Non-nucleoside inhibitors of the norovirus RNA polymerase; scaffolds for rational drug design.

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Running head: Norovirus polymerase inhibitors

Author contribution: Conceived and designed the experiments: AAE, PAW. Performed the experiments: AAE, AGK, JSE, KLL. Analysed the data: AAE, KLL, JMM, PAW. Contributed reagents/materials/analysis tools: JMM. Obtained funding: PAW and JMM. Wrote the paper: AAE, PAW.

Acknowledgments: The authors thank Kim Green and Skip Virgin for reagents, Rowena Bull and Chris Marquis for technical assistance.

Funding: This work was partially funded by a National Health and Medical Research Council project grant APP1010327 and an Australian Research Council discovery project grant DP120104073.

Conflicts of interest: The authors disclose no conflicts of interest.

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Abstract

Norovirus (NoV), is the leading cause of acute gastroenteritis worldwide, causing over 200,000 deaths a year. NoV is non-enveloped, with single-stranded RNA genome and is primarily transmitted person-to-person. The viral RNA-dependent RNA polymerase (RdRp) is critical for the production of genomic and sub-genomic RNA, and is therefore a prime target for antiviral therapies. Using high-throughput screening, nearly 20,000 “lead-like” compounds were tested for inhibitory activity against the NoV Genogroup II, genotype 4 (GII.4) RdRp. The four most potent hits demonstrated half-maximal inhibitory concentrations (IC50) between 5.0 µM and 9.8 µM against the target RdRp. Compounds NIC02 and NIC04 revealed a mixed mode of inhibition, while NIC10 and NIC12 were uncompetitive RdRp inhibitors. When examined using enzymes from related viruses, NIC02 demonstrated broad inhibitory activity while NIC04 was the most specific GII.4 RdRp inhibitor. The antiviral activity was examined using available NoV cell culture models; the GI.1 replicon and the infectious GV.1 murine norovirus (MNV). NIC02 and NIC04 inhibited the replication of the GI.1 replicon, with EC50 values 30.1µM and 71.1µM, respectively, while NIC10 and NIC12 had no observable effect on NoV GI.1 replicon. In the MNV model, NIC02 reduced plaque numbers, size and viral RNA levels, in a dose-dependent manner (EC50 values 2.3 µM to 4.8µM). The remaining three compounds also reduced MNV replication, although with higher EC50 values, (ranging from 32µM to 38µM). In summary, we have identified novel non-nucleoside inhibitor scaffolds which will provide a starting framework for the development and future optimization of targeted antivirals against NoV.

Key words: Norovirus, inhibitors, RNA-dependent RNA polymerase, NS7.
Noroviruses cause around 50% of all gastroenteritis cases worldwide (1), and are associated with the deaths of more than 200,000 people per year, mainly in developing countries (2). Of particular importance are NoVs that belong to Genogroup II, genotype 4 (GII.4) which have been associated with all six major NoV pandemics of acute gastroenteritis in the last two decades and account for 80% of all human NoV infections (3). In addition, NoV is increasingly recognised as an important cause of chronic gastroenteritis in immunocompromised patients (4, 5). Apart from the human costs, NoV infections cause severe economic losses (6). The virus is highly transmissible with a low infectious dose, and high numbers are excreted during acute illness; approximately $10^8$ virions per gram of faeces (7). Following an incubation period of 1-2 days, the clinical features of NoV infection include acute onset of nausea, vomiting, abdominal cramps, headaches and diarrhoea that generally last for 2-4 days (8). A member of the *Caliciviridae* family, NoV is non-enveloped, 27 – 35 nm in diameter with a single stranded RNA genome of 7,400 – 7,700 nucleotides. The genome encodes three open reading frames (ORFs). ORF1 encodes seven non-structural (NS) proteins (NS1-NS7) and includes a viral genome-linked protein (VPg, NS5), a 3C-like protease (NS6) and a RNA-dependant RNA polymerase (RdRp, NS7). ORF2 and ORF3 encode the major capsid protein (VP1) and minor basic protein (VP2), respectively (9).

