In Vitro Characterization of Respiratory Syncytial Virus Inhibitors

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BACKGROUND

- Despite the recent success of prophylactic vaccines and monoclonal antibodies, there remains an unmet need for RSV therapeutic options for vulnerable patient populations
- Favorable clinical trial results of direct-acting antivirals (e.g., ziresovir, zelicapavir, and EDP-323) highlight diverse mechanisms of action (MoA) as potential avenues for therapy
- This study evaluates the in vitro post-infection efficacy and viral resistance profiles of fusion, N, and L inhibitors

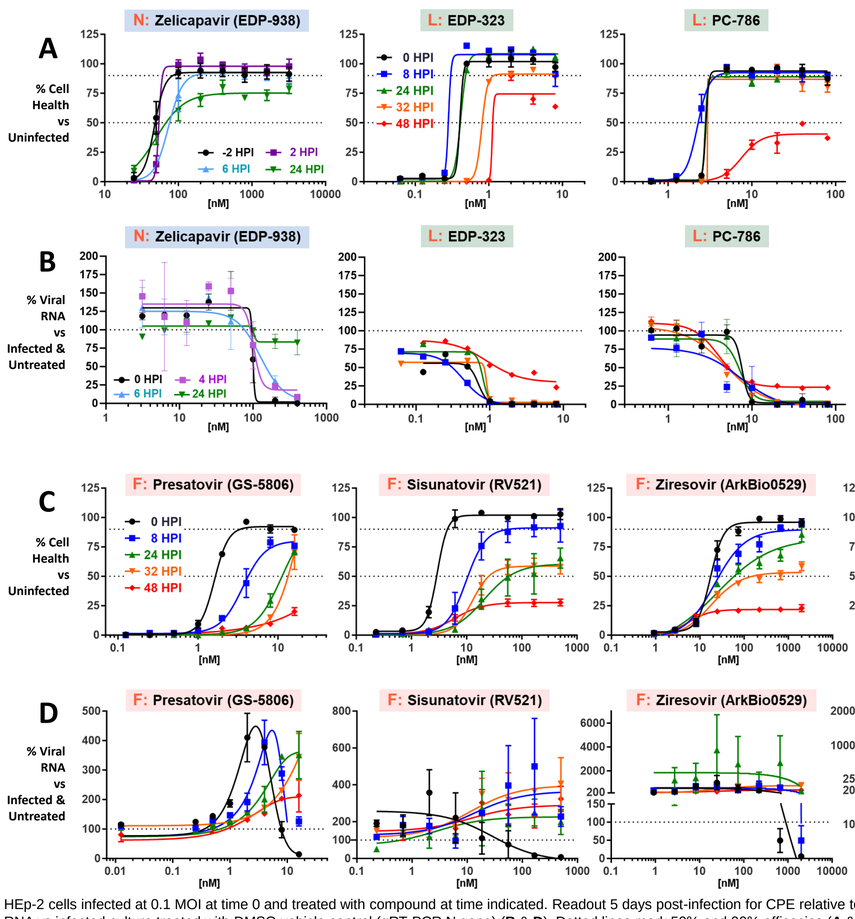
METHODS

- Time-of-addition assays assessed antiviral efficacy using cytopathic effect (CPE) (via ATPlite), viral RNA (via RT-qPCR N gene), protein (via WB), and by infectious virion production ($TCID_{50}$)
- Resistant viruses (^{R}) were generated by serial passage of RSV in HEP-2 cells in the presence of increasing compound concentrations. Viruses^R were full-genome sequenced
  - Reverse genetics was employed, and resistance was evaluated by EC_{50} shifts and viral fitness as measured by cytopathic effect, viral RNA, and infectious virion production rate

