Letters

N-(3,3a,4,4a,5,5a,6,6a-Octahydro-1,3-dioxo-4,6-ethenocycloprop[f]isoindol-2-(1*H*)-yl)carboxamides: Identification of Novel Orthopoxvirus Egress Inhibitors

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Abstract: A series of novel, potent orthopoxvirus egress inhibitors was identified during high-throughput screening of the ViroPharma small molecule collection. Using structure−activity relationship information inferred from early hits, several compounds were synthesized, and compound **14** was identified as a potent, orally bioavailable first-in-class inhibitor of orthopoxvirus egress from infected cells. Compound **14** has shown comparable efficaciousness in three murine orthopoxvirus models and has entered Phase I clinical trials.

Once considered a scourge of mankind, the smallpox (variola) virus has gained notoriety as a potential biological threat. Although mass vaccination remains the primary response by national public health agencies to a terrorist attack using variola virus, concerns over vaccine-related adverse events have prevented institution of a general immunization program.[1] Additionally, the induction period required for a vaccine-induced immune response will necessitate a period of risk before establishment of protective immunity (the "immunity gap"). While several small molecule inhibitors of orthopoxvirus replication have been identified, nonspecific mechanism of virus inhibition has resulted in narrow therapeutic windows.[2] The nucleotide analogue cidofovir (CDV; 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine dihydrate) is the best studied of these.[3] Approved for treatment of cytomegalovirus (CMV) retinitis in AIDS patients, this broad spectrum viral DNA polymerase inhibitor[4] is administered intravenously, and dose-limiting nephrotoxicity requires additional management.[3b] While work continues to address the shortcomings of CDV,[5] there is a recommendation from the Institute of Medicine of the National Academies to identify additional inhibitors with alternate mechanisms of action.[3b,6] Here, we report on the identification, synthesis, and structure−activity relationships (SAR) of a series of novel, orally bioavailable, orthopoxvirus egress inhibitors.

Using vaccinia and cowpox viruses, high-throughput assays were used to measure virus-induced cytopathic effects (CPE) and screen approximately 356 000 commercial, low molecular weight compounds. Examination of hits from the screening revealed a series of tricyclononene carboxamides (Figure 1) with effective concentrations that inhibited 50% of the virus-induced CPE (EC_{50}) ranging from 13 nM to the limit of testing (>5 μM). These novel structures are unique as antiviral agents. Nascent SAR from the screening hits indicated that electron-withdrawing substitution on the carboxamide aryl or heteroaryl enhanced potency of the molecules in the CPE assay. To validate this nascent SAR, a series of analogues were prepared (Scheme 1) and tested against both vaccinia and cowpox viruses in CPE assays. Synthesis of compounds **2**−**14** and **16**−**18** was accomplished in a single step via condensation of an acyl hydrazide with known tricyclononane anhydride,[7] **1a**, or its reduced form **1b**. The structure of example **13** was confirmed by X-ray crystallography (Figure 2). The synthesis of compound **15** was performed in a similar fashion from the fully saturated anhydride via, **1b**.

In particular, electron withdrawing substitution on the carboxamide carbonyl R-group provided the most potent inhibitors of orthopoxvirus CPE (Table 1). The 4-nitrophenyl substituted carboxamide, **2**, was 100-fold more potent than the electron-donating 4-dimethylaminophenyl analogue, **3**, against both vaccinia and cowpox viruses. While the aza-π-deficient 3- and 4-pyridyl examples **6** and **7** displayed potency against vaccinia, the 2-pyridyl analogue, **5**, displayed a dramatic loss of potency. In all cases, heterocyclic substitution provided modest to weak potency against vaccinia virus and exhibited no EC_{50} up to the highest concentrations tested against cowpox virus. For the chloro- and bromo-substituted phenyls (compounds **8**−**13**), a similar pattern was observed for both viruses, where 3- and 4-substitution were more potent than 2-substitution. Reduction of the olefin had little effect on potency (**14** and **15**). Additionally, these compounds displayed excellent activity against a CDV-resistant strain of cowpox virus (Table 1). Examination of selected pairs (**5** and **7**; **11** and **13**; **14** and **15**) in CPE assays against vaccinia, cowpox, monkeypox, and camelpox viruses, and two strains of variola virus, established this broad-spectrum trend (where 4-substitution•3-substitution >>> 2-substitution) for the *Orthopoxviridae* family, and most importantly, confirmed activity against the target threat, smallpox virus (Table 2).

