Obeldesivir and GS-441524 Antiviral Activity Against L Protein Mutants in Respiratory Syncytial Virus (RSV) Minigenome and Recombinant Infectious Virus Systems

P-1169

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Conclusions

- Obeldesivir and GS-441524 inhibited the replication of respiratory syncytial virus RNA-dependent RNA polymerase containing substitutions identified in in vitro resistance assays, with similar activity in both the minigenome and recombinant infectious virus systems
  - I777L was associated with minimal- to low-level reduced susceptibility to obeldesivir and GS-441524 compared with wildtype
  - All other RNA-dependent RNA polymerase substitutions tested demonstrated similar susceptibility to obeldesivir and GS-441524 as wildtype
- These results indicate that obeldesivir and GS-441524 have a high barrier to resistance development and that the optimized minigenome system can be used to evaluate antiviral susceptibility of respiratory syncytial virus RNA-dependent RNA polymerase substitutions

Plain Language Summary

- Obeldesivir is being investigated as a potential treatment for several respiratory viruses, including respiratory syncytial virus
- When taken by mouth, obeldesivir is converted to GS-441524 first and then to an active drug that can stop viruses from multiplying further
- During treatment, viruses can evolve and mutate to escape the antiviral drug inhibition, making treatments like obeldesivir less effective
- In this study, the effects of obeldesivir and GS-441524 were evaluated using 2 different techniques in cells that either expressed a standard version of respiratory syncytial virus proteins or had mutated versions
- Regardless of whether mutations were present, obeldesivir and GS-441524 were able to stop virus multiplication
- These results suggest that different versions of respiratory syncytial virus are unlikely to become resistant to obeldesivir and GS-441524

References: **1.** Pitts J, et al. *Nat Commun*. 2025;16:6437. **2.** Deval J, et al. *PLoS Pathog*. 2015;11:e1004995. **3.** Sourimant J, et al. *Sci Adv*. 2022;8:eabo2236. **4.** Hotard AL, et al. *Virology*. 2012;434:129-36.

Acknowledgments: This study was funded by Gilead Sciences, Inc. Medical writing and editorial support were provided by Pedro de Campos Silva, PhD, of Lumanity Communications Inc., and were funded by Gilead Sciences, Inc.

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Disclosure: **JM**, **JLRZ**, **DH**, **JC**, **NP**, **CR**, **SE**, **JPB**, and **CH** are stockholders and employees of Gilead Sciences, Inc.

Introduction

- Obeldesivir (ODV) is an oral prodrug of the GS-441524 nucleoside analog that targets the RNA-dependent RNA polymerase of respiratory syncytial virus (RSV), which includes the large (L) polymerase catalytic subunit
- ODV has demonstrated antiviral activity in vitro against a diverse set of RSV A and B clinical isolates and in vivo in a nonhuman primate model of RSV infection[1]
- In vitro resistance selections with ODV and GS-441524 resulted in the separate emergence of 3 amino acid substitutions in the L polymerase (ODV, I777L; GS-441524, C319R and L1453W)[1]
- Lumicitabine is a prodrug of the parent nucleoside ALS-8112, which was previously in clinical development for RSV treatment[2]
  - Resistance selections using ALS-8112 resulted in the coemergence of 4 substitutions in the L polymerase (M628L, A789V, L795I, and I796V), referred to as the QUAD mutations
- Generation of RSV amino acid substitutions in the recombinant infectious virus system is labor intensive
  - The RSV minigenome system is a promising higher-throughput alternative for the phenotypic evaluation of amino acid substitutions in the L polymerase

Objective

- To determine the antiviral activity of ODV and GS-441524 against RSV L polymerase substitutions that emerged during in vitro resistance selections with ODV, GS-441524, or ALS-8112 using an optimized RSV minigenome phenotypic assay and a recombinant infectious virus system

Methods

Minigenome Assay (Figure 1A)

- Plasmids used included the RSV minigenome reporter plasmid expressing firefly luciferase (RSV-FLucMG) and 4 helper plasmids encoding codon-optimized RSV L polymerase (RSV-L), nucleoprotein (RSV-N), phosphoprotein (RSV-P), and M2-1 protein (RSV-M2-1)[3]
- L polymerase amino acid substitutions were introduced into the RSV-L plasmid by site-directed mutagenesis using Platinum™ SuperFi II PCR Master Mix (Invitrogen) per the manufacturer's instructions
- HEK293T cells (ATCC) were cotransfected with a DNA mix containing RSV-L (wildtype [WT] RSV A2 or amino acid mutants), RSV-N, RSV-P, RSV-M2-1, and RSV-FLucMG using Lipofectamine 3000 (Invitrogen)
- Cells were treated with 2.5-fold serial dilutions of ODV or GS-441524 and incubated at 37 °C for 48 hours
- Luciferase activity was measured using an EnSpire® Alpha microplate reader after adding ONE-Glo™ EX Luciferase substrate (Promega)