Significant efforts have been made to identify a permissive cell line for human NoV, however, these attempts have largely failed (10). The lack of a cell culture system for human NoV has hindered both replication studies and the identification of NoV therapeutic agents. In 2003, murine NoV (MNV) was identified (11), which led to the development of the first cell culture system and small animal model for NoV infection (12). Wobus et al. demonstrated that MNV replicates in cells of mononuclear origin, such as primary dendritic cells and macrophages (12). Building from this work with MNV, it has been recently shown...
that BALB/c Rag-γc-deficient mice could also support the replication of human GII.4 NoV (13). Another important breakthrough in the establishment of human NoV cell culture models was the development of a human sub-genomic replicon based on the prototype NoV GI strain, Norwalk virus (14). The replicon consists of the complete ORF1, encoding the viral replicative enzymes (NS1-7), while ORF2, which encodes the major structural protein, is disrupted by the insertion of a neomycin resistance gene. This allows replication of autonomous RNA in Huh-7 cells without the expression of viral structural genes and therefore no virions are produced (14). Replicons are invaluable for studying viral RNA replication and provide a very useful platform for antiviral development; for example, they have been instrumental in the recent advances in hepatitis C virus (HCV) antiviral developments, reviewed in reference (15).

The highly infectious nature of NoV, and its association with outbreaks in hospitals, age-care facilities and cruise ships, highlights the need for specific approaches to control NoV infections either through vaccines or antivirals. This is of particular importance for individuals with a high risk of exposure, for immunocompromised patients with chronic NoV infections, and for those who are susceptible to complications and dehydration including young children and the elderly. The NoV infectious cycle offers a number of potential targets for the development of direct-acting antivirals (DAA). One key target for NoV antivirals is the viral polymerase (RdRp) because of its essential role in viral replication and the lack of homologous human enzymes. A number of studies have used recombinant NoV RdRp to characterise its biochemical properties in vitro, and the X-ray crystal structures of RdRps from human GI, GII and mouse GV NoVs have been solved (16-18).

There are only a handful of publications reporting NoV DAA development, reviewed in reference (19). The most advanced pre-clinical studies have so far focussed on protease inhibitors (20, 21), or have repurposed available drugs as NoV RdRp inhibitors which were
originally developed to treat other viral (22-24) and non-viral infections (25). However, thus far no novel molecules or scaffolds have been identified as specific inhibitors of the NoV RdRp. In this study, we conducted a high throughput screen to identify small molecule inhibitors of GII.4 NoV RdRp transcription that may provide a platform for the development of antivirals against this important clinical pathogen. Four scaffolds were identified and their mode of RdRp inhibition was characterised. We further examined specificity of these compounds across a range of calicivirus RdRps, and the antiviral activity was assessed using the human Norwalk GI.1 sub-genomic replicon, and the MNV infectious cell culture model.
Materials and Methods

Recombinant RdRp expression, purification and comparison

Recombinant RdRps with a C-terminal hexahistidine tag, were expressed in *E. coli* and purified by nickel affinity chromatography, as described previously (26, 27). The RdRp of the following caliciviruses (shown along with their corresponding GenBank accession numbers) were used in this study: NoV GII.4 Den Haag 2006b variant (EF684915), NoV GII.4 New Orleans 2009 variant (JQ613573), NoV GI.1 Norwalk virus (NC_001959), NoV GV.1 (MNV, DQ285629) NoV GII.7 (GQ849131) and Sapovirus (SaV) GII (AY237420). Amino acid sequence analysis was performed using the MEGA5 software package (28) and a phylogenetic tree of protein sequences was produced using the Neighbour-Joining method.

