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Discovery of Potent EV71 Capsid Inhibitors for Treatment of HFMD

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KEYWORDS *HFMD, Enterovirus 71, Capsid inhibitor*

ABSTRACT: Enterovirus 71 (EV71) is a major causative agent of hand, foot and mouth disease (HFMD), which can spread its infections to the central nervous and other systems with severe consequences. The viral capsid protein-VP1 is a well-known target for antiviral efficacy because its occupancy by suitable compounds could stabilize the virus capsid, thus prevent uncoating of virus for RNA release. In this paper, design, synthesis and biological evaluation of novel anti-EV71 agents (aminopyridyl 1,2,5-thiadiazolidine 1,1-dioxides) are described. One of the most promising compounds **14** showed excellent antiviral activity against EV71 ($EC_{50} = 4$ nM) and exhibited excellent *in vivo* efficacy in the EV71 infected mouse model.

Introduction

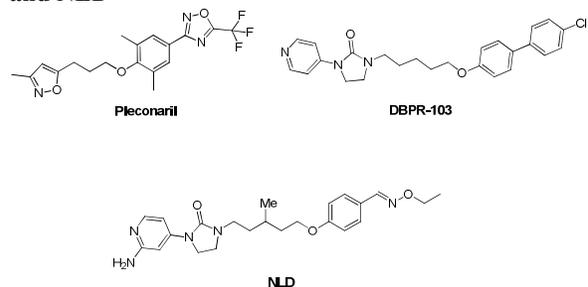
Hand, foot, and mouth disease (HFMD) is a common infectious disease mainly occurring in young children. Clinical symptoms of HFMD are characterized by fever, sore throat and vesicular rash on the hands and feet as well as exanthema on oral mucosa and tongue. For several decades, HFMD has been a very common infection in children all over the world, especially in the Asia-Pacific region. HFMD is caused by a spectrum of pathogens in the enterovirus (EV) family, enterovirus 71 (EV71) and coxsackievirus A16 (Cox A16) are regarded as the major causative agents. In particular, EV71 is more often associated with neurological diseases such as meningitis, encephalitis, monoplegia, acute flaccid paralysis and even death, especially among young children (≤ 5 years old) [1-2]. So far, no marketed anti-enterovirus drugs are available for the treatment of EV71-related disease, though the first EV71 vaccine is now approved by cFDA to prevent this disease [3]. Supportive therapy is still the primary treatment for severe cases of EV71 infection. Ribavirin was used for treating mild HFMD in clinic. But Ribavirin showed no anti-EV71 activity with $EC_{50} > 100$ μ M in the cytopathogenic effect inhibition assay (CPE) in house. Considering the danger of severe EV71 infection in children, there is an urgent need to develop effective antiviral treatment to prevent or treat EV71-related disease before next EV71 outbreak.

EV71 has a non-enveloped, positive-sense, single-stranded RNA encoding capsid proteins (VP1, VP2, VP3, and VP4) and nonstructural proteins (2A, 2B, 2C, 3A, VPg, 3C, and 3D) that are involved in the life cycle of viruses including absorption, invasion, shelling, and replication. All the functional protein involved in the life cycle of viruses can be targets for the development of antiviral drugs [4]. We have previously disclosed the discovery of a series of peptidomimetic 3C^{pro} inhibitors [5].

The viral protein-VP1 is a well-known target for antiviral design, because its hydrophobic pocket can be occupied by suitable compounds, which lead to stabilize the virus capsid and prevent uncoating of virus for RNA release [6]. WIN series

