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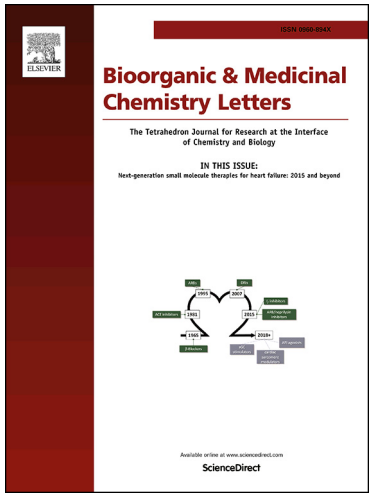

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PII: S0960-894X(19)30111-8
DOI: https://doi.org/10.1016/j.bmcl.2019.02.027
Reference: BMCL 26307

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 1 February 2019
Revised Date: 21 February 2019
Accepted Date: 26 February 2019

Please cite this article as: Patnaik, S., Basu, D., Southall, N., Dehdashti, S., Wan, K.K., Zheng, W., Ferrer, M., Taylor, M., Engel, D.A., Marugan, J.J., Identification, Design and Synthesis of Novel Pyrazolopyridine Influenza Virus Nonstructural Protein 1 Antagonists, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.02.027

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Influenza
NS1
Antagonist
Pyrazolopyridine
Antiviral

ABSTRACT

Nonstructural protein 1 (NS1) plays a crucial function in the replication, spread, and pathogenesis of influenza virus by inhibiting the host innate immune response. Here we report the discovery and optimization of novel pyrazolopyridine NS1 antagonists that can potently inhibit influenza A/PR/8/34 replication in MDCK cells, rescue MDCK cells from cytopathic effects of seasonal influenza A strains, reverse NS1-dependent inhibition of IFN-β gene expression, and suppress the slow growth phenotype in NS1-expressing yeast. These pyrazolopyridines will enable researchers to investigate NS1 function during infection and how antagonists can be utilized in the next generation of treatments for influenza infection.

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Influenza is an acute respiratory disease caused by members of the orthomyxoviridae family of RNA viruses. Recent analysis by the Centers for Disease Control and Prevention (CDC) estimates that influenza has resulted in, on average, about 600,000 hospitalization and 40,000 deaths annually in the US.[1, 2] Coupled with the morbidity and mortality is the substantial financial impact on medical cost, loss of work, and hospitalizations. The "Spanish flu" of 1918, which caused an estimated 20-50 million deaths worldwide, is a stark reminder of a flu pandemic's potential for devastation. The keystones of current public policy remain surveillance and prevention via administration of the seasonal vaccine containing the most commonly circulating viral strains. However, mutations within the viral genome lead to antigenic drift, eventually rendering the flu shots ineffective.[3] Additionally, novel viruses may be formed by antigenic shift when segments of human viral strains reassort with those from other animal reservoirs (e.g. birds and pigs).[4-6] The danger of antigenically shifted pathogens is exemplified by the millions of deaths in the 1957 and 1968 influenza pandemics caused by viruses derived from avian sources for which humans were immunologically unprepared.[6] The dangerous introduction of an antigenically novel virus for which humans have faint immunological memory was felt again in 2009 when a version of the 1918 H1N1 virus, after circulating in pigs for nearly 100 years, led to the emergence of a pandemic strain from Mexico and California.[7] This outbreak exposed shortcomings in global preparedness, as it was clear that it would take at least 6 months to generate a vaccine supply which would be useful, a timeline which could easily be superseded by a rapidly spreading virus.

An effective response to pandemic strains will require a wider variety of antiviral drugs than those that are currently available. Adamantane antivirals, which target the viral M2 protein required for viral uncoating within the host cell, are no longer recommended for use due to widespread resistance. Inhibitors of the neuraminidase protein (i.e. oseltamivir and zanamivir), which block the release of new virions from the host cells, are the most commonly prescribed treatments today.[8] However, given the emergence of oseltamivir-resistant strains[8] and the rapid spread of the highly pathogenic H5N1 strain among birds, it is vital to continue the pursuit of new treatment modalities.[9-10] Recent FDA approval of baloxavir marboxil, which targets the viral polymerase, highlights the need for additional viral targets to be exploited therapeutically.[11]