Biochemical RdRp assays

Polymerase activity was measured by monitoring the formation of double-stranded RNA from a single stranded homopolymeric template, poly(C), using the fluorescent dye PicoGreen (Life Technologies, Carlsbad, USA), as described previously (29) with minor modifications. RdRp assays were performed in 384-well plates and each reaction contained 20 ng enzyme (13.3 nM), 5 µM GTP, 6 µg/mL poly(C) RNA, 2.5 mM MnCl₂, 5 mM DTT, 0.01% bovine serum albumin (BSA) and 0.005% Tween-20 in 20 mM Tris-HCl pH 7.5 with a final volume of 25 µL. Reactions were run for 10 min at 23°C and terminated with 10 mM EDTA followed by PicoGreen staining and dsRNA quantitation. Alternatively, radioactive-GTP incorporation was measured on a scintillation counter as described previously (27).
**High-throughput screening**

A high-throughput screen (HTS) was carried out to identify inhibitors of the NoV using the RdRp of a representative GII.4 variant Den Haag 2006b which was associated with a global pandemic, and was the predominant NoV in circulation between 2006 and 2008 (30). A random selection of 19,956 compounds from the Walter and Eliza lead-like compound library (The Walter and Eliza Hall Institute, Parkville, Australia) were screened at a final concentration of 10 µM, as outlined previously (29). Hits from the HTS were subjected to a confirmatory counter-assay at 10 µM using radioactive-nucleotide incorporation to further exclude false positives that could have affected the fluorescence signal in the primary assay.

**Mode of RdRp inhibition**

To examine the mode of enzyme inhibition by the lead hits, the kinetics of substrate (GTP) incorporation was examined in the presence or absence of inhibitor. Reactions were performed with increasing concentrations of GTP (from 0.2 to 66 µM) and 5, 10, 15, or 20 µM of inhibitor. Kinetic parameters for each compound were determined by non-linear regression and used to generate Lineweaver–Burk double reciprocal plots.

**Cell culture and cytotoxicity**

HG23 cells, a human hepatoma (Huh-7) cell-line bearing the Norwalk virus (GI.1) sub-genomic replicon (31), were kindly supplied by Kim Green (NIAID, NIH, Bethesda, USA). Murine norovirus (MNV) strain CW1 (12) was kindly provided by Herbert Virgin, (Washington University, Saint Louis, USA). MNV stocks were prepared as described previously (32). HG23 and RAW 264.7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 10% foetal bovine serum (FBS,
Sigma-Aldrich, St. Louis, USA), Glutamax (Life Technologies) and 100 U/mL penicillin-streptomycin (Life Technologies), G418 (Geneticin®, Life Technologies) was also added to HG23 cells (750 µg/mL). To examine the cytotoxicity of compounds identified in this study, cell monolayers (1 × 10⁴/well for HG23 or 2.0 × 10⁴/well for RAW 264.7) were treated with various concentrations of each compound for 72 h in 96-well plates. Cytotoxicity was measured by resazurin to resorufin conversion assay (CellTiter-Blue, Promega, Madison, USA) and fluorescence was measured on a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany).

Inhibition of the GI.1 Norwalk replicon

The effect of the identified compounds on the replication of the Norwalk sub-genomic replicon (31) was examined. The nucleoside inhibitor (NI) 2'-C-Methylcytidine (2CM, Sigma-Aldrich), which has previously been shown to inhibit the replication of the Norwalk replicon (23, 33) was used as a positive control in all cell culture experiments, and DMSO (0.5% v/v) was added to untreated cells. HG23 cells were seeded in 96-well plates at a density of 1 × 10⁴ cells/well in antibiotic-free medium and treated with test compounds as described for cytotoxicity analysis. Total RNA was isolated using the RNeasy kit (Qiagen) 72 h later for quantitation of replicons.

Inhibition of murine norovirus replication

Inhibition of MNV replication in RAW 264.7 cells was determined by plaque reduction assays, as described in reference (34) with modifications. Briefly, 6-well plates were seeded with 1 × 10⁶ RAW 264.7 cells/well and incubated overnight at 37°C. Monolayers were then inoculated with 80 plaque-forming units (PFUs) of MNV in DMEM. After a 1h adsorption at 37°C, the medium was removed and wells were overlaid with 0.75%
(w/v) low melting agarose in minimum essential media (MEM, Life Technologies) containing test compounds. Cells were incubated for 48 hours at 37°C then fixed with formaldehyde (4% v/v) and stained with crystal violet (0.2% w/v). Inhibition of MNV in was measured by quantitation of total plaque surface area using the image processing program ImageJ (35). To measure MNV RNA replication, RAW 264.7 cells were seeded in 96-well plates at a density of 2.0 × 10^4 cells/well. Test compounds were added on the following day and cells were incubated for 1 h at 37°C. Cells were then infected with MNV (MOI = 0.1) and incubated for 48 h. Total RNA was extracted for quantitation using QIAamp Viral RNA Mini kit (Qiagen).

**Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)**

Viral RNA was quantitated from either HG23 cells or MNV-infected RAW 264.7 cells by qRT-PCR (7). In brief, cDNA was synthesised using a SuperScript VILO cDNA Synthesis Kit (Life Technologies. Replicon (GI.1) and MNV (GV.1) RNA was measured using an iTaq Universal SYBR Green Supermix (Bio-Rad, California) as described previously (7). NS7-specific primers were used for both GI-based replicon and MNV quantitation and included Replicon Fwd (5´-CCAACTGAAACCCTTTGCGG-3´), Replicon Rev (5´-AGGCATCAGCGTAAGACCAC-3´), MNV Fwd (5´-TGGACGTCGGCGACTATAAG-3´) and MNV Rev (5´-ACCACCTCGTCATCACCATA-3´).

**Structure-activity relationship analysis**

To identify molecules from the remaining 90,000 compounds in the library with structural similarity to leading HTS hits, all functionality was removed from the hit structures and the library was screened using ActivityBase SARview software (IDBS, Guilford, UK), as described previously (36). All identified molecules were screened in triplicate at 10 µM for
their ability to inhibit the NoV GII.4 RdRp, and results were compared to the primary HTS hits.
Results

Identification of RdRp inhibitors by high-throughput screening

Using an *in vitro* HTS, non-nucleoside inhibitors (NNI) of the NoV GII.4 RdRp (Den Haag 2006b variant) were identified from 19,956 compounds randomly selected from a larger 110,000 compound library. Figure 1 shows a summary of the pathway to the identification of the four most potent RdRp inhibitors in this study. Overall, test plates demonstrated suitable measures for HTS quality (37), as quantitated by $Z'$ and $Z$ factors (Figure 2A). Both measures were above the acceptable limit of 0.5 (average $Z' = 0.77 \pm 0.06$, $Z = 0.72 \pm 0.07$) with the exception of two out of 57 plates which had $Z$ factor scores of -0.64 and 0.31. The apparent drop in quality was attributed to two highly fluorescent compounds, which were subsequently omitted from the analysis. Of the 19,956 compounds screened, 35 hits demonstrated greater than $3 \times$ standard deviation (SD) of the average inhibition relative to control reactions, or > 15.5% inhibition of RdRp activity (Figure 2B). All 35 hits were subsequently counter-screened using a radioactive-nucleotide incorporation RdRp assay and 12 compounds demonstrated > 40% inhibition of RdRp activity, while 14 had no inhibitory activity and were most likely fluorescent-quenchers. Four other compounds which appeared to increase the activity of NoV RdRp by ~2-fold (Figure 2B) also had no effect when retested at 10 µM using the fluorescent and radioactive assays (data not shown). Dose-response curves for RdRp inhibition were generated for the 12 most potent hits and half-maximal inhibitory concentrations ($IC_{50}$) ranged from 5 to 30 µM. The most potent of these compounds, a phenylthiazole-carboxamide (NIC02) and a pyrazole-acetamide (NIC04) inhibited the GII.4 NoV RdRp with $IC_{50}$ values of 5.0 and 5.5 µM, respectively (Figure 1, Table 1). NIC10, a triazole and NIC12, a pyrazolidinedione demonstrated slightly higher $IC_{50}$ values of 9.2 and 9.8 µM, respectively (Figure 1, Table 1).
Mode of inhibition

To characterise the mode of NoV GII.4 RdRp inhibition by each of the four lead hits, the kinetics of GTP incorporation was examined in the absence or presence of increasing inhibitor concentrations (Supplementary Figure 1). Double reciprocal Lineweaver–Burk plots indicated a mixed mode of inhibition for NIC02 and NIC04, where both the substrate affinity and the reaction velocity decreased with higher inhibitor concentrations (Figure 4A and 4B). In contrast, the Lineweaver–Burk plots for NIC10 and NIC12 were representative of an uncompetitive mechanism of RdRp inhibition (Figure 4C and 4D). For these two compounds, the apparent substrate affinity increased at higher compound concentrations, as indicated by a decrease in Km values, while the reaction velocity decreased.