from Sterling-Winthrop is a prominent series of capsid-binding compounds. Pleconaril in this series is a typical small molecule inhibitor which has been shown to have a broad spectrum of activities against enteroviruses and rhinoviruses and has been tested in clinical trials [7]. According to what Shia and coworkers reported, Pleconaril showed no antiviral activity against EV71. Nevertheless, using skeletons of WIN compounds as templates, they identified a novel series of imidazolidinone derivatives with significant antiviral activity against enterovirus 71. Among this series of compounds, DBPR103 was identified as a very potent enterovirus 71 inhibitor ($EC_{50} = 54$ nM) with no apparent cytotoxic effect toward RD (rhabdomyosarcoma) cell lines ($CC_{50} > 25$ μ M) [8-9]. During our research, Rao and coworkers reported a paper using structural analysis of the complete virus to guide the design of EV71 inhibitors. Using pyridyl imidazolidinones as a template, they discovered a potent EV71 capsid inhibitor-NLD, which was able to protect cells from EV71 infection at a concentration of 25 pM [10-11]. Based on these studies, here we report the chemical synthesis and biological evaluation of novel aminopyridyl 1,2,5-thiadiazolidine 1,1-dioxides as EV71 capsid inhibitors to treat HFMD.

Scheme 1 Chemical structures of Pleconaril, DBPR-103 and NLD



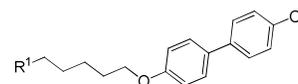
Result and Discussion

Pleconaril and DBPR-103 were tested in the cytopathogenic effect inhibition assay (CPE) in-house. Pleconaril showed no anti-EV71 activity with $EC_{50} > 100 \mu\text{M}$ and DBPR-103 was a potent EV71 inhibitor with $EC_{50} = 91 \text{ nM}$. Data were consistent with those reported in literatures [8-9]. Our research was focused on structure modifications of pyridyl imidazolidone compound DBPR-103. Therefore, the primary task of the project is to identify a novel skeleton based on pyridyl imidazolidone series that could maintain potent antiviral activity. As reported by Chern, the five carbon alkyl linker and terminal pyridine play a very important role in influencing anti-EV71 activity [9]. We keep these 2 positions unchanged.

First, imidazolidone modification was designed and evaluated by the CPE assay. Among this series, 2-ketopiperazines **1** and 1,2,5-thiadiazolidine 1,1-dioxide **5** [12] kept antiviral activity with EC_{50} as 180 nM and 110 nM, respectively. The rest modifications all resulted into loss of antiviral activity. Compound **1** ($CC_{50} = 3.2 \mu\text{M}$) has moderate cytotoxicity while compound **5** ($CC_{50} > 100 \mu\text{M}$) has no such issue.

Another structure modification strategy was the cyclization of phenyl alkyl ether. According to this strategy, several benzoheterocycle compounds were designed and evaluated, as showed in table 2. Most of these compounds lost activity, except the benzodihydropyran **6** ($EC_{50} = 48 \text{ nM}$), which is 2 folds more potent than DBPR-103. Compound **6** also showed moderate cytotoxicity ($CC_{50} = 3 \mu\text{M}$) like **1**.

Table 1 Structure–Activity Relationship of imidazolidone modification.

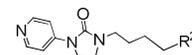


Compd No	R ¹	EC ₅₀ (nM) ^a	CC ₅₀ (μ M) ^b
DBPR103		91	> 100
1		180	3.2
2		1200	-
3		> 1000	-
4		2800	-
5		110	> 100

^a The compound concentration required to reduce the virus-induced cell death by 50% was defined as EC₅₀.

^b The compound concentration required to reduce cell viability to 50% of the tested control culture was defined as CC₅₀.

Table 2 Structure–Activity Relationship of phenyl alkyl ether cyclization.



Compd No	R ²	EC ₅₀ (nM) ^a	CC ₅₀ (μ M) ^b
DBPR103		91	> 100
6		48	3.4
7		540	-
8		1100	> 100
9		1100	4.3

^a The compound concentration required to reduce the virus-induced cell death by 50% was defined as EC₅₀.

^b The compound concentration required to reduce cell viability to 50% of the tested control culture was defined as CC₅₀.

The three potent compounds **1**, **5** and **6** were further evaluated and compared with DBPR103 through *in vitro* ADME and *in*

in vivo PK profiles. The mouse liver microsomal stability of **5** (< 10 uL/min/mg) indicated slower metabolism than the other 3 compounds (40 - 50 uL/min/mg). The PK data with 2 mg/kg IV and 5 mg/kg IP are summarized in Table 3. Following IV/IP administration, the elimination half-life ($T_{1/2}$) of **1**, **5** and **6** ranged from 5 to 6 h, which is better than DBPR103 and would be suitable for once daily dosing. Compound **6** exhibited the lowest clearance and best exposure in these compounds. Compound **5** also showed good exposure which was 2 folds than that of DBPR103 both in intraperitoneal and intravenous injection. However, **1** and **6** showed hERG inhibition issues (IC_{50} = 0.37 μ M and 1.75 μ M, respectively), compared with **5** (IC_{50} > 30 μ M). Compound **5** was selected to further test in oral PK. The oral exposure of **5** indicated it is also suitable for oral dosing. Taking all factors into consideration, compound **5** is believed to be more suitable as a lead compound.

