Global Burden and Novel Treatment of Noroviruses

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Outline

- NoV epidemiology
- Clinical course of NoV disease
- Pathology of NoV infection
- Virion structure
- Organization of the NoV genome
- NoV classification in genogroups and genotypes
- Single-cell replication cycle of NoV
- **Recent advances in NoV research:** recombinant NoV proteins and structures, cell based NoV assays, animal models of human NoV disease
- NoV protease as a drug target
- NoV polymerase as a drug target
- NoV vaccine trials
- Animal models of human NoV disease
Background

• NoVs are the leading cause of gastroenteritis in humans worldwide:
  • **Hallmarks of the disease:** watery diarrhea, vomiting, abdominal cramps, nausea, and sometimes fever
  • 700 million infections (Aron et al., Expert Review of Vaccines, 2016)
  • 200,000 deaths (Bartsch et al., PLoS One, 2016)

• Each year in the United States:
  • 21 million infections
  • 71,000 hospitalizations
  • 800 deaths

• On average, people will experience 4 to 5 episodes of NoV in their life (CDC, 2016).

• Children, immunocompromised individuals, and the elderly are at higher risk for long-term or fatal infections.

• Currently there is no antiviral therapy or vaccine to treat or prevent NoV infections.
Features of NoV epidemiology

http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus.htm
http://rivn.nl/en/aboutrivm/projects.index
**Clinical course of NoV disease**

**Various treatment protocols tested in normal and immunocompromised patients with NoV gastroenteritis**

<table>
<thead>
<tr>
<th>Patient status at time of norovirus illness</th>
<th>Age</th>
<th>Norovirus treatment</th>
<th>Dose or modification of Immunosuppressive therapy (IST)</th>
<th>Reported outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Adult</td>
<td>Bismuth subsalicylate</td>
<td>420 mg</td>
<td>Reduced abdominal cramping</td>
</tr>
<tr>
<td>Normal</td>
<td>12–60 years</td>
<td>Nitazoxanide</td>
<td>Oral, 500 mg, 2× daily</td>
<td>Shorter duration of illness</td>
</tr>
<tr>
<td>AML</td>
<td>36 years</td>
<td>Nitazoxanide</td>
<td>Oral, 500 mg, 2× daily</td>
<td>Improvement</td>
</tr>
<tr>
<td>Immunocompromised</td>
<td>All</td>
<td>Immunoglobulin</td>
<td>Oral, 25 mg/kg, 4× daily</td>
<td>Decreased stool output</td>
</tr>
<tr>
<td>HIV</td>
<td>56 years</td>
<td>Immunoglobulin</td>
<td>IV, 400 mg/kg, 1× daily</td>
<td>Minimal 2-day improvement</td>
</tr>
<tr>
<td>Stem cell/lung transplant</td>
<td>&lt;2 years</td>
<td>Modify IST</td>
<td>Drug switch</td>
<td>Improvement</td>
</tr>
<tr>
<td>Intestinal transplant</td>
<td>Adult</td>
<td>Modify IST</td>
<td>Dose reduction</td>
<td>Improvement</td>
</tr>
<tr>
<td>Renal transplant</td>
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</table>

AML, acute myelogenous leukemia; HIV, human immunodeficiency virus; IST, immunosuppressive therapy.

*Two study groups: one with average age of 2 years old, the other with male adults.

*Long term follow-up information unavailable for most patients.

Pathology of NoV infection in the jejunum

A. Normal jejunal tissue from biopsy of a volunteer
B. Broadened and flattened villi in jejunal tissue from the same volunteer during illness with NoV induced gastroenteritis.
C. Scanning electron micrograph (EM) showing normal jejunal tissue from biopsy of pig.
D. Scanning EM showing shortening, blunting, fusion, or absence of villi in jejunal biopsy from the same pig following challenge with NoV.

Clinical and public health intervention scenarios for the use of new anti-NoV agents

• Treating immunocompromised patients with chronic NoV infection (e.g., HIV infection or AIDS, cancer, solid organ transplantation, stem cell transplantation, asplenia and congenital immune deficiencies).