RESULTS

MoA Differences in Antiviral Effect when Administered Post-Infection

Figure 1: Fusion inhibitors generate excess viral RNA when dosed post-infection

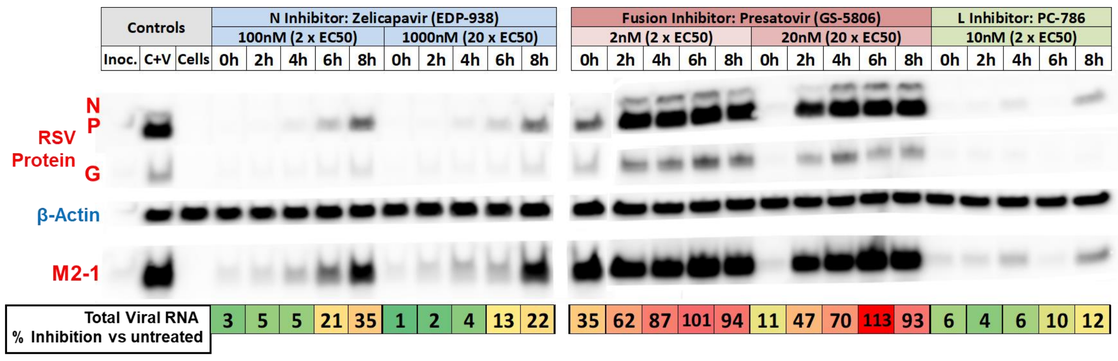


- N, fusion, and L inhibitors all prevent viral-induced CPE post-infection in a time-dependent manner
- N and L inhibitors also inhibit viral RNA production post-infection
- Fusion inhibitors increase viral RNA production over untreated levels when underdosed or administered post-infection

HEp-2 cells infected at 0.1 MOI at time 0 and treated with compound at time indicated. Readout 5 days post-infection for CPE relative to uninfected culture (ATPlite) (**A** & **C**), or % viral RNA vs infected culture treated with DMSO vehicle control (qRT-PCR N gene) (**B** & **D**). Dotted lines mark 50% and 90% efficacies (**A** & **C**), or RNA levels seen in DMSO-treated infected cultures (**B** & **D**).

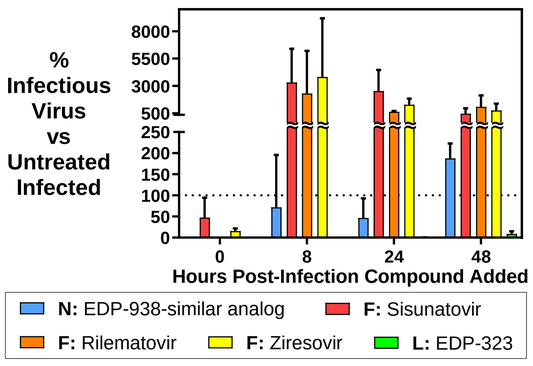
Figure 2: Post-infection N and L inhibitors suppress viral protein production while fusion do not

- The fusion inhibitor rapidly loses the ability to inhibit viral protein and RNA production
- N and L inhibitors inhibit viral replication post-infection



High MOI of 3 (~95% infection @ start), pre-incubated 1 hour at 4ºC (adsorption but not fusion), move to 37ºC (0h, fusion begins), compounds added at indicated times post-fusion. At 24h, washed wells 2x with media and then read using full-well RT-qPCR or discarded supernatant and collected for WB (~6ug total protein per lane loaded for WB except for inoculum which at max load was only 3ug). C+V = untreated cells + virus.

Figure 3: Unlike N or L, fusion inhibitors result in excess virions when administered post-infection



- Post-infection, fusion inhibitors generate increased amounts of infectious virions in vitro versus untreated cultures
  - Fusion inhibitors appear to keep infected cells alive by preventing lysis through syncytial formation, but do not prevent viral replication, leading to increased viral RNA and virion production versus untreated cells
- EDP-323 shows robust antiviral activity even at 48h post-infection