Table3 Single Dose Mouse *in vivo* PK Properties.

Cpd.	DBPR103	1	5	6	
iv@2mpk					
Cl (mL/min/kg)	28	21	12	6	
$T_{1/2}$ (h)	4	6	6	5	
V_d (L/kg)	9	10	5	3	
AUC (nM*h)	2731	3344	5710	10999	
ip@5mpk					
C_{max} (nM)	453	1255	682	617	3362
T_{max} (hr)	2	2	2	2	1
AUC (nM*h)	4275	8947	9011	6166	23240
%F	64	112	63	43	86

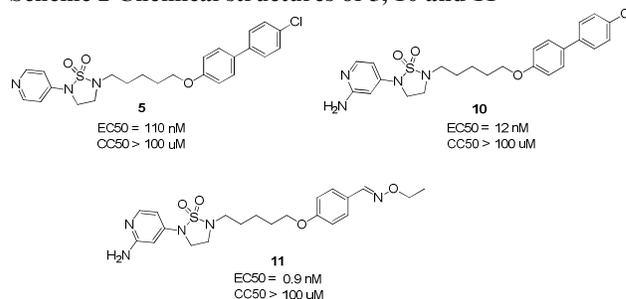
(A) Data reported are the means from the dosing cohorts (Male CD-1 mouse, n = 3/dose). (B) Dosed at 2 mg/kg iv (20% solutol in water), 5 mg/kg ip (20% solutol in water) and 5 mg/kg po (20% solutol in water). (C) Cl is clearance. V_d is volume of distribution. C_{max} represents the highest observed concentration. T_{max} is the time taken to reach the maximum concentration. $T_{1/2}$ is the half-life of the compounds exposure in plasma. AUC is the area under the curve. %F is percent bioavailability.

Since the PK profiles of **5** is good, the next goal of the project is to further increase activity of compounds based on **5** (EC_{50} = 110 nM). According to Chern reported, antiviral activity of DBPR compounds could be improved by replacing chlorobenzene with oxime ether [13-14]. So, we planned to introduce these moieties into 1,2,5-thiadiazolidine 1,1-dioxides to improve the activity. Coincidentally, Rao and coworkers reported a series of EV71 capsid inhibitors (NLD as a representative) during our research process. As shown in NLD structure (scheme 1), the methyl group in the linker and oxime ether has been confirmed to make contribution to activity. The main difference between NLD and DBPR series is the amine group in the 2 position of pyridine. With such slight alteration, NLD was able to protect cells from EV71 infection at a concentration of 25 pM, which is an amazing improvement in activity. To confirm this, NLD was synthesized and tested in CPE assay. NLD exhibited excellent antiviral activity (EC_{50} = 0.03 nM), as previously reported [10]. But NLD shows moderate cytotoxicity (CC_{50} = 5 μ M), and also suffers from severe hERG inhibition (0.8 μ M). We planned to introduce amine group into our 1,2,5-thiadiazolidine 1,1-dioxide serials to check whether this modification can improve activity.

The amine group was added to compound **5** to generate **10** [12], which showed 10 folds more potent than **5**. This indicated the amine made a great contribution to the activity. Compound **11** (EC_{50} = 0.9 nM), which replaced the chlorobenzene of **10** with oxime ether, is 10 folds more potent than **10**. In

total, compound **11** exhibited 100 folds improvement in activity compared to **5** with these 2 steps modification. Furthermore, **10** and **11** have no cytotoxicity (CC_{50} > 100 μ M).