• Preventing/reducing NoV shedding from acute infection in patients with an intact immune system -- limiting the spread of the virus in the hospital, nursing home, school, or military settings.

• Use as a prophylactic anti-NoV agent in uninfected persons during NoV outbreaks to prevent/contain the spread of NoV infection (less than 20 virus particles cause disease, difficult to disinfect NoV contamination due to the relative resistance of particles to disinfectants and high environmental stability).

Organization of the NoV major capsid protein, VP1. A. Linear representation of the NoV VP1 protein. B. Crystal structure of the NoV whole capsid determined at 3.4 Å resolution, Prasad et al., Science, 1999. C. Ribbon representation of the VP1 dimer derived from the 3.4 Å crystal structure. P2 domain arch; P1 domain arms; S domain internal scaffold surrounding the RNA genome; D. P domain only dimer at 1.4 Å resolution, Choi et al., PNAS, 2008.
Comparative features of calicivirus genomes

A. The positive-sense RNA genome is covalently linked to a virion protein (VPg) at the 5’ end and polyadenylated at the 3’ end. B. Calicivirus genomes are organized so that the major capsid protein is either in frame or not with the upstream nonstructural protein sequence. C. A large polyprotein is translated from the viral RNA genome, and it is processed into final products by a virus encoded protease (NS6). Dark arrows represent cleavage sites preserved among all calicivirus genera; cleavage sites that vary among the family are indicated with a light arrow. Adapted from Green, Virology, 2013.
Genetic classification of noroviruses in 9 genogroups

GI: 5-10%
GII: 90-95%
GIV: < 0.1%
GVII: ?

Chhabra & Vinjé, 2016
Single-cell replication cycle of noroviruses

Adapted from Thorne and Goodfellow (2014).

Adapted from Green, Virology, 2013.
A. General structure of norovirus protease
B. Surface representation of a homology model of the Minerva virus protease
**Cleavage junctions**

- Proteolytic processing by NoV Pro occurs at 5 sites
- Q-G, E-G, E-A serve as the cleavage junctions
- Q-G sites are preferentially processed first
- Neighboring residues implicated in substrate recognition

Norovirus cleavage product: a starting point for inhibitor design

**Compound 7**

**Anti-norovirus activity:**
- EC$_{50}$ = 0.09 μM (replicon)
- EC$_{90}$ = 0.33 μM (replicon)
- IC$_{50}$ = 0.204 μM (enzyme)
- IC$_{90}$ = 1.59 μM (enzyme)

**Toxicity (CC$_{50}$) in:**
- Huh7 >10 μM
- PBM >100 μM
- CEM = 39 μM
- Vero > 100 μM
Inhibition of NoV PR by compound 2

<table>
<thead>
<tr>
<th>Compound 2</th>
<th>IC$_{50}$ (nM)</th>
<th>K$_i$ (nM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>112 ± 25</td>
<td>49.7 ± 2.3</td>
</tr>
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</table>

Incubation time: 90 min (time required to form the covalent bond between the enzyme and the inhibitor)
Antiviral drug design strategy targeting the NoV PR

Diagram showing chemical structures and labels for Cap, P3, P2, P1, and Warhead.
Aldehydes vs. Michael-acceptors: aldehyde based compounds are the most potent warheads
Summary of our NoV PI studies

We have identified 3 new norovirus PIs for further characterization and optimization.

- EC$_{50}$ is in the low nanomolar range (EC$_{50}$ = 90 nM).
- Aldehyde based compounds are the most potent warheads.
- Mechanism of action: irreversible inhibition of the NoV PR
- Favorable toxicity profile in 4 different cell types (PBM, CEM, Vero, Huh7 cells)
- CC$_{50}$/EC$_{50}$ ratio = 1,111 for compound 7
- Favorable ADME/Tox prediction (2 to 4 star rating)
- Specificity studies: the lead compound (compound 7) is specific for NoV PR.

