

Supporting Information

1'- and 4'-cyano modified adenosine analogs against prototypic flavivirus RNA-dependent RNA polymerases

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Table S1: Selectivity values for GS-443902 against flavivirus RdRps.

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Figure S1. Selective incorporation of GS-646939 and GS-443902.

Figure S2. WNV, YFV, and ZIKV RdRp-catalyzed RNA synthesis and inhibition patterns following incorporation of a single GS-443902.

Figure S3. Product formation over time following ATP or GS-443902 incorporation by DENV-2 and ZIKV RdRps.

Figure S4. WNV, YFV, and ZIKV RdRp-catalyzed RNA synthesis and inhibition patterns following incorporation of a single GS-646939.

Figure S5. Template-dependent inhibition of DENV-2 and WNV RdRps by a single embedded GS-646939 residue at position 11.

Table S1: Selectivity values for GS-443902 against flavivirus RdRps.

	ATP			GS-443902			
Virus	V _{max} ^a (product fraction)	K _m ^b (μM)	V _{max} /K _m	V _{max} (product fraction)	K _m (μM)	V _{max} /K _m	Selectivity ^e (fold)
DENV-2 ^f							11.1
	0.90	0.18	5.0	0.89	2.0	0.45	
± ^d	0.018	0.016		0.025	0.23		
JEV ^g							6.8
	0.74	5.5	0.13	0.27	14	0.019	
±	0.035	1.0		0.029	4.8		
WNV ^h							6.8
	0.83	0.18	4.6	0.75	1.1	0.68	
±	0.014	0.014		0.0072	0.036		
YFV ⁱ							10.4
	0.84	0.16	5.3	0.76	1.5	0.51	
±	0.0024	0.0021		0.0084	0.052		
ZIKV ^j							6.5
	0.73	0.086	8.5	0.69	0.55	1.3	
±	0.011	0.0069		0.016	0.049		
h-mtRNAP ^k							508 ^l

^a $V_{\max}$ is a Michaelis–Menten parameter reflecting the maximal velocity of nucleotide incorporation.

^b K_m is a Michaelis–Menten parameter reflecting the concentration of the nucleotide substrate at which the velocity of nucleotide incorporation is half of $V_{\max}$.

^c All reported values have been calculated based on an 8–data point experiment repeated at least 3 times (n=3).

^d Standard error associated with the fit.

^e Selectivity of a viral RNA polymerase for a nucleotide substrate analog is calculated as the ratio of the $V_{\max}/K_m$ values for NTP and NTP analog, respectively.

^f Dengue virus

^g Japanese encephalitis virus

^h West Nile virus

ⁱ Yellow fever virus

^j Zika virus

^k Human mitochondrial RNA polymerase

^l Data from Tchesnokov et al. (1)

Table S2: Selectivity values for GS-646939 against flavivirus RdRps.

	ATP			GS-646939			
Virus	$V_{\max}^a$ (product fraction)	K_m^b (μM)	$V_{\max}/K_m$	$V_{\max}$ (product fraction)	K_m (μM)	$V_{\max}/K_m$	Selectivity ^e (fold)
DENV-2 ^f							7.0
	0.90	0.18	5.0	0.92	1.3	0.71	
± ^d	0.018	0.016		0.022	0.097		
JEV ^g							3.4
	0.74	5.5	0.13	0.35	9.3	0.038	
±	0.035	1.0		0.034	3.1		
WNV ^h							5.1
	0.83	0.18	4.6	0.75	0.82	0.91	
±	0.014	0.014		0.0039	0.015		
YFV ⁱ							5.8
	0.84	0.16	5.3	0.82	0.89	0.92	

±	0.0024	0.0021		0.016	0.061		
ZIKV ⁱ							4.0
	0.73	0.086	8.5	0.75	0.36	2.1	
±	0.011	0.0069		0.018	0.036		
h-mtRNAP ^k							1540 ¹

^a $V_{\max}$ is a Michaelis–Menten parameter reflecting the maximal velocity of nucleotide incorporation.

^b K_m is a Michaelis–Menten parameter reflecting the concentration of the nucleotide substrate at which the velocity of nucleotide incorporation is half of $V_{\max}$.

^c All reported values have been calculated based on an 8–data point experiment repeated at least 3 times (n=3).

^d Standard error associated with the fit.

^e Selectivity of a viral RNA polymerase for a nucleotide substrate analog is calculated as the ratio of the $V_{\max}/K_m$ values for NTP and NTP analog, respectively.