Activity of identified NNIs across related calicivirus RdRps

To determine the breadth of the inhibitory activity for the four identified compounds, purified recombinant RdRps were used from five caliciviruses of varying relatedness to the target GII.4 Den Haag 2006b (Figure 5A). These represented a different variant within GII.4 (GII.4, New Orleans 2009 variant), a different genotype within the same genogroup (GII.7) and different genogroups within the genus (GI.1 Norwalk and GV.1 MNV). Additionally, an RdRp from Sapovirus (GII), which represents another genus within the Caliciviridae family, was examined. A phylogenetic analysis of the protein sequences reveals the relationship of the enzymes examined in this study (Figure 5A), with amino acid identity ranging from 32.6 % (Sapovirus) to 97.1% (New Orleans 2009), when compared to the target GII.4 Den Haag 2006b RdRp sequence (Figure 5B).

All four lead hits demonstrated inhibitory activity against both GII.4 enzymes (New Orleans 2009 and Den Haag 2006b variants, 97% identity) with similar inhibitory activities.
for each NIC (Figure 5C), as assessed by in vitro RdRp assays. Of the four lead hits, NIC02 showed the broadest inhibitory activity, with similar levels of inhibition across RdRps from all six caliciviruses (±0.19 fold change in IC_{50}, Figure 5C). Given its inhibitory activity against distantly related RdRps, we examined the inhibitory activity of NIC02 against different classes of polymerases. NIC02 did not demonstrate any inhibition of enzyme activity, even at concentrations up to 100 µM, against an RNA dependent DNA polymerase (Avian Myeloblastosis Virus RdDp) or a DNA-dependent DNA polymerase (Taq DdDp) (data not shown).

NIC10 demonstrated a narrower spectrum of inhibitory activity, with an 11-fold and 56-fold reduction in inhibitory activity against SaV and GI.1 RdRps, respectively when compared to the GII.4 RdRp, and a 1.9-fold reduction against MNV, but had comparable activity against the GII.7 RdRp (Figure 5C). NIC12 showed a modest loss of potency against the GI.1 (2.3-fold) and GII.7 (4.6-fold) RdRps, compared to GII.4, and had little inhibitory activity against the SaV RdRp, with 33.9% ± 14.1 inhibition of RdRp activity at 100 µM (Figure 5C). Interestingly, however, NIC12 was 4-fold more active against the MNV RdRp, compared to the GII.4 enzymes. Finally, NIC04 appeared to be the most specific scaffold for the GII.4 enzymes with significant losses of inhibitory activity against GII.7 (4.6-fold), GI.1 (3.1-fold) and SaV RdRp (8.7-fold), while no inhibition was observed against the MNV RdRp (Figure 5C).

**Inhibition of the NoV Replicon**

The antiviral activity of the identified compounds was assessed by monitoring the replication of the GI.1 Norwalk virus replicon in Huh-7 cells (31). Cells were treated with increasing concentrations of each compound (1 to 100 µM) and replicon RNA levels were quantitated 72 h later by qRT-PCR. NIC02 and NIC04 inhibited replicon replication in a
dose-dependent manner, with EC\textsubscript{50} values of 30.1 µM (95% CI 19.6 to 45.9 µM) and 71.1 µM (95% CI 56.7 to 89.1 µM), respectively (Figure 6A, Table 1). NIC10 and NIC12 had no effect on the replication of the GI.1 replicon when tested at concentrations up to 100 µM. Compound cytotoxicity was assessed simultaneously and compared to vehicle (0.5% DMSO) treated cells. NIC02 was toxic to the Huh-7 cells (Figure 6B, Table 1) at concentrations greater than 10 µM, with a half-maximal cytotoxic concentration (CC\textsubscript{50}) of 134 µM. In contrast, NIC04, NIC10 and NIC12 had no cytotoxic effects at concentrations up to 100 µM. The nucleoside analogue 2CM was used as a positive control and at 10 µM reduced RNA replicon levels by 84.9% (± 1.4) relative to untreated cells, with no observable cytotoxicity (data not shown).

