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MOUSE AND HUMAN NOROVIRUS HIGH-THROUGHPUT SCREENS TO IDENTIFY A NOVEL NOROVIRUS ANTIVIRAL (CMX521)

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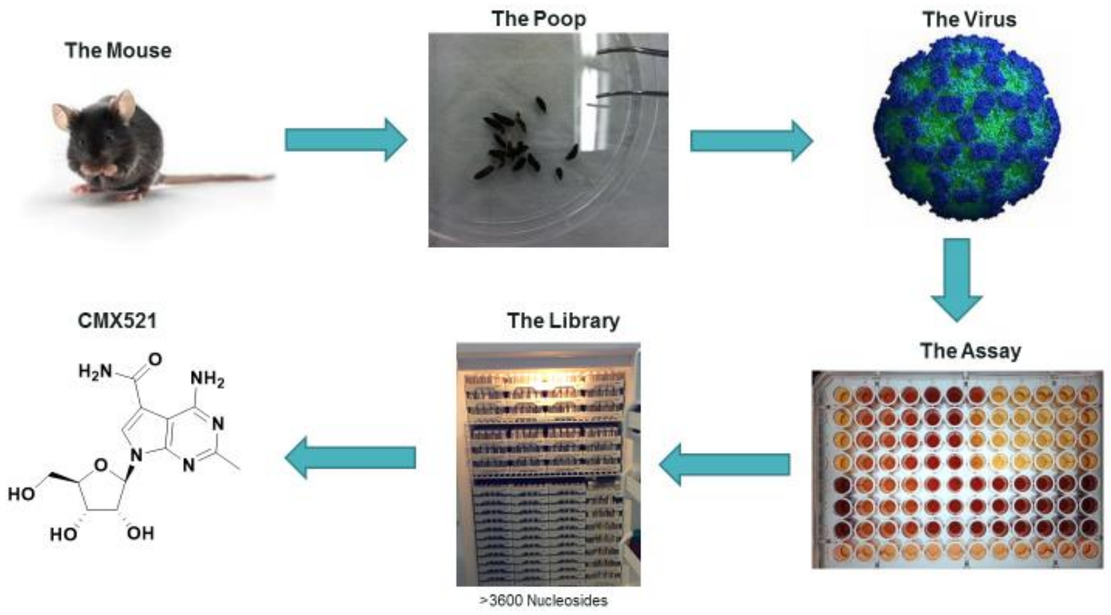
INTRODUCTION

Human norovirus (HuNoV) is the most common cause of epidemic acute gastroenteritis worldwide with an estimated total cost of $64 billion per year (Bartsch, 2016). While acute HuNoV infection typically presents as short-term vomiting and diarrhea, longer-term sequelae have been recognized (e.g., GERD, IBS) and chronic HuNoV is relatively common in transplant patients where clinical symptoms can last for many months causing significant morbidity and even mortality (Bok, 2013). Currently, there are no FDA-approved vaccines or small molecule therapeutics available for treatment or prophylaxis of norovirus. Genetic drift and diversity has made the development of a HuNoV vaccine difficult. The discovery of broad-spectrum norovirus antivirals has been hindered by the lack of good in vitro culture systems for HuNoV. Given the high sequence conservation in the active site of the norovirus RNA polymerase (93% amino acid identical, Table 1), we reasoned that mouse norovirus (MNV) could be used to identify nucleoside antivirals with the desired mechanism of action to have broad activity against noroviruses. A novel MNV was isolated from mouse feces and characterized. This highly cytolytic MNV isolate was used in a high-throughput cell-based, cytopathic effect (CPE) assay to screen our nucleoside compound library. Compounds showing cytoprotective activity in 3-shot screens were confirmed in a full dose-response assay against MNV and HuNoV. The best compound found was CMX521. Toxicity assessments demonstrated a lack of mitochondrial toxicity, no genotoxicity and a favorable selective index. Additional assays with CMX521 revealed broad activity across all MNV and HuNoV strains tested to date. CMX521 is currently in Phase 1 clinical studies.



METHODS

MNV 3-Shot and EC_{50} Assays

- The Chimerix nucleoside analog library was screened for norovirus activity using 2'-C-methylcytidine (2'-CMC, Rocha-Pereira, 2013) as a positive control.
- MNV was isolated at Chimerix from mouse feces and sequenced (GenBank #MF326938). Clone (DS-2) was purified through limited dilutions and plaque purification in RAW cells, and stored frozen at -80°C.
- RAW 264.7 cells were obtained from the ATCC. Cells were maintained and assayed in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, 1% MEM NEAA, 1% GlutaMAX , and 1% HEPES.
- 96-well plates were seeded with 5E4 RAW cells/well and infected at an MOI of 0.0005. Compounds were tested in 3-shot format at 100, 10, or 1 µM. Plates were incubated for 2 days at 37ºC and 5% CO_2. CPE protection was determined using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay.
- Compounds showing significant protection of infected cells were progressed to a dose-response curve assay. EC_{50} values were calculated using Gen5 software with a 4-parameter curve-fitting function (Table 2).
- Protein-binding effect was tested by adding physiological concentrations of human serum proteins, 2 mg/mL alpha-1-acid glycoprotein (AAG) and/or 40 mg/mL human serum albumin (HSA) added to the cell culture medium.
- Compound-treated, uninfected RAW cells were used to generate cytotoxicity data (CC_{50}) using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Table 3).