A proposed antiviral mechanism targets the viral nonstructural protein 1 (NS1), which plays a key role in virus replication by repressing the innate host immune system.[12-16] NS1 is a well conserved, 230-237 amino acid multifunctional protein that contains an RNA binding domain and an effector domain that are connected by a variable linker,[17] and it is highly expressed during infection.[18] NS1 is the centerpiece of the viral response to the host interferon (IFN) system, functioning as a key component in the temporal regulation of viral RNA synthesis, its splicing, and translation. Specifically, NS1: i) suppresses the host IFN-β response to viral infection,[19-23] ii) inhibits the function of 2'-5'-oligoadenylate synthase and protein kinase R, iii) inhibits the maturation of host pre-mRNAs,[24-25] iv) interferes with the host RNAi pathway, adaptive immune response, and the apoptotic response,[26] and v) binds to the human PAF1 transcription elongation complex (hPAF1C), a crucial player in transcription of antiviral genes.[27] In doing so, NS1 promotes and enhances viral replication, making it a compelling target for influenza treatment. As such, a number of chemical series that can disrupt NS1 function have been reported.[14, 28-33] Here we report a structurally distinct class of pyrazolopyridine antagonists of NS1 function, discovered using a yeast-based screening strategy,[29] their structure-activity relationship (SAR), and their ability to reduce virus replication, rescue infected cells from virus-induced cytotoxicity and restore the NS1-inhibited expression of IFN mRNA.

Expanding on a previous approach,[29, 34] which uses a NS1-pYES yeast strain that exhibits a pronounced slow-growth phenotype upon expression of NS1 protein, we have performed a quantitative high-throughput screen (qHTS) against a library of 269,572 compounds from the NIH Molecular Libraries-Small Molecule Repository (MLSMR) collection for activities that specifically reversed the NS1-induced slow-growth phenotype. The published protocol[29] was adapted for 1536-well plate format at 4 doses (100 nM-57 μM). The screening effort and subsequent cherry-picking experiments identified 200 hits, representing 0.08% of the chemical library.[35] These hits were then evaluated for their antiviral activity and cytotoxicity. Their antiviral activity was examined by testing the compounds ability to slow replication of the A/PR/8/34 strain of influenza A virus (H1N1) in MDCK cells infected at a multiplicity of infection (MOI) of 0.1 for 48 hours. The reduction of viral titer was measured by a hemagglutination assay of the supernatant and standard $TCID_{50}$ analysis. Cytotoxicity was tested in MDCK cells using the CellTiter Glo ATP cell viability assay. Twenty-three compounds belonging to two structural clusters, and seven singletons showed antiviral activity. The pyrazolopyridine hit **1 (Figure 1)** demonstrated the most robust antiviral activity without toxicity towards the host MDCK cells. Based on this activity and its chemical tractability, hit **1** was chosen for further optimization.[36]

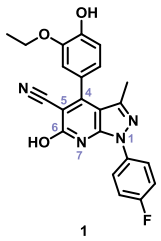

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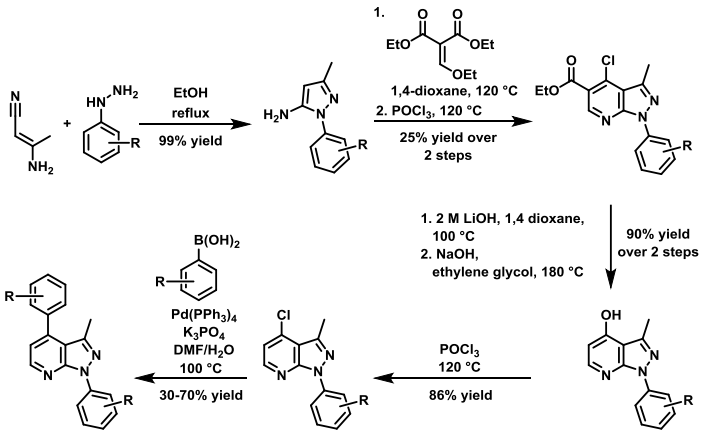
Figure 1. Pyrazolopyridine hit from qHTS screen.