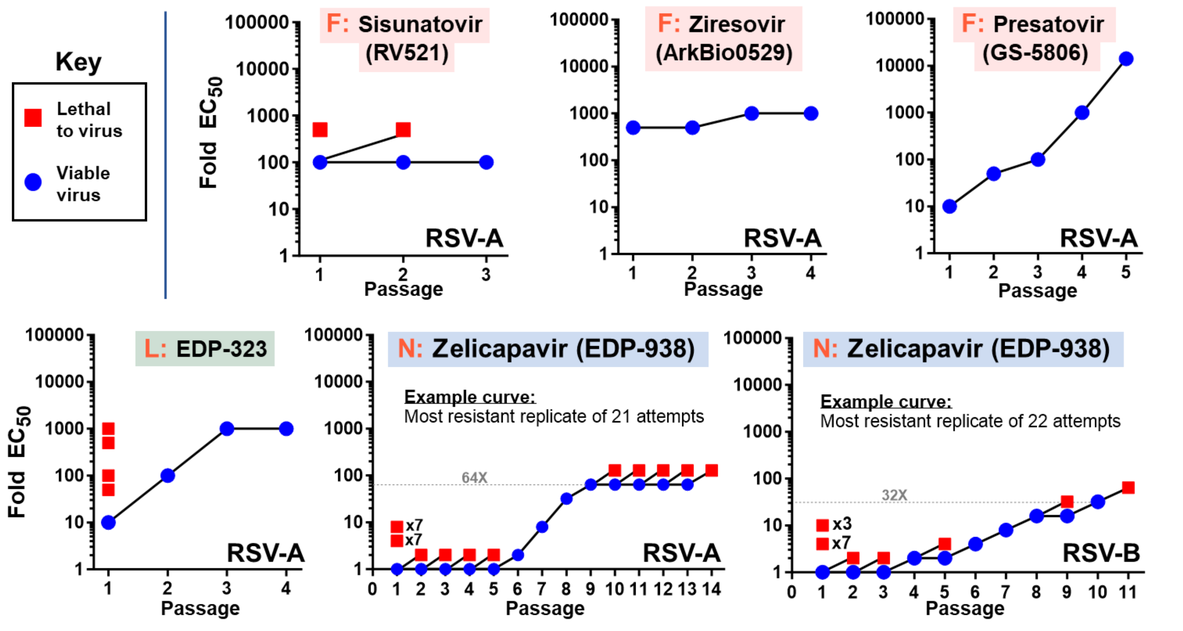
HEp-2 cells infected at an MOI of 0.1. Compound (100x EC_{50}) was added at indicated time. 5 days post-infection cultures were collected, and live virus was assessed by $TCID_{50}$. Data are mean ± SEM from 3 independent biological replicates. Dotted line depicts infectious virion levels in infected DMSO-treated control cultures.

RESULTS (cont.)

Drug Resistance Profiling

Figure 4: The N inhibitor zelicapavir demonstrates a very high barrier to resistance

- Fusion inhibitors rapidly develop breakthrough infection, demonstrating low barriers to resistance
- EDP-323 develops resistance quickly, but prevents resistance when dosed at ≥50X EC_{50}
  - Such drug exposure levels have been achieved in Ph.1 studies of EDP-323[1]
- N inhibitor zelicapavir displays a high barrier to resistance



All additional attempts to generate resistance to zelicapavir resulted in earlier loss of virus and/or lower fold EC_{50}s attained than are shown here.

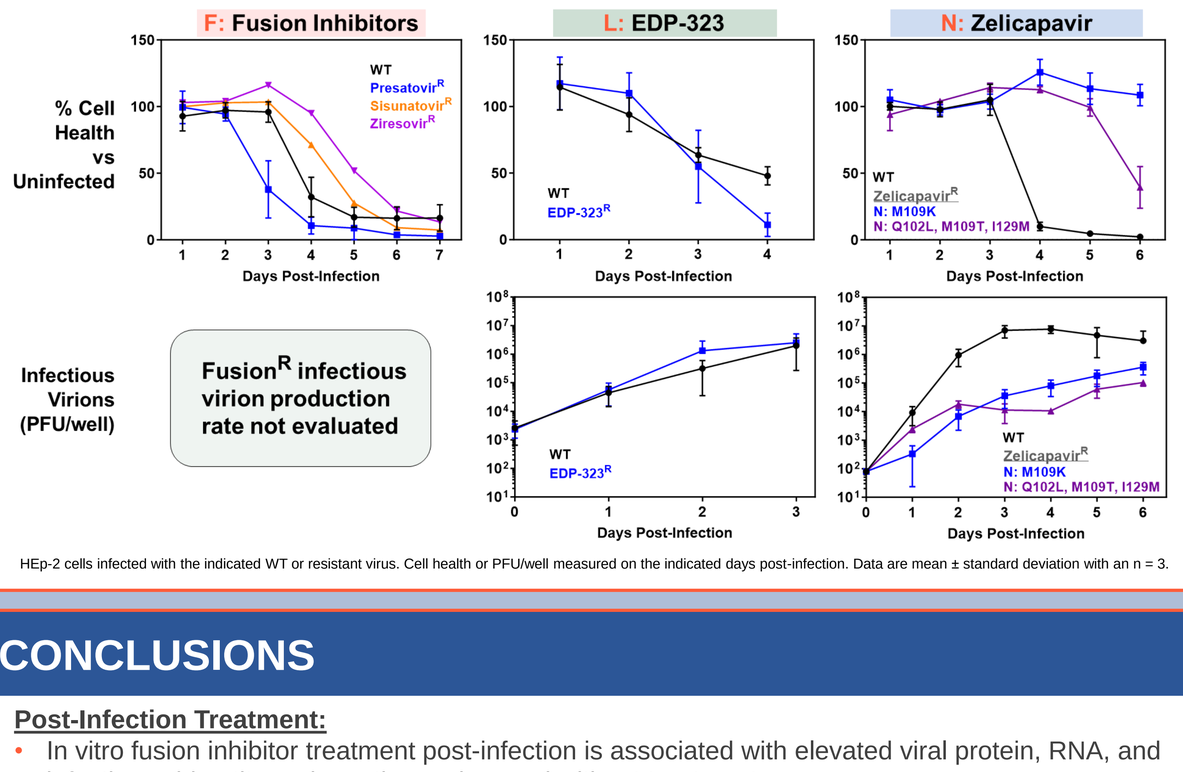
Table 1: Drug-Resistant (^{R}) Viruses Identified