^f Dengue virus

^g Japanese encephalitis virus

^h West Nile virus

ⁱ Yellow fever virus

^j Zika virus

^k Human mitochondrial RNA polymerase

¹ Data from Gordon et al. (2)

Table S3: Inhibitory effect of the incorporated analog-monophosphate on the incorporation of the subsequent nucleotide.

Virus	Primer 3'-end (base) ^a	Substrate	$V_{\max}^b$ (product fraction)	K_m^c (μM)	$V_{\max}/K_m$	Inhibition ^d (fold)
DENV-2 ^h	AMP	UTP	$0.77^e \pm 0.0080^f$	0.029 ± 0.0013	27	Ref. ^g
	GS-646939 (MP)		0.57 ± 0.016	132 ± 12	0.0043	>1000
	GS-443902 (MP)		0.73 ± 0.011	0.33 ± 0.020	2.2	12
JEV ⁱ	AMP	UTP	0.45 ± 0.022	0.35 ± 0.071	1.3	Ref.
	GS-646939 (MP)		0.0082 ± 0.0029	127 ± 149	0.000065	>1000
	GS-443902 (MP)		0.18 ± 0.0058	1.5 ± 0.15	0.12	11
WNV ^j	AMP	UTP	0.63 ± 0.0024	0.022 ± 0.00039	29	Ref.

	GS-646939 (MP)		0.57 ± 0.0050	116 ± 3.4	0.0049	>1000
	GS-443902 (MP)		0.56 ± 0.0066	0.38 ± 0.018	1.5	19
YFV ^k	AMP	UTP	0.50 ± 0.0058	0.016 ± 0.00089	31	Ref.
	GS-646939 (MP)		0.18 ± 0.051	406 ± 268	0.00044	>1000
	GS-443902 (MP)		0.38 ± 0.0020	0.90 ± 0.017	0.42	74
ZIKV ^l	AMP	UTP	0.58 ± 0.0066	0.026 ± 0.0013	22	Ref.
	GS-646939 (MP)		0.11 ± 0.0054	11 ± 2.6	0.010	>1000
	GS-443902 (MP)		0.32 ± 0.016	0.053 ± 0.010	6.0	3.7

^a Denotes that the 3'-end of the primer is terminated by the monophosphate form of the respective nucleotide.

^b $V_{\max}$ is a Michaelis–Menten parameter reflecting the maximal velocity of nucleotide incorporation.

^c K_m is a Michaelis–Menten parameter reflecting the concentration of the nucleotide substrate at which the velocity of nucleotide incorporation is half of $V_{\max}$

^d Inhibition of subsequent nucleotide incorporation is calculated as the ratio of $V_{\max}/K_m$ values for UTP determined

on primers terminated with AMP, GS-646939, or GS-443902

^e All reported values have been calculated based on an 8–data point experiment repeated at least 3 times (n=3)

^f Standard error associated with the fit

^g Reference

^h Dengue virus

ⁱ Japanese encephalitis virus

^j West Nile virus

^k Yellow fever virus

^l Zika virus

Table S4: Inhibitory effect of templated RDV-MP against JEV, WNV, YFV, and ZIKV RdRps.

Enzyme	Template	UTP (μ M) ^a	Relative inhibition ^b (Ratio of concentrations, fold)
JEV ^c	"A"	0.11	Ref.
	"GS-443902"	65.9	599
WNV ^d	"A"	0.042	Ref.
	"GS-443902"	13.3	317
YFV ^e	"A"	0.033	Ref.
	"GS-443902"	12.2	370
ZIKV ^f	"A"	0.029	Ref.
	"GS-443902"	2.13	73.4

^a Concentration of UTP at which 50% of RNA products generated are beyond position 10.

^b Fold excess of UTP required to overcome the inhibitory effect of GS-443902 embedded in the template, relative to the natural nucleotide.

