High Throughput Screen to Identify Non-Nucleoside Small Molecule Inhibitors of SARS-CoV-2 RNA-dependent RNA polymerase

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BACKGROUND

- Developing direct-acting antivirals against SARS-CoV-2, the causative agent of COVID-19, is important for preventing severe disease.
- Remdesivir, a nucleoside analog targeting the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) has been successfully used for the treatment of hospitalized or high-risk patients with COVID-19 but its utility is limited by the parenteral delivery route.
- Identification of non-nucleoside RdRp inhibitors could be useful additions to the SARS-CoV-2 treatment arsenal.
- Here, we describe a high throughput screen to identify non-nucleoside inhibitors of the SARS-CoV-2 RdRp. A library of ~400,000 small molecules was screened for RdRp inhibition which resulted in identification of a novel class of nsp12-nsp8 disrupting small molecules.

METHODS

Materials. Recombinant SARS-CoV-2 nsp7 and nsp8 were expressed and purified from *E. coli*. Recombinant SARS-CoV-2 nsp12 was expressed and purified from Sf21 cells. RdRp activity was determined using the AmNS-UTP utilization assay which included an RNA primer (5'-GCUAUGUGAGAUUAAGUUAU-3') and an AmNS RNA template (5'-$(A)_{20}$UGCUGCAUAACUUAAUCUCACAUAGC-3'). The Kinase-Glo assay RNA template was 5'-$(U)_{30}$AUAACUUAAUCUCACAUAGC-3'.

AmNS-UTP utilization. RdRp activity assays were assembled in 1536-well plates (6 µL) for HTS or 384-well (20 µL) plates for hit confirmation. Reactions were incubated at 30ºC for 3 hours and readout with excitation/emission 340/460 nm. Assays contained 50 mM Tris HCl (pH 8), 10 mM KCl, 0.01% Triton X-100, 1 mM DTT, 6 mM $MgCl_2$, 20 µM ATP, 20 µM CTP, 20 µM GTP, 13 µM AmNS-UTP, 500 nM primer/template pair, 500 nM nsp7, 1000 nM nsp8, 100 nM nsp12.

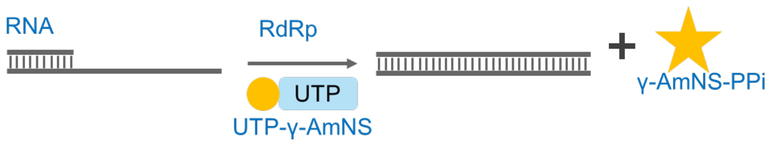

Figure 1. AmNS-UTP RdRp assay schematic.

Kinase-Glo assay. RdRp activity assays were assembled in 384-well (15 µL) plates, incubated at 30ºC for 30 minutes followed by luminescence detection on an Envision plate reader after addition of Kinase-Glo (Promega). Assays contained 50 mM Tris HCl (pH 8), 10 mM KCl, 0.01% Triton X-100, 1 mM DTT, 6 mM MgCl2, 5 µM ATP, 500 nM primer/template pair, 500 nM nsp7, 1000 nM nsp8, 100 nM nsp12.

Figure 2. Kinase-Glo RdRp assay schematic.

RESULTS

High throughput screen summary

- 400K • ~400,000 compounds from library screened at 30 µM
- 5143 • 5143 confirmed hits at 30 µM
- 857 • 857 compounds selected for dose response
- 233 • 624 compounds removed due to purity rating, activity in intercalator counter-screens, redox activity, or assay interference
- 127 • 106 compounds removed after final visual inspection identified undesired substructures, low quantitative estimate of drug-likeness (QED), or high potency efficiency index (PEI)
- 94 • Cmax >50% filter followed by efficiency driven selection afforded 94 compounds for powder confirmation - lipophilic efficiency (LiPE) & ligand efficiency (LE)

Figure 3. Screening funnel from library to compounds selected for hit confirmation.

Hit confirmation

IC_{50} range (µM)	# compounds AmNS	# compounds Kinase-Glo
<10	11	13
10-30	13	18
30-50	10	12
50-75	8	1
75-100	5	2
Total compounds with confirmed activity	47	46

Table 1. Number of compounds with IC_{50}s <10, 10-30, 30-50, 50-75, and 75-100 µM in AmNS and Kinase-Glo RdRp assays. Compounds used were prepared from fresh powder.

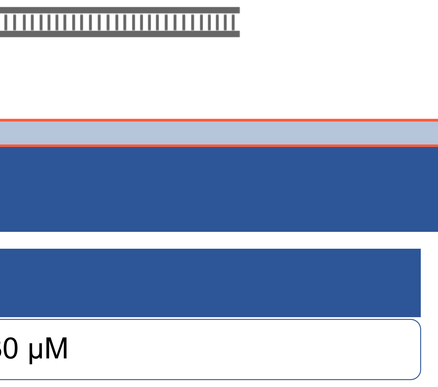

Figure 4. Correlation of pIC_{50}s of Kinase-Glo vs. AmNS RdRp assays.