HuNoV Replicon EC_{50} Assay

- HG23 cells containing the GI.1 HuNoV replicon were obtained from Kyeong-Ok Chang (Kansas State Univ., 2006). Cells were maintained in DMEM containing 10% heat-inactivated FBS, 1% penicillin / streptomycin / glutamine, and 0.5 mg/mL G418. G418 was not included during antiviral assays.
- 96-well plates containing compound dilutions were seeded with 1E4 HG23 cells/well and incubated for 4 days at 37°C, 5% CO_2. Media was then removed, wells were rinsed with 4ºC PBS and cells were lysed with 100 µL lysis buffer (0.5 mg/mL protease K, 50 mM KCl, 10 mM Tris-Cl pH 8.0, 2.5 mM $MgCl_2$, 0.45% IGEPAL 0.45% Tween-20, DEPC-treated water) for 30 minutes at 65°C, then 2 minutes at 98°C.
- Intracellular HuNoV RNA was measured by RT-qPCR using SensiFAST Probe Lo-ROX polymerase, forward and reverse primers COG1F (CGY TGG ATG CGN TTY CAT GA), COG1R (CTT AGA CGC CAT CAT CAT TYA C), and probe RING1a-TP (56-FAM/AGA TYG CGA /ZEN/TCY CCT GTC CA/3IABkFQ). Viral copy number was determined using DNA standards. RT-qPCR conditions were: 1 cycle at 48ºC for 10 minutes, 1 cycle at 95ºC for 2 minutes, followed by 45 cycles of 95ºC for 10 seconds and 56ºC for 60 seconds using an Applied Biosystems 7500 Real-Time PCR System.
- Compound-treated HG23 cells were used to generate cytotoxicity data (CC_{50}) using CellTiter Glo® Reagent (Table 3).

Additional Assays

- **MT4 CC_{50} Assay:** MT4 cells were obtained from the NIH AIDS Reagent Program and grown in RPMI-1640 Medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The assay was performed as described above for the HG23 CC_{50} assay with the exception of a 6-day incubation (Table 3).
- **Antiviral Assays Against Various DNA and RNA Viruses:** Methods for the in vitro activity/cytotoxicity profiles shown in Table 4 were those used by the DMID In Vitro Antiviral Screening Services reference laboratories. Chimerix gratefully acknowledges the In Vitro Screening Services from the Virology Branch of the Division of Microbiology and Infectious Disease, NIAID, NIH.
- **Mitochondrial Toxicity Assay:** HepG2/C3A cells obtained from ATCC were maintained and assayed in EMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. 96-well plates were seeded with 2E3 HepG2/C3A cells/well. The assay was run in the presence and absence of physiological concentrations of human serum proteins, 2 mg/mL alpha-1-acid glycoprotein (AAG) and/or 40 mg/mL human serum albumin (HSA). ddC was used as a positive control in the assay. Plates were incubated at 37ºC in 5% CO_2 for 7 days to generate cytotoxicity data (CC_{50}). Mitochondrial toxicity was evaluated using the MitoBiogenesis In-Cell ELISA colorimetric test kit according to the manufacturer instructions, and the IC_{50} was determined using Gen 5 software using a 4-parameter curve-fitting function (Table 5).
- **Ames Assay** (BioReliance/MilliporeSigma)
- **In Vitro Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes** (BioReliance/MilliporeSigma.)
- **In Vivo Mammalian Micronucleus Assay in Mice in Peripheral Blood Reticulocytes** (BioReliance/MilliporeSigma.)

Table 1. Percent amino acid identity of the RdRp active sites between MNV and HuNoV (GenBank)

MNV Polyprotein Codon Position	1422	1423	1424	1425	1426	1427	1480	1481	1521	1522	1523	1524	1571	1572
Amino Acid	D	Y	T/S	R/A	W	D	S	G	Y	G	D	D	L	R
GV (MNV, N=64)	100	100	72/28	99/**R**	100	100	100	100	100	100	100	100	100	100
GII (HuNoV, N=656)	100	100	100/**S**	100/**R**	100	100	99.8	100	100	100	100	100	100	100
GI (HuNoV, N=40)	100	100	95/**T**	100/**A**	100	100	100	100	100	100	100	100	100	100

RESULTS

HTS and anti-norovirus activity of CMX521
Over 3,600 nucleoside analogs in the Chimerix compound library were screened in a 3-shot MNV assay. The hits were retested and confirmed hits progressed to an EC_{50} assay. Of the 89 confirmed hits, 35 had an EC_{50} <100 µM. The best compound found was CMX521. The activity of CMX521, 2'-CMC, and nitazoxanide against MNV and HuNoV is shown in Table 2.