In total, 40 pyrazolopyridines were evaluated in the aforementioned influenza virus replication and cytotoxicity assays. The non-commercially available analogs were synthesized via the route shown in **Scheme 1**.



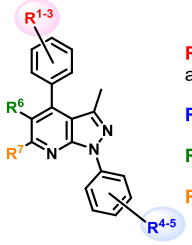
Scheme 1. Synthetic route to pyrazolopyridine analogs for SAR evaluation.

Most of the compounds were non-cytotoxic, and several compounds reduced viral titer >100-fold at 10 μM. **Table 1** summarizes the fold reductions in viral titer after incubating the compounds at 10 μM for 48 hours. The fold decrease in viral replication was determined by calculating the inverse log of the difference between the $logTCID_{50}$ value for each compound and the DMSO control. The SAR summary of the series is illustrated in **Figure 2**.

Table 1. SAR around hit **1**

Cmpd	R^1	R^2	R^3	R^4	R^5	R^6	Viral fold reduction at 10 μM[a]
2	OH	OH	H	H	H		32
3	OH	OH	H	Me	H		64
4	OH	OH	H	Me	Me		256
5	OH	OH	H	F	H		16
6	OH	OH	H	Cl	H		256
7	OMe	OH	H	H	H		32
8	OMe	OH	H	Me	H		4
9	OMe	OH	H	Me	Me		4
10	OMe	OH	H	F	H		256
11	OMe	OH	H	Cl	H		32
12	OMe	OMe	H	H	H		2
13	OMe	OMe	H	Me	H		0
14	OMe	OMe	H	F	H		2
15	OMe	OMe	H	Cl	H		0
16	OMe	OMe	OMe	H	H		16
17	OMe	OMe	OMe	Me	H		32
18	OMe	OMe	OMe	Me	Me		0
19	OMe	OMe	OMe	F	H		0
20	OMe	OMe	OMe	Cl	H		0
21	OMe	OH	H	H	H		16
22	OMe	OMe	H	H	H		2
23	OMe	OMe	OMe	H	H		16
24	OMe	OMe	H	F	H		0
1 (hit)	**OEt**	**OH**	**H**	**F**	**H**		**2**
25	OMe	OH	H				2
26	OMe	OMe	H				4
27	OMe	OH	OMe				4
28	OH	OMe	H				2
29	-OCH_2O-		H				4
30	OMe	OH	H	H	F		0
31	OMe	OH	H	H	OM		0
32	OMe	OH	H	CF_3	H		128
33	OMe	OH	H	H	CF_3		256
34	OMe	OH	H	H	H		2
35	OMe	OH	H	OMe	H		32
36	OMe	OH	H	F	H		2
37	OMe	H	H	F	H		4
38	OMe	OMe	H	F	H		2
39						CO_2H	4
40						CO_2Et	16

[a]MDCK cell lines infected at a MOI of 0.1 for 48 h (time) (with or without compounds) were used to determine virus titer using $TCID_{50}$ analysis.



R^{1-3}: One H-Bond donor (e.g. OH) necessary for activity

R^{4-5}: Lipophillic group necessary for activity

R^6: CN not necessary for activity

R^7: H, NH_2, OH tolerated

Figure 2. Structure activity relationship summary for chemical series.

Within the context of the 5-cyano-6-amino pyrazolopyridine core with a catechol-like substituent in the C4 position (i.e. **2-6**), the presence of a lipophilic methyl or chloro group at R^4 and/or R^5 seemed to cause potent antiviral activity, with **4** and **6** showing 256-fold reduction in virus production. Capping one of the phenols (R^1) with a methyl group (i.e. **7-11**) led to compounds that did not strictly recapitulate this trend, but still produced **10** with similar potency. The antiviral activity of analogs with a *m*,*p*-dimethoxy phenyl (i.e. **12-15**) or *m*,*p*,*m*-trimethoxy phenyl (i.e. **16-20**) at C4 was attenuated in general. Analogs with a 6-hydroxy core (i.e. **21-24**) was evaluated briefly as it was present in hit **1**, and modest 16-fold reductions were observed with compounds **21** and **23**. A limited examination of a pyridinedione tricyclic core (i.e. **25-29**) with an unsubstituted phenyl ring at N1 revealed modest antiviral activity. The necessity of substitutions at C5 and C6 of the core was also interrogated using synthesized analogs **30-38**. Significant reductions in viral titer were observed with **32** and **33**, reiterating the ideal presence of a small lipophilic group at R^3 or R^4. This potent activity was obtained with a *m*-OMe-*p*-OH phenyl substituent at C4, implying that the presence of a catechol-like substituent, like that in analogs **2-6**, is not necessary for potent activity. Modest activity was also observed with a carboxylic acid **39** or ester **40** at C5 in the context of a 4-F phenyl group at N1.