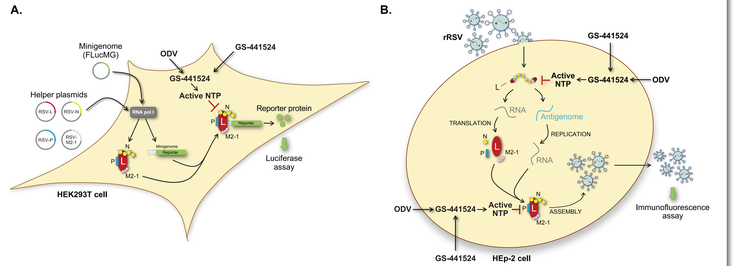
Recombinant Infectious Virus System (Figure 1B)

- RSV containing either WT sequences or the previously specified L polymerase substitutions were generated via cloning, virus rescue, propagation, and titration as previously described[1,3,4]
- For the antiviral assay, HEp-2 cells (ATCC) were treated with 3-fold serial dilutions of ODV or GS-441524, infected with WT recombinant RSV (rRSV) or rRSV bearing L polymerase substitutions at a multiplicity of infection of 0.05, and incubated at 37 °C for 72 hours
- RSV immunofluorescence signal was measured using a Cellomics CellInsight™ machine (CellHealthProfiling.V4 assay) via RSV fusion protein staining[1]

Half-Maximal Effective Concentration (EC_{50}) Calculations

- ODV and GS-441524 EC_{50} values were determined in both systems from curve fits using variable slope nonlinear regression as the concentration where there was a 50% decrease in the normalized fluorescence signal (GraphPad Prism 10.1.2)
- In the recombinant infectious virus system, assay variability thresholds for reduced susceptibility were determined using WT EC_{50} values against ODV and GS-441524 from 17 independent experiments following a normal distribution and were calculated as follows: assay variability = 95th percentile EC_{50}/5th percentile EC_{50}. Mutant mean EC_{50} fold change values below the threshold were deemed within the variability of the assay
- In the minigenome system, preliminary assay variability thresholds were similarly determined with a limited available dataset of 6 independent experiments. Minigenome assay variability thresholds are subject to change in future work upon the availability of additional data

Figure 1. Schematic of the (A) Minigenome and (B) Recombinant Infectious Virus Systems



NTP, nucleotide triphosphate; ODV, obeldesivir; RNA pol I, RNA polymerase I; rRSV, recombinant RSV; RSV, respiratory syncytial virus; RSV-FLucMG, RSV minigenome reporter plasmid expressing firefly luciferase; RSV-L, RSV large polymerase; RSV-M2-1, RSV M2-1 protein; RSV-N, RSV nucleoprotein; RSV-P, RSV phosphoprotein.

Results

Antiviral Activity of ODV and GS-441524 Against RSV WT and L Polymerase Mutants in the Minigenome and Recombinant Infectious Virus Systems

- The mean EC_{50} values of ODV and GS-441524 against RSV WT and L polymerase mutants in both the minigenome and recombinant virus systems are shown in **Table 1**, and EC_{50} fold changes from the WT reference are shown in **Figure 2**

Phenotypic Results in Minigenome System

- C319R, A789V, L795I, I796V, L1453W, and QUAD mutants had EC_{50} fold changes within the variability of the assay (ODV, 1.73-fold change; GS-441524, 1.47-fold change), indicating no impact on ODV or GS-441524 antiviral activity
- I777L showed minimal- to low-level reduced susceptibility to ODV and GS-441524 compared with WT (EC_{50} fold change: 2.1 for both)

Phenotypic Results in Recombinant Infectious Virus System

- C319R, A789V, L795I, I796V, L1453W, and QUAD mutants had EC_{50} fold changes within the variability of the assay (ODV, 2.33-fold change; GS-441524, 1.85-fold change), indicating no impact on ODV and GS-441524 antiviral activity
- I777L showed a low reduction in susceptibility to ODV and GS-441524 compared with WT (EC_{50} fold change: 3.8 and 3.3, respectively)