Next Step? Proof of concept In Vivo - Animal Studies in collaboration with the Christiane Wobus lab at the University of Michigan
A. The NoV RdRP adopts a classic “right hand” structure characteristic of polynucleotide polymerases as shown in the stick model. The fingers (blue) and palm (green) domains form a rigid unit, while the thumb (red) domain is flexible and can assume either a “closed” or “open” conformation. B, C, and D. The front, top, and side views, respectively, of the NoV RdRP bound to a primer-RNA duplex, cytidine triphosphate (CTP), and metal divalent cation, Mn$^{2+}$. Ng et al. J. Biol. Chem., 2002, Ng et al. J. Biol. Chem., 2004, Zamyatkin et al., J. Biol. Chem., 2008.
Table 1. Anti-norovirus activity and cytotoxicity of nucleoside analogue inhibitors

<table>
<thead>
<tr>
<th>Nucleoside analogue inhibitor</th>
<th>EC_{50}\text{a}, \mu M</th>
<th>IC_{50}\text{a}, \mu M</th>
<th>EC_{50}\text{b}, \mu M</th>
<th>IC_{50}\text{b}, \mu M</th>
<th>SI\text{c}</th>
<th>EC_{50}\text{c}, \mu M</th>
<th>IC_{50}\text{c}, \mu M</th>
<th>EC_{50}\text{d}, \mu M</th>
<th>IC_{50}\text{d}, \mu M</th>
<th>SI\text{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>3TC</td>
<td>-</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>3.2 ± 1.5</td>
<td>N/A</td>
<td>49.0 ± 2.9</td>
<td>&gt;100</td>
<td>&gt;31.4</td>
</tr>
<tr>
<td>2'-F-2'-C-MeC</td>
<td>12.7 ± 2.9</td>
<td>33.9 ± 1.1</td>
<td>91.2 ± 2.8</td>
<td>&gt;100</td>
<td>&gt;7.9</td>
<td>1.29 ± 0.8</td>
<td>2.77 ± 1.4</td>
<td>8.9 ± 1.5</td>
<td>&gt;100</td>
<td>&gt;77.5</td>
</tr>
<tr>
<td>2'-C-MeC</td>
<td>6.9 ± 3.9</td>
<td>15.7 ± 1.2</td>
<td>25.4 ± 3.6</td>
<td>&gt;100</td>
<td>&gt;14.6</td>
<td>1.47 ± 1.0</td>
<td>4.0 ± 1.2</td>
<td>8.6 ± 1.7</td>
<td>&gt;100</td>
<td>&gt;68.0</td>
</tr>
<tr>
<td>NHC</td>
<td>8.8 ± 4.2</td>
<td>-</td>
<td>&gt;100</td>
<td>&gt;11.4</td>
<td>&gt;1.6</td>
<td>1.47 ± 1.1</td>
<td>6.0 ± 1.7</td>
<td>16.6 ± 2.1</td>
<td>&gt;100</td>
<td>&gt;68.0</td>
</tr>
<tr>
<td>RBV</td>
<td>63.5 ± 2.3</td>
<td>73.5 ± 3.1</td>
<td>104.7 ± 1.5</td>
<td>&gt;100</td>
<td>&gt;1.6</td>
<td>4.0 ± 2.3</td>
<td>96.3 ± 2.5</td>
<td>96.3 ± 2.5</td>
<td>&gt;100</td>
<td>&gt;5.0</td>
</tr>
</tbody>
</table>

*Compound concentration required to reduce murine norovirus (MNV) RNA copy number by 50% as determined by RT-qPCR. *Compound concentration required to reduce the MNV infectivity in RAW 264.7 cells by 50% as determined by 50% tissue culture infectious dose. *Compound concentration required to reduce MNV RNA copy number by 90% as determined by RT-qPCR. *Compound concentration required to reduce the viability of RAW 264.7 cells by 50% as determined by the CytoTox 96 Non-radioactive Cytotoxicity assay (Promega) and MTT proliferation assay (Promega). *Selective index (SI)=EE_{50}/EE_{50}. *Selective index required to reduce Norwalk virus (NV) RNA copy number in H23 cells by 50% as determined by RT-qPCR. *Selective index required to reduce NPT II expression in H23 cells by 50% as determined by western blot assay. *Selective index required to reduce NV RNA copy number in H23 cells by 90% as determined by RT-qPCR. *Selective index required to reduce the viability of H23 cells and Huh-7 cells by 50% as determined by the CytoTox 96 Non-radioactive Cytotoxicity assay (Promega) and MTT proliferation assay (Promega). EC_{50}, 50% cytotoxic concentration; IC_{50}, 50% effective concentration; EC_{50}, 90% effective concentration; IC_{50}, 50% inhibitory concentration; N/A, not applicable; NHC, β-D-N-Acetylhexosaminidase; RBV, ribavirin; 3TC, lamivudine.