Our efforts at exploring the SAR of the screening hit **1** had therefore resulted in several compounds with potent antiviral activity. It was also noteworthy that, besides compound **37**, no other analogs exhibited cytotoxicity towards the host MDCK cells. Key potent compounds with ≥128 fold reduction in viral titer (i.e. **4**, **6**, **10**, **32**, and **33**) were analyzed for dose response in the $TCID_{50}$ assay, and they successfully decreased viral titer with increasing concentration. As an illustrative example, analog **32** had an IC_{90} of 155 nM (**Figure 3**), and since it did not affect cell viability at 5, 10, 50 and 100 μM, it had a greater than 100-fold selectivity index (SI).

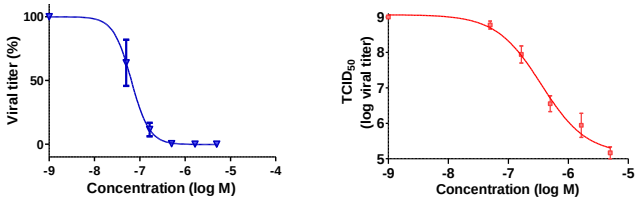


Figure 3. The activity of **32** on viral titer. Triplicate cultures were infected with A/PR/8/34 at a MOI of 0.1 and treated with the indicated concentrations of compound. After 48 h (time), the supernatants were collected and analyzed to determine the reduction in virus by assaying the supernatant infectivity. The data are presented (left) as a percent decrease in titer back-calculated from $TCID_{50}$s where IC_{90} is 155 nM and (right) the actual $TCID_{50}$s where 3 log units of viral suppression are observed at 1.6 μM concentration.

Because the A/PR/8/34 is a mouse adapted laboratory strain, we wanted to evaluate these compounds in other viral strains that are more relevant to human infection. To that end, we utilized resources available at the National Institute of Allergy and Infectious Diseases (NIAID) and evaluated key compounds (**1, 4, 6, 10, 19, 32 and 33**) in an assay that measured their ability to protect host cells from cytopathic effects of various viral strains. The compounds were tested against 3 additional human influenza viruses: H1N1 (strain: *A/California/7/2009)*, H3N2 (strain: *Influenza A/Brisbane/10/20070*, and H5N1 (strain: a recombinant A/PR/8/34 strain that encoded NS1 from avian A/Vietnam/1203/2004), and the results are presented in **Table 2**. The best activity was observed against a seasonal A/H1N1 strain,

Table 2. Selected compounds' ability to rescue virus-mediated cytopathy of MDCK cells

Virus Screened	Influenza A virus H1N1				Influenza A virus H3N2				Influenza A virus H5N1			
Virus Strain	*Influenza A/California/7/2009*				*Influenza A/Brisbane/10/2007*				*Influenza A/Vietnam/1203/2004 x A/PR/8/34 (recombinant H5N1)*			
cmpd	EC_{50}[a]	EC_{90}[a]	CC_{50}[a]	SI_{50}[a]	EC_{50}	EC_{90}	CC_{50}	SI_{50}	EC_{50}	EC_{90}	CC_{50}	SI_{50}
1	1.7	4.9	>50	>29	47.6	>50	>50	>1	28.5	>50	>50	>1
4	1.2	3.4	39.3	34	>50	>50	39.0	<1	>50	>50	24.5	<1
6	>50	>50	31.9	<1	>50	>50	20.4	<1	>50	>50	24.5	<1
10	2.1	4.6	48.3	23	10.1	>50	45.8	5	3.6	9.9	24.7	7
19	18.3	42.4	5.6	<1	>50	>50	6.0	<1	>50	>50	0.87	<1
32	0.7	>50	25.3	38	17.0	>50	27.8	2	>50	>50	19.8	<1
33	2.4	5.7	49.0	21	2.4	>50	48.6	20	>50	>50	24.5	<1
Ribavirin	2.2	3.5	>100	>44	28.8	34.9	>100	>3	32.2	68.6	>100	>3