	Fusion			L	N
	Sisunatovir^R	Ziresovir^R	Presatovir^R	EDP-323^R	Zelicapavir^R
Resistance (fold-shift)	**11,175**	**>13,628**	**>40,000**	**10,453**	**67 & 60**
Mutation	F: F488L	F: K394T	F: L141V+ N197T	L: L1372V+C1388G	N: M109K N: Q109L+M109T+I125M
Cross-Resistance	**Resistant to other fusion inhibitors** No cross-resistance with other non-fusion inhibitors			No cross-resistance with other inhibitors	

Fold-shift resistance based on HEp-2 culture at MOI 0.1 with CPE readout 5 days post-infection and compared to WT virus run in parallel. 8 additional zelicapavir^R mutations identified during 67 more rounds of selection[2]. 7 displayed potency shifts ranging from 3 – 7-fold, while 1 mutant had a 42-fold shift to zelicapavir. None of the 8 had cross-resistance to other compounds.

Figure 5: Fusion and L inhibitor^R viruses are fit, while N inhibitor zelicapavir^R viruses are not

- Fusion^R viruses vary in fitness from slightly more cytopathic than WT to slightly less so
- L inhibitor EDP-323^R virus maintains WT fitness levels
- N inhibitor zelicapavir^R viruses are heavily attenuated with reductions in CPE and virion production



HEp-2 cells infected with the indicated WT or resistant virus. Cell health or PFU/well measured on the indicated days post-infection. Data are mean ± standard deviation with an n = 3.

CONCLUSIONS

Post-Infection Treatment:

- In vitro fusion inhibitor treatment post-infection is associated with elevated viral protein, RNA, and infectious virion titers above those observed without treatment
  - Possibly due to reduced CPE, keeping cells alive but not halting viral replication in infected cells
- N and L inhibitors suppress replication post-infection, with L inhibitors improving on N inhibitors

Resistance Profiling:

- Fusion inhibitors have the lowest barrier, with little impact to viral fitness observed
- EDP-323 has a higher barrier to resistance compared to fusion inhibitors
- N inhibitor zelicapavir has the highest barrier to resistance and is associated with viral fitness defects

Take-away:

- These unique distinctions among fusion, N, and L inhibitors in antiviral effect and resistance profiles suggest benefits for N and L inhibitors over fusion inhibitors, and may translate to outcome differences in clinical trials and patient populations

REFERENCES

1) Mills, K. 2023. (17-20 September 2023). EDP-323, a First-in-Class, Once-Daily, Oral L-Protein Inhibitor for the Treatment of RSV: Results from a Phase 1 Study in Healthy Subjects and Correlation with In Vitro Antiviral Activity [Poster Presentation]. ESWI Influenza Conference; Valencia, Spain.

2) Rhodin, M., et al. *EDP-938, a novel nucleoprotein inhibitor of respiratory syncytial virus, demonstrates potent antiviral activities in vitro and in a non-human primate model.* PLoS Pathog. 2021 Mar 15;17(3):e1009428. doi: 10.1371/journal.ppat.1009428.

DISCLOSURES & ACKNOWLEDGEMENTS

- Rachel E. Levene, Nicole M. Kelly, Nalini Bisht, Joyce Sweeney Gibbons, Yat Sun Or, and Michael H. J. Rhodin are or were employees of Enanta Pharmaceuticals, Inc. and may be stockholders.

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