Scheme 2 Chemical structures of **5**, **10** and **11**



To the best of our knowledge, no aldoxime ether structures were tested in clinical therapies. While ketoxime ether structures were widely used in pharmaceutical molecules in the market, like fluvoxamine, ceftriaxone sodium, cefetamet pivoxil and cefuroxime (Scheme 3). This might be because aldoximes ether is more easily metabolized to aldehydes, which are potential genotoxic agents. In this respect, we speculate it will be more drug-like and safer to replace aldoxime ether of **11** to ketoxime ether. So we designed several compounds on the basis of **11**, including acetophenone oxime ether (**12**), propiophenone oxime ether (**13**), benzocyclopentanone oxime ether (**14**), benzocyclohexanone oxime ether (**15**) and some heterocyclic-fused ring as bioisosteres of oxime ether (**16**, **17**, and **18**).

As showed in table 4, most of these compounds lost antiviral activities in CPE assay. Fortunately, benzocyclopentanone oxime ether (**14**) kept excellent anti-EV71 activity (EC_{50} = 4 nM).

Scheme 3 Marketed drugs contain ketoxime ether structure

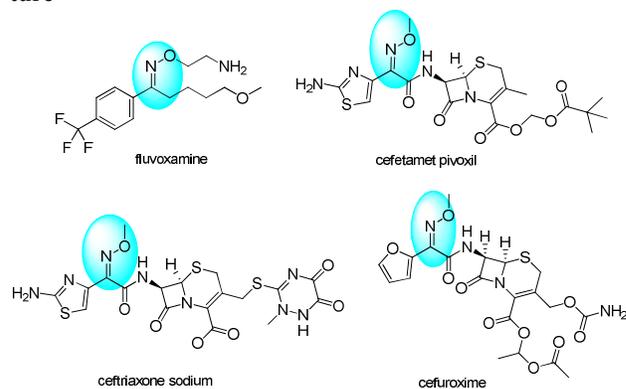
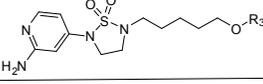
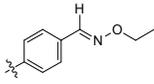
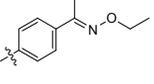
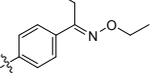
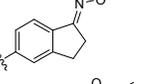
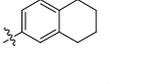
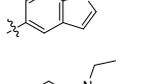
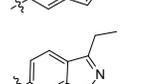
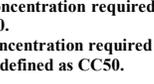


Table 4 Modifications of compound 11.


Compd No	R ³	EC ₅₀ (nM) ^a	CC ₅₀ (μ M) ^b
11		0.9	> 100
12		600	> 100
13		554	> 100
14		4	42
15		309	> 100
16		> 1000	> 100
17		> 1000	> 100
18		352	> 100

a The compound concentration required to reduce the virus-induced cell death by 50% was defined as EC₅₀.

b The compound concentration required to reduce cell viability to 50% of the tested control culture was defined as CC₅₀.

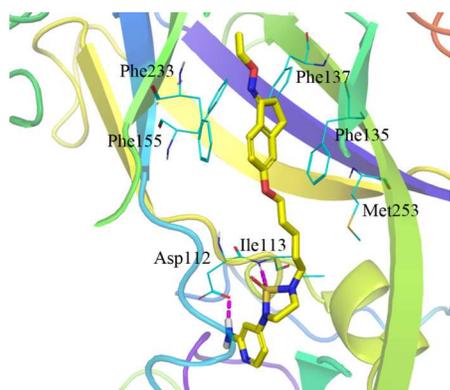


Figure 1. Predicted binding mode of compound **14** on EV71 capsid. X-ray structure of NLD (PDB code 4CEY) was used as docking template. Pink dash stands for hydrogen bonding. The figure was generated with PyMOL Molecular Graphics System, version 1.5.0.4 Schrödinger, LLC.