[a]The EC_{50}, EC_{90} (Effective compound Concentration that reduces viral mediated cell death by 50% and 90% respectively) and CC_{50} (compound concentration that reduces cell viability by 50%) are in µM and derived after analysis at four concentrations (0.8, 4, 20, 100 µM). The selectivity index SI_{50} is CC_{50}/EC_{50}.

with most compounds (i.e. **1**, **4**, **10**, **33**) able to rescue cytopathic effects caused by the H1N1 virus at single digit micromolar concentrations. Compound **32** was most potent with a sub-micromolar EC_{50} value. The antiviral activity of these compounds was in general attenuated towards H3N2 and H5N1, and all the compounds were inactive against a common Influenza B strain, B/Brisbane/60/2008 (data not shown). Notably, compound **10** showed broad-spectrum antiviral activity against all three strains of influenza A viruses, and compound **33** showed potent activity against H1N1 and H3N2 with high selectivity indices. Curiously, we noticed that the sensitivity of this NIAID assay was reduced compared to our primary assay.

The most promising compounds (i.e. **4**, **6**, **10**, **32**, **33**) were then cross-examined for their ability to inhibit NS1 function in the yeast-based assay that discovered the screening hit, which demonstrated compound-dependent restoration of yeast growth. The data for the lead molecule **32** is presented in **Figure 4** (compounds **4**, **6**, **10** & **33** show the same behavior and are not shown). Examination at three time intervals (22, 42 & 60 h) clearly indicated a reversal of the slow growth phenotype that was observed with the NS1-pYES strain. Thus, **32** antagonized NS1 function in yeast, confirming that structural changes to the hit had not compromised the functional activity. Moreover, restoration of yeast growth indicates that the compound does not display acute toxicity in this assay.

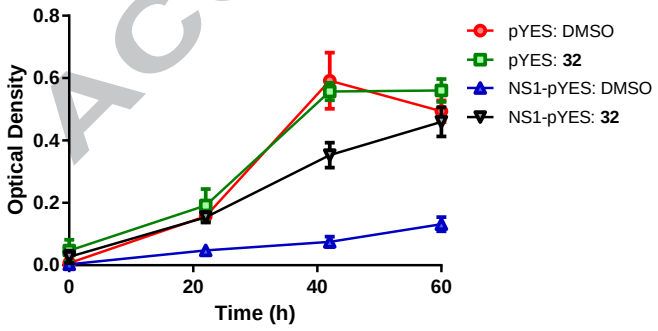


Figure 4. Compound-dependent restoration of yeast growth: Growth of the control yeast strain pYES (without NS1) and the NS1-expressing stain NS1-pYES measured as OD600nm at 22, 42 and 60 h. pYES shows growth in the presence of DMSO and **32** (50 µM), while the NS1-pYES strain exhibits a slow growth phenotype, which is reversed by the presence of **32** (50 µM).

One of the ways that NS1 enhances viral replication is by blocking the cellular IFN response.[12, 15, 26] Consequently, antagonists of NS1 function might be expected to restore IFN-β