The mechanism of action for the observed activity was systematically uncovered through virus yield and resistance mapping of cowpox drug-resistant variants.[8] These studies indicated that compound **14** inhibited formation of extracellular forms of virus. Marker rescue of compound-resistant cowpox virus variants localized the reduced susceptibility phenotype to the cowpox virus V061 gene, which is homologous to the vaccinia virus F13L gene. This gene encodes a major envelope protein necessary for the formation and egress of extracellular virus particles. Based on these data and the fact that compound **14** inhibited plaque formation and virus-induced CPE, the effects of compound **14** on virus egress were examined. Virus yield

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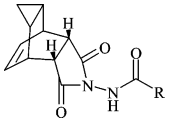


Figure 1. General structure of orthopoxvirus egress inhibitors.

Scheme 1. Synthesis of Compounds **2**−**18**

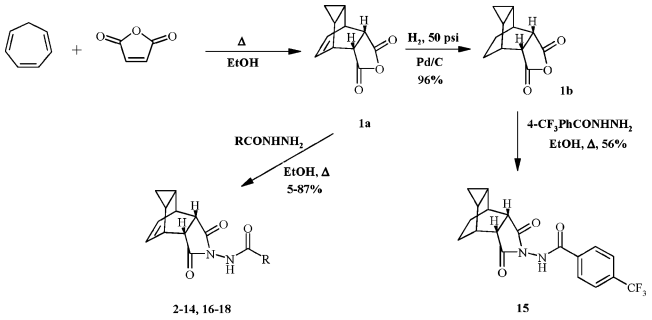
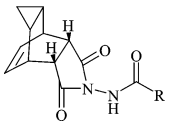


Table 1. CPE Assay Screening Results



	R	vaccinia EC_{50} (μM)	cowpox EC_{50} (μM)	CDV^r cowpox EC_{50} (μM)	CC_{50} (μM)
2	4-nitrophenyl	0.02	0.15	0.10	>86 ± 20
3	4-Me_2N-phenyl	2.0	15.5	3.5	>100 ± 0
4	4-aminophenyl	7.7	>20	>50	>92 ± 11
5	2-pyridyl	>20	>20	>20	>100 ± 0
6	3-pyridyl	0.74	>20	>5	>100 ± 0
7	4-pyridyl	0.5	17.2	1.8	>100 ± 0
8	2-chlorophenyl	3.0	>20	ND	>100 ± 0
9	3-chlorophenyl	0.04	0.6	ND	>100 ± 0
10	4-chlorophenyl	0.02	0.77	ND	>100 ± 0
11	2-bromophenyl	2.3	>20	4.0	>100 ± 0
12	3-bromophenyl	0.05	0.6	0.03	>100 ± 0
13	4-bromophenyl	0.02	1.6	0.1	>100 ± 0
14	4-CF_3phenyl	0.04	0.6	0.07	>100 ± 0
15	satd 4-CF_3phenyl	0.02	0.3	0.02	>100 ± 0
16	4-methoxyphenyl	2.2	>20	3.1	>100 ± 0
17	2-(1-methyl)-pyrrolyl	15.8	>20	4.7	>100 ± 0
18	5-(3-methyl)-pyrazolyl	7.1	>20	4.6	>100 ± 0

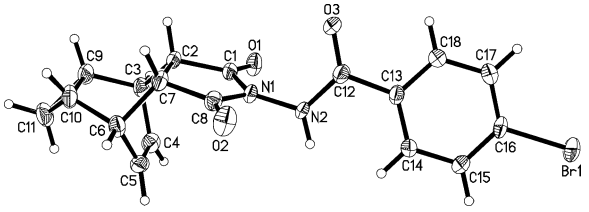


Figure 2. X-ray crystal structure of compound **13**.

assays were conducted to measure the amount of intracellular and extracellular virus produced in the presence and absence of compound. The results show that compound **14** reduced extracellular virus titers by approximately 158-fold at 24 h post-infection while reducing the level of intracellular virus titers relative to untreated controls by 11-fold (Figure 3). These results suggest that compound **14** inhibits extracellular virus formation and are consistent with F13L being the target of antiviral activity. Furthermore, orthopoxvirus selectivity for compound **14** was confirmed in plaque reduction EC_{50} values > 40 μM

