen on interactive viewers.

***Nsp3 Macrodomain 2 (Mac1) of SARS-CoV-2 and MERS***

*Construction of mutation in glycosyl transferase active site*

The laboratories of Brett Lindenbach and Farren Isaacs (Yale) devised a reverse genetics scheme in which SARS-CoV-2 virus could be launched from a yeast artificial chromosome in DNA repair-deficient yeast. Mutations could then be introduced via the addition of oligonucleotides that serve as primers for DNA replication and can thus put in focused bursts of mutagenesis. One of the viruses they constructed destroyed the glysosyl transferase active site of Mac1. RNA encoding this virus was sent to the ASAP Virology Core to serve as a **negative control for the Mac1 program**. This virus was shown to be highly attenuated in animal models, but not in cultured cells (manuscript in preparation). Unfortunately, the passing of PI Brett Lindenbach in January 2024 has prevented the further work planned, deep mutational scanning of EV-A71 and EV-D68 proteases.

***3C protease of Enterovirus A-71 and Enterovirus-D68***

Medicinal chemists in ASAP and elsewhere optimize protease inhibitors using fluorogenic peptide substrates. For the proteases of positive-strand viruses, however, the protease targets are often within polyproteins, and can even occur exclusively intramolecularly, as we have shown for the 3C protease of EV-D68 (Fig. 2A, B). Thus, it is important to demonstrate that inhibitors can block the cleavage of natural substrates. As shown below for rupintrivir, a potent 3C inhibitor that has not been successfully developed as a pharmaceutical, intramolecular cleavage of the 3ABC precusor to 3AB and 3C is inhibited by rupintrivir with an IC50 of approximately 200 nM (Fig. 3CD). As potent 3C inhibitors are developed by ASAP, Project 1 will use this assay to test both inhibition of intra-molecular 3AB cleavage and of control substrates known to be cleaved inter-molecularly to determine which activities correlate with viral inhibition.

A graph with red lines and numbers

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**Figure 2. Assay to determine effectness of ASAP 3C inhibitors on intramolecular polyprotein cleavage**. A) Enteroviruses A71 and D68 genomes are translated as a single polyprotein that is processed by 2A and 3C protease to individual proteins. (B) Pulse-chase exeriments demonstrated that the cleavage of EV-D68 precursor 3AB is dilution insensitive and, thus, intra-molecular. (C, D) Intra-molecular cleavage of 3ABC can be inhibited by 3C inhibitor rupintrivir with an IC50 of approximately 200 nM (PMID 38047713). Such pulse-chase assays will be used to assist ASAP in determining which inhibitory activities best correlate with viral inhibition.

***NS2B-NS3 protease of Dengue and Zika viruses***

The flavivirus viral protease was chosen as an ideal target for the ASAP consortium due to the pre-existing expertise in viral proteases and the potential for sub-stoichiometric inhibition that results from the dominant inhibition provided by uncleaved precursors (PMID: 30228122). This is an excellent opportunity to test the hypothesis that small molecules that inhibit the intra-molecular cleavage of NS2B-NS3 that allows the accumulation of a dominant inhibitor will be the most potent inhibitors of viral growth. Assays to test this hypothesis are described below.

*Deep mutational scanning of protease domain of Zika NS2B-NS3.* The laboratory of Dr. Matthew Evans was funded by ASAP to perform deep mutational scanning on Zika NS2B-NS3 protease. This work was performed by postdoctoral fellow Dr. Blake Richardson in collaboration with Jesse Bloom (manuscript in preparation). Figure 3A shows a single view of the Fitness Viewer analysis of these DMS data in the context of the NS2B-NS3 complexed with a bound fragment. That this DMS work was performed at the same time as the fragment screen in Project 2 has provided the novel opportunity for the viral geneticists, structural biologists and medicinal chemists to work together to optimize NS2B/3 inhibitors. Project 1 is currently working with Dr. Nathaniel Kenton of Project 3 to test novel NS2B-NS3 inhibitors for their effects on polyprotein cleavage.

A close-up of a person's face

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**Figure 3. NS2B/3 Fitness Viewer and assays for dengue virus polyprotein cleavage.**  A) Fitness Vewer of NS2B-NS3- binding fragment. B) Pulse-chase analysis of cleavage patterns of NS2B/NS3/NS4A polyprotein in the absence and presence of f NS2B-NS3 activity designed in Project 3 by Nathaniel Kenton. Control patterns can be seen in the DMSO lane and in the indistinguishable lanes 2 and 4, which display reactions containing ASAP-0014875 and ASAP-0015372, respectively. Lanes 1, 3 and 6, displaying reactions that contained ASAP-0015682, ASAP-0013916 and ASAP-0016829, respectively, are lacking the band corresponding to uncleaved NS2B/NS3 and an increase in the full-length band, arguing that these molecules inhibit cleavage at the NS3-4A junction. In lane 5 (ASAP-0015919), several cleaved products are reduced, including that resulting from the internal cleavage of NS3. Most compounds were present at 500 μM. This assay will be further optimized to determine which patterns of cleavage inhibition best correlate with viral inhibition.

***2A proteases of Enterovirus A-71 and D-68***

The 2A proteases of enteroviruses are excellent antiviral targets because the dominant inhibition of viral growth by accumulated uncleaved precursors offers the opportunity for sub-stoichiometric inhibiton. As with other polyprotein-cleaving proteases, however, it is not always the case that complex substrates are as effectively inhibited as the model substratres used in antiviral discovery. As with 3C and NS2B/3 proteases, the Kirkegaard laboratory has established pulse-chase analyses to monitor the intra-molecular cleavages of 2A proteases. Figure 4 shows that telaprevir, a known inhibitor of EV-D68 growth and of 2A protease activity, is a more effective inhibitor of the inter-molecular cleavage of host protein eIF-4G than of viral polyprotein cleavage.