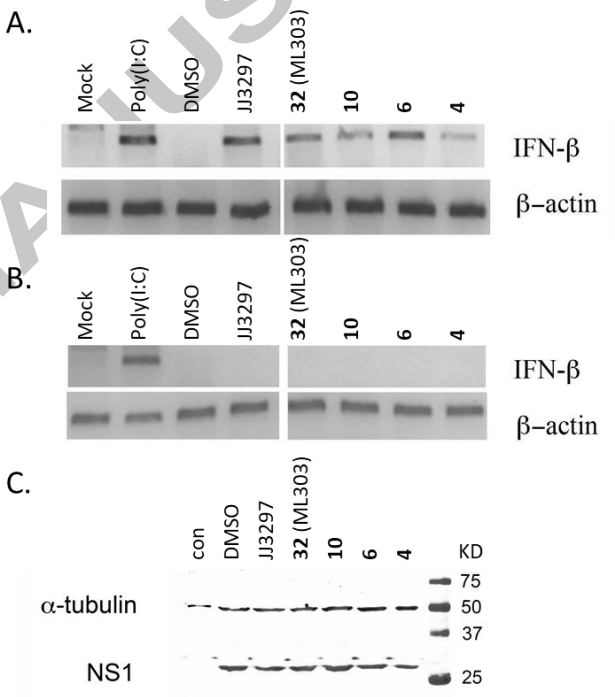


Figure 5. A) MDCK cells were infected with A/PR/8/34 at a MOI of 2 and incubated in the presence of 20 µM concentration of indicated compounds for 6 h (time). Cells were harvested for RT-PCR analysis of IFN-β and β-actin mRNA. Second lane showing induction of IFN-β in the presence of poly(I:C) represents a positive control for the RT-PCR and induction of IFN- β mRNA; **B**) Cells were uninfected, except for the third lane, where cells were infected with A/PR/8/34. Drug treatment and RT-PCR were as described for panel (A); **C**) Cells were uninfected (first lane) or infected with A/PR/8/34 in the presence of DMSO or 20 µM of the indicated compounds. Western blots were performed for NS1 and α-tubulin as loading control.

mRNA levels in infected cells. As such, compounds **4, 6, 10** and **32** were further evaluated for their ability to restore IFN-β mRNA levels in MDCK cells. MDCK cells were infected with A/PR/8/34, at a MOI of 2, for 6 hours (enough time to allow an initial round of viral replication in a majority of cells), and then harvested to detect IFN-β mRNA via RT-PCR analysis. The four representative pyrazolopyridines significantly restored IFN-β mRNA levels in infected cells (**Figure 5A**). Compounds **6** and **32** were the best, with **6** able to restore IFN-β level similar to the known NS1 antagonist JJ3297[31, 37] (lane 4) or the known IFN-β inducer poly(I:C)[38] (lane 2). Importantly, the compounds did not induce IFN-β in the absence of A/PR/8/34 infection (**Figure 5B**),

demonstrating that they were antagonizing specific functions of response. Also, as shown in **Figure 5C**, none of the compounds directly affected the steady-state level of NS1 protein in infected cells, which indicates that the inhibitors act functionally, and not at the level of NS1 production or stability.

We then set out to demonstrate that the ability for the pyrazolopyridines to restore IFN-β mRNA levels in MDCK cells is dependent on NS1. Poly(I:C) is a strong inducer of IFN-β mRNA, and NS1 is known to specifically block this induction. We recapitulated this activity by co-transfection of an NS1 expression plasmid and an IFN-β luciferase reporter plasmid, where poly(I:C)-induced luciferase activity was abrogated in the presence, but not in the absence, of NS1 (**Figure 6A**). Treatment with NS1-inhibitory compounds in the presence of both the plasmids efficiently restored poly(I:C)-induced luciferase activity, demonstrating that that they block NS1 function. Importantly, in the absence of NS1 transfection and poly(I:C) treatment (**Figure 6B**), the compounds had no effect on luciferase activity, indicating that they do not induce the IFN-β reporter on their own and that their action strictly requires NS1 expression. Thus, the compounds are able to exert their effect only in the presence of the NS1 protein.

Together, these experiments strongly suggest that the key pyrazolopyridines interfere with the function of NS1, specifically its ability to ameliorate the host innate immune response. Because our goal is to provide the research community with tool compounds that can be used in therapeutically relevant, advanced *in vivo*, proof-of-concept studies, and because no previous NS1 antagonists have been evaluated *in vivo* in the literature, we carried out preliminary examination of the key analogs' *in vitro* ADME properties. To that end, most compounds showed good stability in mouse liver microsomes (MLM), moderate solubility, and moderate permeability (**Table 3**). The compounds also showed low efflux in a Caco-2 monolayer, though the absolute permeability numbers in the Caco-2 assay were also low, which is characteristic of compounds with low solubility. In order to position this series towards *in vivo* evaluation, we selected **32** and **33** as two representative members for a pharmacokinetics (PK) study.