Table 2. CPE Results against a Panel of Orthopoxviruses

	EC_{50} (μM)					
					variola	
example no.	vaccinia	cowpox	monkeypox	camelpox	BUT	BSH
5	>20	>20	>16	4.2	10	>20
7	0.5	17.2	0.15	0.11	0.22	0.4
11	2.3	>20	3.6	3.8	1.4	3.3
13	0.02	1.6	0.01	0.01	0.02	0.05
14	0.04	0.6	0.01	0.01	0.02	0.05
15	0.02	0.3	0.08	0.03	0.01	0.2

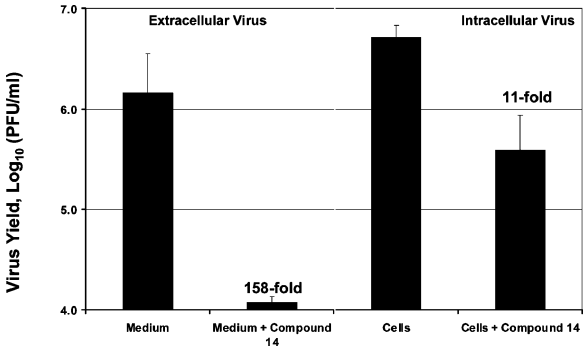


Figure 3. Inhibition of virus egress by compound **14**. BSC-40 cells were infected with 2 pfu/cell of vaccinia virus strain IHD-J and incubated in the presence and absence of 5 μM compound **14**. At 24 h post-infection, extracellular virus in the medium and cell-associated virus were quantified by plaque assay. *Y* axis = log virus titer. Error bars represent the standard deviation of the mean of triplicate virus yield determinations.

Table 3. S9 Metabolic Stability of Compounds **13** and **14**

	$T_{1/2}$[a] (min)				
cmpd	rat	mouse	dog	monkey	human
13	>200	>200	>200	>200	57
14	>200	>200	>200	>200	>200

[a] NADPH added.

against other double-stranded DNA viruses (HSV-1, CMV) and RNA viruses (RSV, BVDV, rotavirus).[8]

With SAR defined, we began to examine the pharmacokinetics for these inhibitors. Metabolic stability was measured in vitro by incubating compounds **13** and **14** with microsomal (S-9) extracts derived from the livers of multiple animal species and measuring the loss of the parent compound by LC/MS. From the decay curve, the half-life of compound was calculated and used as a measure of metabolic stability. As shown in Table 3, the metabolic stability ($T_{1/2}$) for each compound was greater than 200 min. Ultimately, the superior human S-9 stability for compound **14** led to the advancement of this compound into pharmacokinetic (PK) studies.

Oral and intravenous administration of compound **14** in rats demonstrated oral bioavailability of 31% with a clearance rate and plasma drug exposure (area under the concentration time curve (AUC)) at levels above the in vitro EC_{50} values (Table 4). Subsequent dose ranging and tolerability studies in mice supported proof-of-concept animal studies. Compound **14** was tested in three murine models of orthopoxvirus disease and demonstrated efficacy comparable to CDV.[8] Compound **14** was found to be safe and well tolerated after 28-day repeat dosing in mice and cynomolgus macaques with no observable adverse effect levels of 2000 mg/kg and 600 mg/kg, respectively. Based on the PK profile and safety profile, compound **14** (ST-246) is currently being evaluated in human Phase I clinical trials.

Table 4. Oral Pharmacokinetics of Compound **14** in the Rat at 10 mg/kg (0.75% Methocell/1% Tween)

dose	2 mg/kg
T_{max}	2.2 h
C_{max}	0.92 μg/mL
AUC	6.10 μg·h/mL
$t_{1/2}$	4.2 h
CL	0.52 L/h/kg
V_{ss}	0.6 L/kg
%*F*	31

A series of potent, selective orthopoxvirus inhibitors that act by inhibiting virus egress from infected cells has been discovered. Compound **14** demonstrated favorable PK properties, efficacy in murine models of orthopoxvirus disease following oral administration, and a safety profile that supports use of this compound in humans. SAR around the aryl carboxamide indicates correlation between electron withdrawing substituents and potency. Additional SAR studies around this scaffold will be discussed in a future report.

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Supporting Information Available: Experimental details of the synthesis and characterization of the orthopoxvirus inhibitors and crystallographic information for compound **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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