Concordance Between Phenotypic Systems

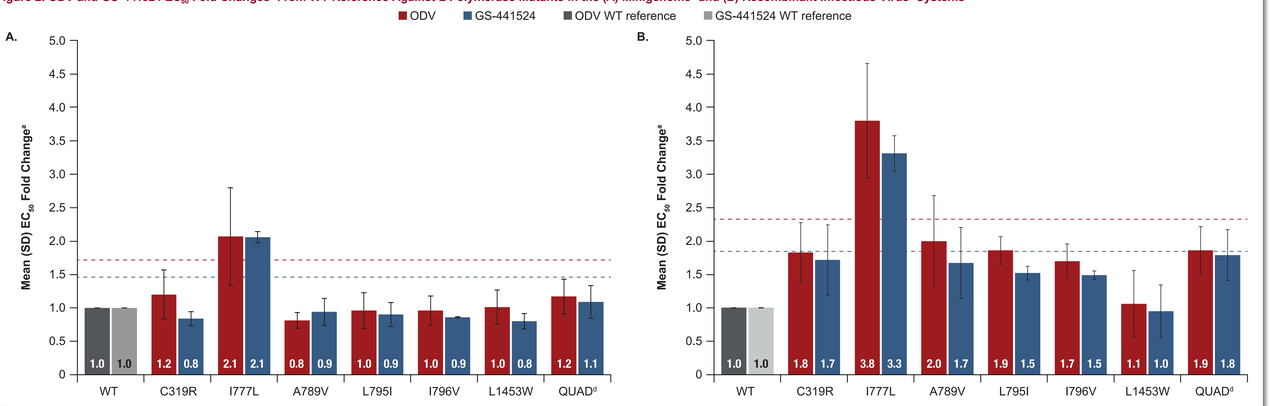
- Both the minigenome and recombinant virus systems demonstrated that the C319R, A789V, L795I, I796V, L1453W, and QUAD mutants had susceptibility to ODV and GS-441524 similar to that of the WT reference (**Table 1** and **Figure 2**)
- The I777L mutant was associated with EC_{50} fold changes ranging from 2.1 to 3.8 across both phenotypic systems, indicating minimal- to low-level reduced susceptibility to ODV and GS-441524 compared with the WT reference

Table 1. Antiviral Activity of ODV and GS-441524 Against RSV WT Reference and RSV L Polymerase Mutants in the Minigenome and Recombinant Virus Systems

	Minigenome System		Recombinant Virus System	
L Polymerase	Mean (SD) ODV EC_{50}, µM	Mean (SD) GS-441524 EC_{50}, µM	Mean (SD) ODV EC_{50}, µM	Mean (SD) GS-441524 EC_{50}, µM
WT	4.9 (1.1)	5.9 (0.6)	0.3 (0.1)	0.7 (0.1)
C319R[a]	4.5 (<0.1)	4.6 (0.1)	0.7 (<0.1)[a]	1.0 (0.1)[a]
M628L[b,c]	No replication		No rescue	
I777L[a]	7.7 (0.4)	11.4 (0.6)	1.5 (0.5)[a]	2.3 (0.5)[a]
A789V[c]	4.1 (0.8)	5.8 (0.7)	0.8 (0.3)	1.2 (0.5)
L795I[c]	5.4 (0.7)	4.9 (0.5)	0.7 (0.2)	0.8 (<0.1)
I796V[c]	3.7 (0.3)	4.8 (0.4)	0.6 (0.2)	0.8 (0.1)
L1453W[a]	3.8 (0.2)	4.4 (0.2)	0.3 (0.1)[a]	0.7 (0.3)[a]
QUAD[c,d]	4.4 (0.3)	6.0 (0.8)	0.5 (<0.1)	1.4 (0.2)

[a]L polymerase mutations and EC_{50} values in the recombinant virus system are described in Pitts J, et al. *Nat Commun*. 2025;16:6437.
[b]The M628L mutant could not be tested in either system due to limited replicative capacity.
[c]L polymerase mutations are described in Deval J, et al. *PLoS Pathog*. 2015;11:e1004995.
[d]QUAD mutations included M628L, A789V, L795I, and I796V.
EC_{50}, half-maximal effective concentration; ODV, obeldesivir; RSV, respiratory syncytial virus; SD, standard deviation; WT, wildtype.

Figure 2. ODV and GS-441524 EC_{50} Fold Changes[a] From WT Reference Against L Polymerase Mutants in the (A) Minigenome[b] and (B) Recombinant Infectious Virus[c] Systems



[a]EC_{50} fold change was calculated for each experimental replicate, and the mean fold change was calculated with these values.
[b]Dashed lines in Figure 2A indicate preliminary thresholds for assay variability of the minigenome system against ODV (1.73-fold) and GS-441524 (1.47-fold).
[c]Dashed lines in Figure 2B indicate thresholds for assay variability of the recombinant infectious virus system against ODV (2.33-fold) and GS-441524 (1.85-fold).
[d]QUAD mutations included M628L, A789V, L795I, and I796V.
EC_{50}, half-maximal effective concentration; ODV, obeldesivir; SD, standard deviation; WT, wildtype.