^c Japanese encephalitis virus

^d West Nile virus

^e Yellow fever virus

^f Zika virus

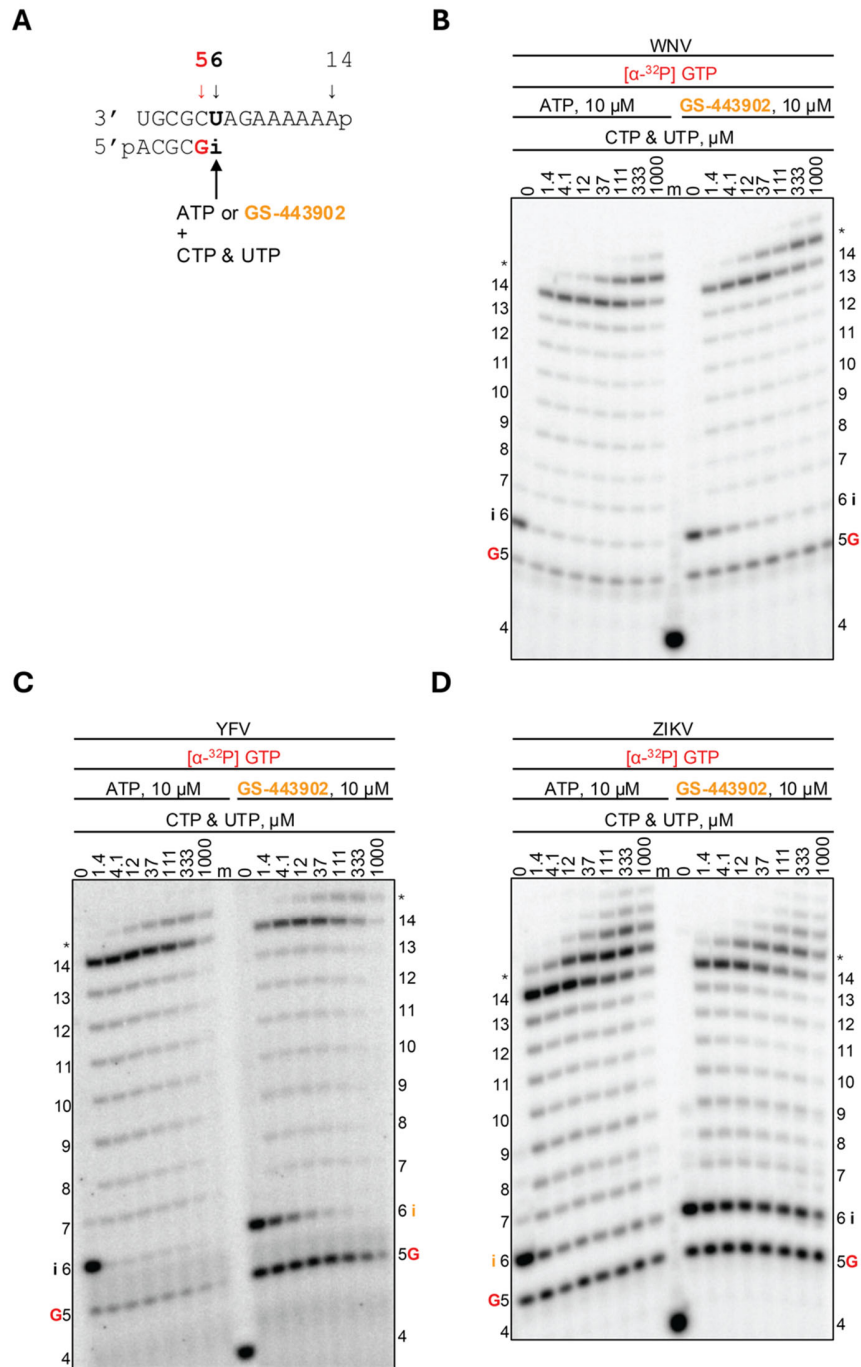


Figure S2. WNV, YFV, and ZIKV RdRp-catalyzed RNA synthesis and inhibition patterns following incorporation of a single GS-443902. **A**, RNA primer/template supporting a single incorporation of ATP or GS-443902 at position 6 (i). G indicates incorporation of [α -³²P]-GTP at position 5. **B**, migration pattern of the products of RNA synthesis catalyzed by WNV RdRp. A 5'-³²P-labelled 4-nt primer (4) serves as a size marker (m). RNA products formed at and beyond the asterisk indicate slippage products that may be a result of RdRp nucleotide transferase activity. **C**, migration pattern of products of RNA synthesis catalyzed by YFV RdRp. **D**, migration pattern of products of RNA synthesis catalyzed by ZIKV RdRp. WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