Table 2. Antiviral activity of CMX521 and 2'-CMC against mouse and human norovirus

Virus	CMX521 EC_{50} (µM)*	2'-CMC EC_{50} (µM)	Nitazoxanide EC_{50} (µM)
MNV (DS-2)	1.9 ± 0.8 (n = 79)	3.4 ± 1.3 (n = 22)	> 12.4 (n = 2)
HuNoV (GI.1 Replicon)	1.6 ± 0.7 (n = 27)	5.7 ± 2.2 (n = 4)	2.7 ± 0.1 (n = 4)

- CMX521 was more active against MNV and HuNoV in vitro than 2'-CMC or nitazoxanide.
- Mean EC_{50} +/- SD

* Physiological concentrations of added human serum proteins (AAG and HSA) did not increase EC_{50} values, suggesting low protein-binding properties of CMX521 (data not shown).

Table 3. In vitro cytotoxicity of CMX521

Cell Line	CMX521 CC_{50} (µM)	CMX521 Selective Index	2'-CMC CC_{50} (µM)	2'-CMC Selective Index	Nitazoxanide CC_{50} (µM)	Nitazoxanide Selective Index
RAW	>200 (n = 7)	>105	36.9 (n = 7)	10.8	12.4 (n = 2)	~1
MT4	56.8 (n = 7)	35.5	21.2 (n = 7)	3.7	6.7 (n = 3)	2.5
HG23	>200 (n = 10)	>125	>100 (n = 4)	>17.5	14.8 (n = 16)	5.5

- CMX521 had higher CC_{50} values and higher selective indexes in RAW, MT4, and HG23 cells than 2'-CMC or nitazoxanide

Table 4. Activity of CMX521 against various DNA and RNA viruses
(DMID In Vitro Antiviral Screening Services)

DNA Virus	EC_{50} (µM)	CC_{50} (µM)	Selectivity Index
AdV, BKV, HSV2, HCMV, MCMV, VZV, Vaccinia	>50	>60	~1
EBV	>20	65	<3.3
HHV-6B	40	45	~1
HHV-8	>12	47	<3.9

RNA Viruses	EC_{50} (µM)	CC_{50} (µM)	Selectivity Index
Dengue virus-2, Eastern equine encephalitis, EV 71, Flu, Measles virus, Poliovirus-3, Rift Valley fever virus, West Nile, Yellow fever	>100	>100	~1
Hepatitis C	18	>20	> 1.1
RSV	31	33	~1
SARS	37	>100	>2.7

- Specificity of CMX521 was demonstrated by activity against all noroviruses, but low/no activity against all DNA and RNA viruses tested to date.

Table 5. Lack of mitochondrial toxicity associated with CMX521 in HepG2 cells

Condition	CMX521 mt IC_{50} (µM)	ddC mt IC_{50} (µM)
No AAG or HSA	>100 (n = 2)	0.20 (n = 2)
2 mg/mL AAG	>100 (n = 2)	0.22 (n = 2)
40 mg/mL HSA	>100 (n = 2)	0.26 (n = 2)
2 mg/mL AAG + 40 mg/mL HSA	>100 (n = 2)	0.25 (n = 2)

- No mitochondrial toxicity was observed up to the maximum tested concentration of 100 µM in cells treated with CMX521 for 7 days in the presence or absence of AAG, HSA, or both, at their respective physiological concentrations.

Table 6. Lack of genotoxicity associated with CMX521

Genotoxicity Assay	Results
Bacterial Reverse Mutation (Ames) Assay	Negative for mutagenicity ≤5000 µg + /- S9 activation
In vitro Micronucleus Assay	Negative for induction of micronuclei ≤1 mM + /- S9 activation
In vivo Mouse Micronucleus Assay	Negative for induction of micronuclei ≤2000 mg/kg/day

CONCLUSIONS

- **A novel strain of MNV was isolated, characterized, and utilized in a MNV HTS to identify the novel nucleoside analog CMX521**
- **CMX521 shows specific antiviral activity against MNV and HuNoV, and exhibits better activity and less toxicity than 2'-CMC or nitazoxanide in vitro**
- **CMX521 shows no evidence of mitochondrial toxicity, genotoxicity, or protein binding**
- **Favorable pre-clinical data supported the progression of CMX521 to ongoing phase I clinical studies**

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