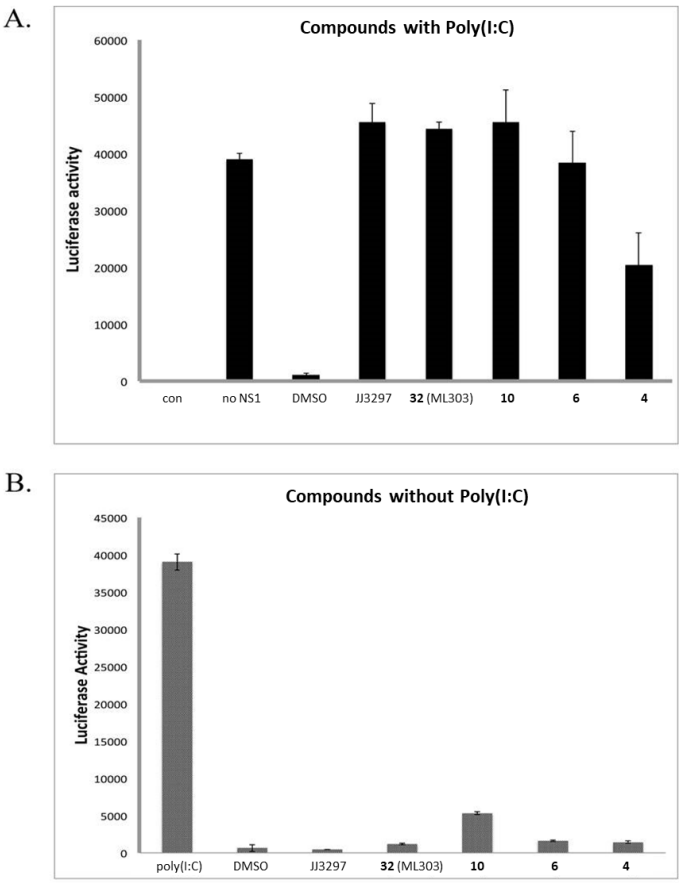


Figure 6. A) 293 cells were co-transfected with a firefly luciferase reporter driven by the human IFN-β promoter and NS1-encoding expression constructs. At 16 h after transfection, cells were treated with 50 µg of poly(I:C)/mL and the indicated compounds (20 µM), and incubated for an additional 24 h. The cells were harvested, and their luciferase activity was measured using a Glo-max luminometer (Promega). "con" indicates the luciferase activity of the untransfected cells; "DMSO" indicates treatment with 1% DMSO; **B**) 293 cells were transfected with the IFN-β reporter and treated with poly(I:C), 1% DMSO, or 20 µM of the indicated compounds in the absence of poly(I:C).

Table 3. *In vitro* ADME data for key analogs.

cmpd	Aqueous Kinetic Solubility	Mouse Liver Microsomal Stability (% remaining)			Caco-2 Permeability (10^{-6} cm/s after 2 h)			Efflux Ratio
	(µM)	0 min	15 min	30 min		$P_{app\ A\ to\ B}$	$P_{app\ B\ to\ A}$	
32	3.1	100	66	45		0.10	0.23	2.29[a]
					3% BSA	0.22	0.27	1.20[b]
33	1.7	100	25	16		0.13	0.38	2.88
6	1.9	100	93	90		0.34	2.64	7.78
4	11.6	100	78	64				
10	3.0	100	74	60				

[a]The mass recovery was 5 and 9%, respectively
[b]The mass recovery was 49 and 81%, respectively, with 3%BSA.