The molecular modeling method was used to illustrate the binding mode of compound **12**, **14** and **16**. The results indicated that the overall binding mode of compound **14** was similar to that of the NLD on EV71 capsid. The amino-pyridine group could form hydrogen bonding with Asp112. The sulphonyl group could form hydrogen-bonding to the backbone NH of residue Ile113 as that of the carbonyl oxygen of the imidazole

of NLD. The phenoxy group sandwiched between Phe135 and Phe155. And the binding mode of tail oxime group of compound **14** could overlay well with that of NLD in the co-crystal structure (pdb code: 4CEY). The aminopyridine group of compound **12** could overlay with NLD, but its phenoxy group could not overlay well with NLD (see SI figure S2). Both the aminopyridine group and phenoxy group of compound **16** could not overlay well with NLD (see SI figure S3). The binding results are consistent with the cell-based bioactivities of **12**, **14** and **16**. This implies that the inhibition mechanism of compound **14** is targeting on EV71 capsid and blocking viral uncoating as NLD does.

Since NLD suffers from severe hERG inhibition (0.8 μM), we tested the hERG inhibition of two potent compound **11** and **14**. The hERG activities of **11** (9 μM) and **14** (6 μM) are much better than NLD. Compounds **11**, **14** and NLD were further tested and compared by PK characterization (table 5). Pharmacokinetic studies were conducted in mouse to assess the intraperitoneal and intravenous exposure for **11**, **14** and NLD. The elimination half-life (T_{1/2}) of **11**, **14** are 1.6h and 1.5h, respectively. NLD showed high clearance and its half-life (T_{1/2}) is only 0.3h. The IV/IP exposure of **14** is better than **11** and NLD. Also considering the poor drug-like property of aldoxime ether in compound **11**, compound **14** was selected for further exploration in *in vivo* efficacy model.

Table 5 Single Dose Mouse *in vivo* PK Properties

Cpd.	11	14	NLD
iv@1 mpk			
Cl (mL/min/kg)	36	34	105
T _{1/2} (h)	1.6	1.5	0.3
V _d (L/kg)	3.2	2.8	2.4
AUC (nM*h)	592	821	371
ip@5 mpk			
C _{max} (nM)	1443	2152	1558
T _{max} (h)	0.5	0.7	0.3
AUC (nM*h)	2449	3868	1711
%F	79	88	92

(A) Data reported are the means from the dosing cohorts (Male CD-1 mouse, n = 3/dose). (B) Dosed at 2 mg/kg iv (20% solutol in water) and 5 mg/kg ip (20% solutol in water). (C) Cl is clearance. V_d is volume of distribution. C_{max} represents the highest observed concentration. T_{max} is the time taken to reach the maximum concentration. T_{1/2} is the half-life of the compounds exposure in plasma. T_{last} is the time taken to reach the last measurable concentration. AUC is the area under the curve. %F is percent bioavailability.

ICR mouse model (3 days old) with lethal-dose (1 × 10⁶ PFU) EV71 strain 695F infection was used to imitate the severe complications of EV71 infected children. Lycorine, as reported in an EV71 infected model, was used as a positive control [15-16]. Ribavirin is a broad-spectrum antiviral agent and was used for treating mild HFMD in clinic. Compound **14** was dosed 10 mg/kg/d *via* intraperitoneal injection route at different time. One is dosing before EV71 infection (group 3), the other is dosing after EV71 infection (group 4). The vehicle, lycorine and ribavirin groups were dosed before EV71 infection. The results showed that mice in vehicle group all died at day 8. Ribavirin group mice all died at day 5 (100 mg/kg) and day 6 (50 mg/kg), due to toxicity of Ribavirin. Lycorine group mice protection was only 30% after 14 days dosing. Compound **14** showed excellent protection on EV71 infected mouse, which was 100% in both two groups (Figure 2). The

body weight and clinical score also showed **14** was much better than lycorine.