RESULTS

12 compounds disrupt nsp12:nsp8 protein:protein interactions

Structural cluster	Compound ID	IC_{50} fold-shift with increasing: nsp8	nsp7	RNA	NTP
A	EPS-1604	5.5	1.6	0.9	0.53
	EPS-1970	6.9	1.1	0.7	0.65
B	EPS-1731	6.6	1.1	1.0	0.60
	EPS-1975	5.8	1.1	0.8	0.63
C	EPS-1978	11.0	1.6	0.9	0.59
	EPS-1979	12.0	2.2	1.4	0.51
	EPS-1980	>5.3	1.5	2.2	0.51
Individual hits	EPS-1955	13.2	1.3	0.7	0.61
	EPS-1956	3.4	1.4	0.8	0.78
	EPS-1605	4.3	1.4	0.7	0.61
	EPS-1984	1.7	1.3	1.5	0.80
	EPS-1987	NA	NA	1.1	0.58
	EPS-2000	>1.4	1.6	1.2	NA
	EPS-2003	>8.3	1.1	1.2	0.62
	EPS-2004	13.2	1.5	1.1	0.63
RNA competitive control	suramin	1.2	1.3	5.0	0.66
NTP competitive control	3' dGTP				>12

Table 2. IC_{50} fold-shifts in RdRp biochemical assays under different concentrations of nsp8 (1 or 8 µM), nsp7 (0.125 or 1 µM), RNA template (0.5 or 5 µM), or NTPs (0.6 or 20 µM) for compounds with IC_{50}s <10 µM in at least one biochemical RdRp assay. Predicted fold shifts were ~5.7, 5.7, 5.7, and 22 for nsp8, nsp7, RNA, and NTP competitive inhibitors respectively. 12 compounds disrupt nsp12:nsp8 protein:protein interactions.

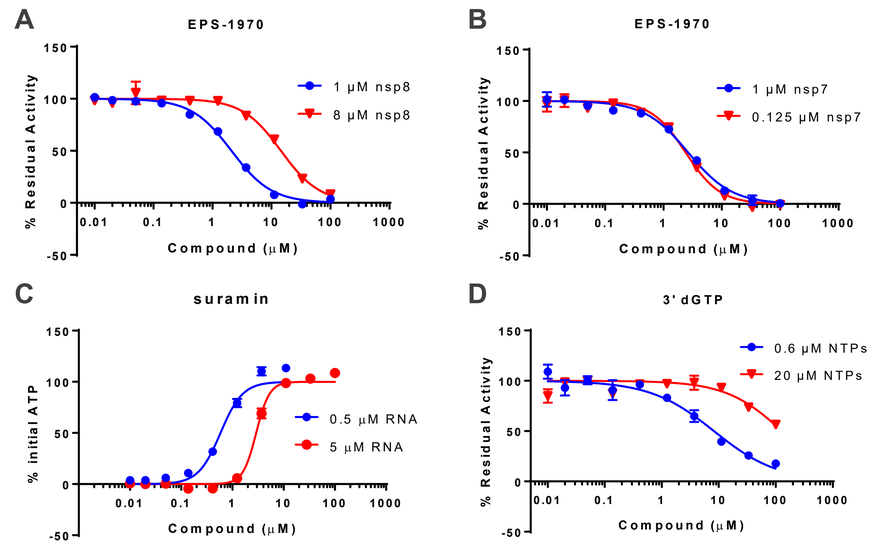

Figure 5. Representative curves of RdRp biochemical assays under different concentrations of (A) nsp8, (B) nsp7, (C) RNA, and (D) NTPs. IC_{50} shifts observed for EPS-1970 for with increasing nsp8, but not nsp7 suggest competition with the nsp12:nsp8 protein:protein interactions. IC_{50} shifts with suramin and 3' dGTP with RNA and NTPs respectively as expected for competitive inhibitors.

Compound ID	NanoDSF ΔTºC
EPS-1970	-3.5
EPS-1979	2
EPS-1987	-0.9
EPS-2000	2
EPS-2003	-2
suramin	-3.5

Table 3. Nano differential scanning fluorimetry with nsp12 and compounds. Five compounds destabilize or stabilize nsp12, suggesting binding to nsp12.

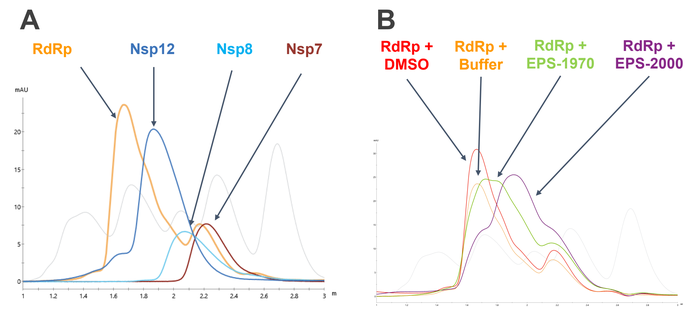

Figure 6. Analytical size-exclusion chromatography of (A) the RdRp complex, nsp12, nsp8, and nsp7 and (B) the RdRp complex with EPS-1970 and EPS-2000. EPS-1970 and EPS-2000 disrupt the RdRp complex.

CONCLUSION

- After counter-screens to remove RNA intercalators, redox cyclers, and compounds with undesirable medicinal chemistry properties, 15 compounds from 11 structural families with half-maximal inhibitory concentrations (IC_{50}s) <10 µM were selected for mechanism of inhibition studies.
- None of the compounds were RNA or NTP competitive inhibitors.
- Biochemical assays and analytical size-exclusion chromatography showed 12 compounds disrupted nsp12-nsp8 protein-protein interactions.
- Nano differential scanning fluorimetry analysis suggested 5 compounds target nsp12 directly.
- This high throughput screen identified multiple, structurally diverse, non-nucleoside SARS-CoV-2 RdRp inhibitors as potential starting points for hit optimization.

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DISCLOSURE: All authors are either current or former employees of Enanta Pharmaceuticals, Inc. and received salary and stock compensation.