Table 4. Pharmacokinetics parameters for **32** and **33.**

PK parameters	Unit	Plasma	Liver	Lung	Plasma	Liver	Lung
		Compound 32			Compound 33		
T_{max}	h (time)	0.250	0.250	0.250	0.083	0.083	0.083
C_{max}	ng/mL	2437	49600	18567	4470	64000	36200
Terminal $t_{1/2}$	h	4.6	4.7	4.5	5.2	5.3	5.0
AUC (AUC_{last})	h*ng/mL	7655	80218	53854	5960	119000	49100
AUC_{INF}	h*ng/mL	53894	80282	53894	5970	120000	49100
AUC_{organ}/AUC_{plasma}	%		1048	704		2010	823

A single intraperitoneal (IP) dose of 30 mg/kg of **32** and **33** in male C57BL/6 mice led to an AUC_{INF} of 53894 and 49100 h*ng/mL, respectively, and >1 μM concentration in the lungs, the primary site of infection, for over 12 hours (**Table 4, Figure 7**). The overall exposures in the liver and lungs were ~10-20 and ~7-8 times that in plasma. The half-life was the same ($T_{½}$ ~ 4-5 h) in all organs for both compounds in plasma, liver and lung, with no signs of drug accumulation or toxicity. There were also no adverse effects observed at this single dose. The reasonable PK profile of these pyrazolopyridines should allow future *in vivo* experiments to examine NS1 antagonism.

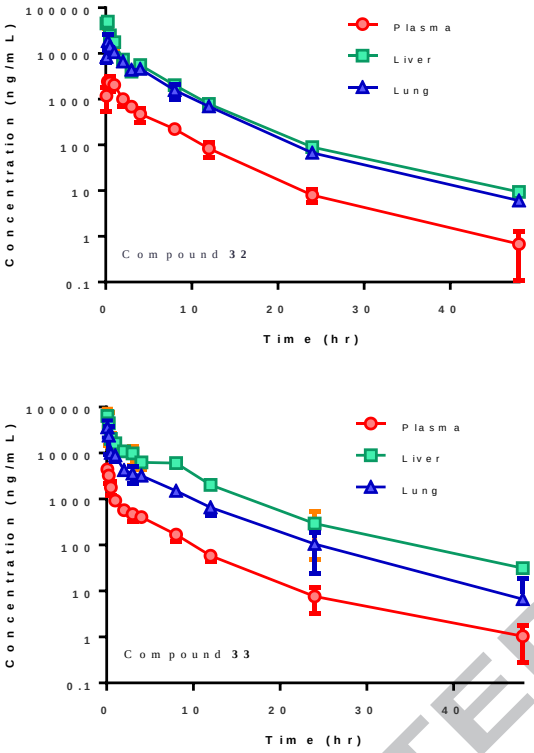


Figure 7. Mean plasma, liver, and lung concentrations-time profiles for compounds **32** and **33** after IP dose of 30 mpk in male C57BL/6 mice (N=3).

In summary, following a qHTS campaign for NS1 antagonists, we discovered a novel class of pyrazolopyridines that inhibits NS1 function. SAR evaluation produced several compounds that can potently reduce virus replication, cytoprotect against seasonal influenza A H1N1, and restore IFN-β mRNA levels in infected MDCK cells. Two of these analogs, **32** and **33**, have acceptable pharmacokinetic profiles, making them amenable for *in vivo* experiments. Compound **32** has previously been disclosed as the probe **ML303** in a report deposited in the public domain.[39] We believe that this pyrazolopyridine series should represent useful tools that will allow future researchers to gauge NS1 antagonism in mouse models of influenza, and determine if NS1 antagonism can either reduce viral load or rescue animals from toxic effects of the influenza virus to validate NS1 as a therapeutic target.

Acknowledgments

This work was supported by NIH grant R03MH085680 to DAE and R44AI084244 to D.B. This work was also supported by contract HHSN272201100019I from the Respiratory Diseases Branch, Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA.

Competing Interests Statement

The authors declare that they have no competing interests.

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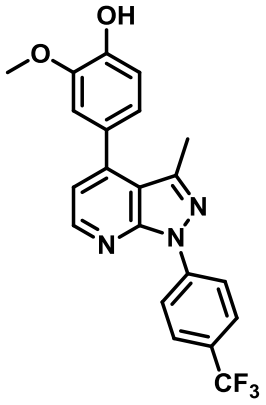

Novel pyrazolopyridine NS1-antagonists for influenza A

Reduces viral titer by 128-fold at 10 μM

Inhibits cytopathic effects of a seasonal influenza A strain (EC_{50} = 660 nM)

Restores IFN-β mRNA levels in virus-infected MDCK cells

Pharmacokinetics: > 1μM concentration in lungs for over 12 h after 30mpk IP single dose in mouse