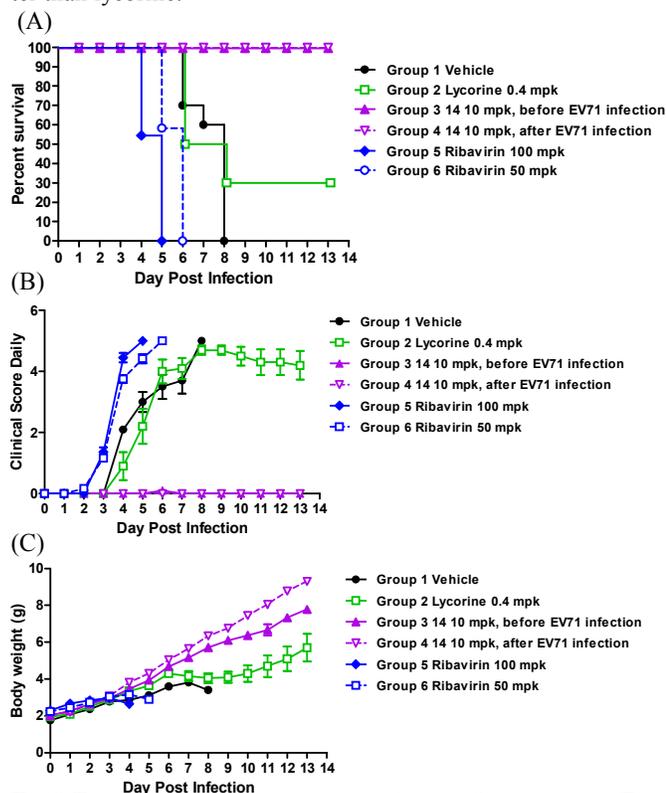
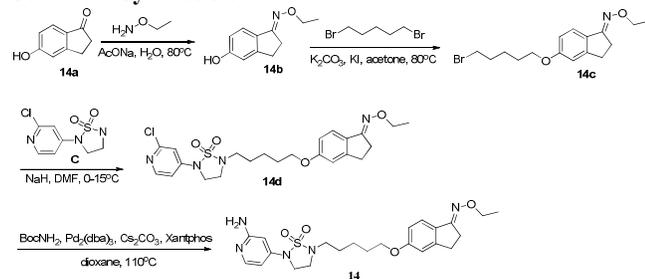


Fig.2 The *in vivo* antiviral activity of **14**. (A) Survival rate. (B) Mice body weight. (C) Clinical score. 3-day-old ICR mice are i.p. inoculated with EV71 and administrated with vehicle (N=10), lycorine (0.4 mpk, N=10), **14** before EV71 infection (10 mpk, N=10), **14** after EV71 infection (10 mpk, N=9), Ribavirin (100 mpk, N=11, or 50 mpk, N=12).

Chemistry

The chemical synthesis of **14** is shown in Scheme 4. Starting from 5-hydroxy-1-indanone **14a**, condensation of **14a** with ethoxyamine gave oxime ether **14b**. Monosubstitution of **14b** with 1,5-dibromopentane afforded intermediate **14c**, which then reacted with **C** to give **14d**. Final product **14** was obtained by the reaction of chloropyridine intermediate **14d** with BocNH₂ and Boc deprotection *in situ*. The synthesis of other compounds can be found in the supporting information.

Scheme 4 Synthesis of 14



Conclusion

Considering the danger of severe EV71 infection in children and no marketed antiviral drugs are available, there is an un-

met medical need to develop effective antiviral treatment. The novel aminopyridyl 1,2,5-thiadiazolidine 1,1-dioxides were first synthesized and their anti-EV71 activities were evaluated by CPE assay. In this series, compound **14** was identified to have excellent antiviral activity against EV71 ($EC_{50} = 4$ nM) and more potent than DBPR103. Furthermore, **14** showed excellent *in vivo* efficacy in EV71 infected mouse model. Thus, **14** is a promising drug candidate for potential application in the clinical therapy of HFMD.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, analytical data, assay protocol are available free of charge on the ACS Publications website.

AUTHOR INFORMATION

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Author Contributions

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ABBREVIATIONS

EV71, Enterovirus 71; HFMD, hand, foot and mouth disease; Cox A16, coxsackievirus A16; CPE, cytopathogenic effect; RD, rhabdomyosarcoma; ADME, absorption, distribution, metabolism, and excretion; PK, pharmacokinetics; CYP, Cytochrome P450; hERG, human ether-a-go-go related gene; MMS, microsomes metabolic stability; IV, intravenous; IP, intraperitoneal; PO, per oral; AUC, area under curve; CL, clearance; T1/2, half-life; Vd, volume of distribution; PFU, plaqueforming unit.

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