A diagram of a protein

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**Figure 4. Pulse-chase analysis of telaprevir inhibition of polyprotein and host protein substrates of EV-D68 2A protease.** (A) To assay the intra-molecular cleavage between VP1 and 2A that is the only 2A substrate in the viral polyprotein, the VP1-2A polyprotein was made in translation extracts. Processing of VP1-2A was monitored by pulse-chase and of host protein eIF-4G by immunoblot. (B). The percentage of substrate cleaved in the presence of increasing amounts of telaprevir was determined.

A guiding hypothesis of these studies is that measuring the inhibition of viral proteases on viral substrates will contribute to the selection of optimal inhibitors by the ASAP consortium. Now that inhibitory compounds of enteroviral 2A proteases are available from Project 4, they will be tested in the assays described here to identify those most likely to inhibit polyprotein cleavage.

**C. Significance**

New medicines directed against RNA viruses are desperately needed, both for those that are current threats to human health such as flaviviruses and enteroviruses and those that are threats for future pandemics. Successful, lasting antiviral effectiveness will require the ability to suppress the outgrowth of drug-resistant viruses, either by combination therapy or more novel approaches. The goal of Project 1 is to bring understanding of viral genetics to the earliest stages of antiviral discovery. The choice of positive-strand RNA viruses was made due to their obvious threats to human health. In addition, their relatively facile genetics and plentiful sequence data has allowed us to test two ideas for the suppression of drug resistance.

We are testing whether a systematic approach to exacting a fitness cost to drug resistance can be effective. By analyzing the millions of SARS-CoV-2 variants, the most thorough phylogenetic analysis of any virus has been made possible. Furthermore, deep mutational scanning of one of the ASAP targets, Zika virus NS2B-NS3, has allowed relative fitness to be determined for each mutation at each amino acid. Using a novel computational method, Fitness Viewer to allow medicinal chemists to design molecules that deliberately target mutationally invariant residues, we predict that a systematic approach to fitness cost is being developed and will lead to compounds with high barriers to drug resistance.

The choice of positive-strand RNA viruses as targets also allows the deliberate inhibition of a unique aspect of their biology: polyprotein processing. Given that the cascade of proteolytic events is the major form of gene regulation in these viruses, it is not surprising that uncleaved precursors can be potent inhibitors of subsequent stages of viral infection. By deliberately inhibiting intra-molecular polyprotein cleavages, we plan to cause the accumulation of such precusors. Project 1 has devised assays for the intra-molecular cleavage of enteroviral and flaviviral proteases and will use these assays to illuminate those inhibitory events that best correlate with both the inhibition of viral growth and the suppression of drug resistance due to the creation of dominant suppressors of growth of all viruses.

The Kirkegaard laboratory is now completely focused on using the principles of viral genetics in collaborative work to reduce the spectre of drug resistance in antiviral development. In Project 1, an interdisciplinary team to systematically invoke fitness costs and the sub-stoichiometric inhibition that can lead to the genetic dominance of drug-susceptible viruses has been assembled to achieve this goal in collaboration with the talented structural and chemical biology ASAP team.

**D. Plans**

**1. For dengue and Zika virus,** we will contribute to the ASAP effort to develop effective inhibitors by:

A. Assay the different chemotypes of NS2B/3 protease inhibitors discovered in ASAP for their ability to inhibit polyprotein cleavages (Fig. 3). The specific patterns of protease inhibition will be tested for correlation with viral inhibition, as assayed by the Virology Core. **We anticipate that, as we have observed previously, inhibition of the internal NS3 cleavage is the most potent approach to viral inhibition for dengue serotype 2 virus.**  This will be tested for the novel inhibitors developed by ASAP as well as for other dengue serotypes and for Zika virus.

B. For viral polyprotein precursors found to be responsible for sub-stoichiometric cleavage, we will determine the inhibited step by time-of-addition experiments and single-cycle infections.

C. Selecting for compound-resistant viruses and determining whether their fitness could be predicted by Fitness View analysis. These experiments will be performed at low multiplicities of infection to assess the genotype-phenotype relationship for each virus.

D. Determine whether the presence of selection for compound-resistant variants can be suppressed by the presence of compound-susceptible variants by passage at higher MOIs or from deliberate co-infections to mimic the mileiu of a newly minted mutant genome within an infected cell.

**2. For enteroviruses A-71 and D-68,** we will perform similar experiments to assay the different chemotypes of 2A inhibitors discovered in ASAP for their ability to inhibit the intra-molecular cleavage of the VP1/2A precusor (Fig. 3), known to be a dominant inhibitor of viral growth. We will test the effects of 3C inhibitors identified by ASAP on cleavages of 3ABC-containing polyproteins (Fig. 4) and determine which sites of inhibition correlate with viral inhibition and test whether the predicted losses of fitness and dominant inhibition were accomplished.

**3. Utilize and distribute Fitness Viewer** as a method to render viral genetic data more useful to structural biologists and medicinal chemists. We have recently initiated a collaboration with the Rice laboratory (Rockefeller University) to accomplish this goal in their deep mutational scanning studies of the hepatitis B polymerase.