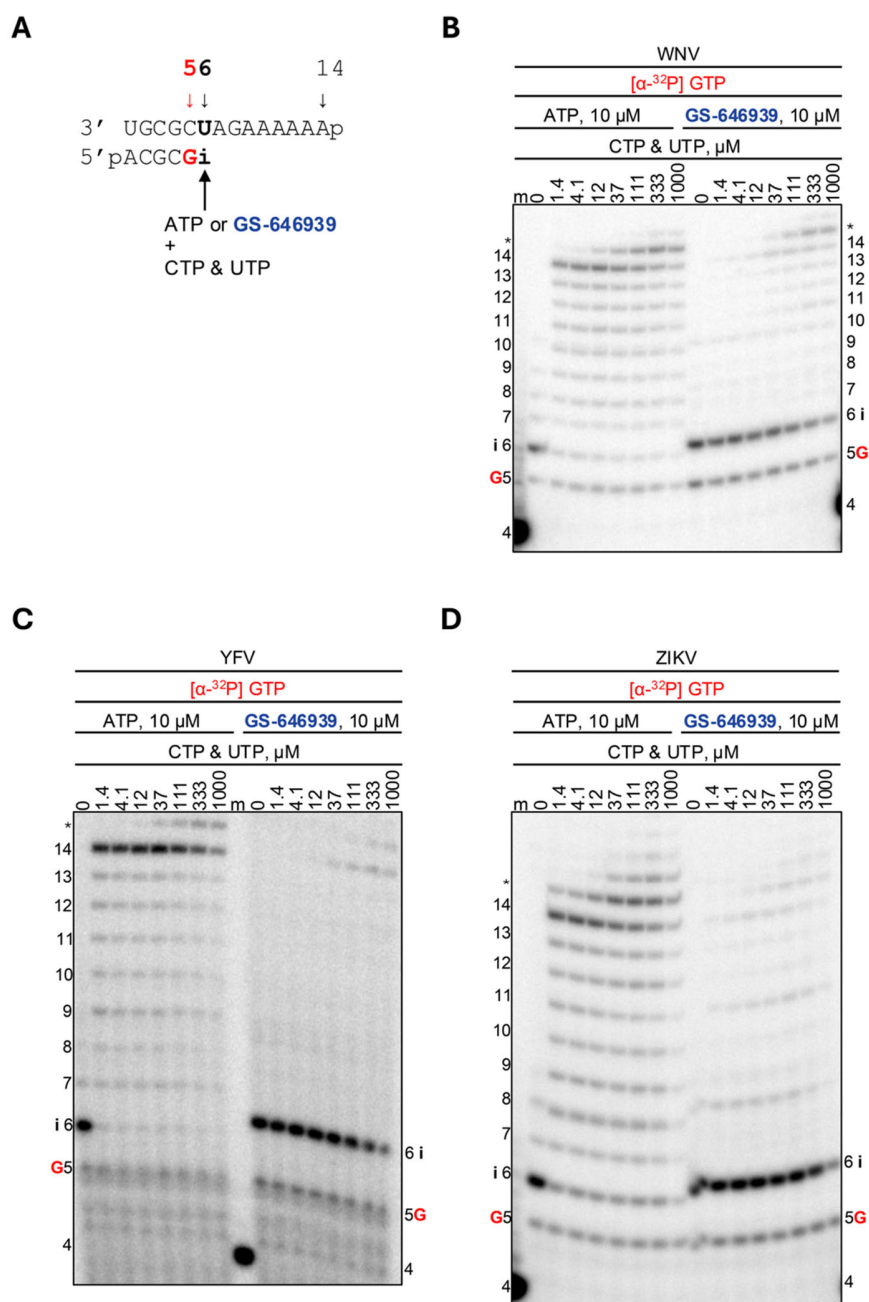


Figure S4. WNV, YFV, and ZIKV RdRp-catalyzed RNA synthesis and inhibition patterns following incorporation of a single GS-646939. **A**, RNA primer/template supporting a single incorporation of ATP or GS-646939 at position 6 (i). G indicates incorporation of [α - 32 P]-GTP at position 5. **B**, migration pattern of the products of RNA synthesis catalyzed by WNV RdRp. A 5'- 32 P-labelled 4-nt primer (4) serves as a size marker (m). RNA products formed at and beyond the asterisk indicate slippage products that may be a result of RdRp nucleotide transferase activity. **C**, migration pattern of products of RNA synthesis catalyzed by YFV RdRp. **D**, migration pattern of products of RNA synthesis catalyzed by ZIKV RdRp. WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

1. Tchesnokov, E. P., Feng, J. Y., Porter, D. P., and Gotte, M. (2019) Mechanism of Inhibition of Ebola Virus RNA-Dependent RNA Polymerase by Remdesivir *Viruses* **11**,
2. Gordon, C. J., Walker, S. M., Tchesnokov, E. P., Kocincova, D., Pitts, J., Siegel, D. S. *et al.* (2024) Mechanism and spectrum of inhibition of a 4 -cyano modified nucleotide analog against diverse RNA polymerases of prototypic respiratory RNA viruses *J Biol Chem* 10.1016/j.jbc.2024.107514107514