Table 1. Activities of the identified inhibitors using the NoV RdRp and cell culture models

<table>
<thead>
<tr>
<th>Compound</th>
<th>CLogP\text{a}</th>
<th>GI4 RdRp IC_{50} (\mu M)^{b}</th>
<th>GI1 replicon EC_{50} (\mu M)^{c}</th>
<th>GV.1 MNV EC_{50} (\mu M)^{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIC02</td>
<td>3.6</td>
<td>5.0 (3.6–6.9)</td>
<td>30.1 (19.6–45.9)</td>
<td>4.8 (1.7–13.3)</td>
</tr>
<tr>
<td>NIC04</td>
<td>3.5</td>
<td>5.5 (4.5–6.7)</td>
<td>71.1 (56.7–89.1)</td>
<td>32.8 (22.0–48.9)</td>
</tr>
<tr>
<td>NIC10</td>
<td>0.8</td>
<td>9.2 (7.4–11.3)</td>
<td>&gt;100</td>
<td>34.5 (22.6–52.5)</td>
</tr>
<tr>
<td>NIC12</td>
<td>0.4</td>
<td>9.8 (7.4–13.0)</td>
<td>&gt;100</td>
<td>38.1 (17.7–82.4)</td>
</tr>
</tbody>
</table>

\text{a} CLogP, calculated partition coefficient.
\text{b} IC_{50}s were determined by \textit{in vitro} radioactive-GTP incorporation RdRp assays (95% CI in parentheses).
\text{c} EC_{50}s were determined using cell-based replicon and infectious-NoV model systems (95% CI in parentheses).
NoV vaccine trials

• The goal of the vaccine trials: reduction in disease symptoms and severity in human challenge studies.

• A bivalent intramuscular vaccine is in Phase 2b clinical trial in male and female adults, age 18-49 (Takeda Pharmaceuticals).

• The vaccine relies on a virus-like particle that is made of the NoV capsid proteins in order to mimic the external structure of the virus.

• Since there is no RNA in the particle, it is incapable of reproducing and cannot cause infection.

• The vaccine includes antigens from genotypes GI.1 and GII.4 to represent both of the genogroups that cause the majority of human illness.

• Oral recombinant vaccine that is administered by tablet rather than injection is in Phase 1 trials in 66 healthy adult volunteers (Vaxart, Inc.).

• Challenge: the fast evolving NoV will likely require annual vaccinations in an approach similar to vaccinations against the influenza virus.
Animal models to study human NoV disease

- Most data derived from human volunteer studies - Inherent limitations
- Studies in animal models are critical

Animal models of Human NoV infection – each model has strengths and weaknesses

- **Immunocompromised mice** (low cost, used for antiviral development)
  Balb/c Rag/gamma chain-deficient (Rag-yc−/−) mice
  Taube et al. mBIO (2013); Karst el al. Cell Host & Microbe (2014)

- **Gnotobiotic pigs** (diarrhea, high cost, used for antiviral and vaccine development)

- **Chimpanzees** (intravenous route, asymptomatic infection, no more NIH funding)
  Bok et al. PNAS (2011)

- **Gnotobiotic calves** (diarrhea, high cost)

- **Rhesus macaques** (asymptomatic infection, high cost, Biomedical Primate Research Center, Netherlands)
Wayne State University
Benjamin Kuiper
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Prasanna Medapureddy
Joshua Holcomb
Nicholas Spellmon
Zhe Yang
Iulia Kovari
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U. of Michigan
Christiane Wobus

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