**Inhibition of infectious murine norovirus GV.1**

To examine the antiviral activity of the four lead hits (NIC02, NIC04, NIC10 and NIC12) in an infectious norovirus cell culture system, the GV.1 murine norovirus (MNV strain CW1) was used. Monolayers of RAW 264.7 cells were treated with test compounds and infected with MNV for 48 h. MNV replication was assessed either by plaque reduction assays or by viral RNA genome quantitation by qRT-PCR (NIC02 only). Inhibitory activity was demonstrated by a reduction in both plaque size and numbers with all four compounds compared to DMSO-treated MNV infected cells (Supplementary Figure 2). Quantitation of inhibitory activity, based on reduction in plaque area, revealed an EC\textsubscript{50} of 4.8 µM for NIC02 (95% CI 1.7-13.3), the most potent of the four inhibitors (Figure 7A). The quantitation of MNV RNA genomes from infected cells also revealed an EC\textsubscript{50} value of 2.3 µM for NIC02 (Figure 7C). Higher concentrations were required to inhibit the replication of MNV with NIC04, NIC10 and NIC12, with EC\textsubscript{50} values 32.8 (22.0-48.9), 34.5 (22.6-52.5) and 38.1 µM (17.7-82.4), respectively (Table 1).
The reduction in plaque size for NIC04, NIC10 and NIC12 was not due to cytotoxic effects of the test compounds as no cell death was observed with concentrations up to 100 μM (Figure 7B). However, toxicity to RAW 264.7 cells was observed with NIC02, with a CC₅₀ of 57.1 μM, which was more than 10-fold higher than the EC₅₀ of NIC02. The nucleoside analogue 2CM was also cytotoxic to RAW 264.7 cells at concentrations above 1 μM and demonstrated a CC₅₀ value of 12.2 μM; however, at 1 μM MNV plaque area was reduced by 93.5% (± 0.3) relative to untreated cells (data not shown).

**Preliminary structure-activity analysis**

In order to gain initial insights into the structure-activity relationships for the four molecules, the compound library used in the primary HTS was searched to identify relevant analogues to NIC02, NIC04, NIC10 and NIC12. A total of 182 molecules were identified which were examined for inhibitory activity (10μM) against the NoV GII.4 RdRp (Table S1). Only three analogues of NIC02 were found, and one retained moderate inhibitory activity at 10 μM; 17% compared to 63% enzyme inhibition for the original hit (Table S1). None of the analogues for NIC04 (112 compounds) or NIC10 (33 compounds) demonstrated increased activity when compared to the original hit molecules (Table S1). Of 34 NIC12 analogues identified, three were more potent than the original hit (48% inhibition at 10 μM) with 55%, 62% and 76% inhibition of RdRp activity at 10 μM, respectively (Table S1).
Discussion

There is a clear unmet need for safe, effective antiviral therapies to combat norovirus infections, both for prophylactic use to prevent NoV transmission in an outbreak setting, and to treat immunocompromised patients with chronic NoV infections. Four small “drug-like” molecules were identified as inhibitors of NoV RdRp activity and represent new scaffolds for the development of therapeutic molecules (Figure 3). The compounds demonstrated inhibitory activity in the low micromolar range and included; a phenylthiazole-5-carboxamide (NIC02), a pyrazole-4-acetamide (NIC04), a triazole (NIC10) and a pyrazolidine-3,5-dione (NIC12). Three of these compounds were novel scaffolds that have not been previously reported as viral NNIs, however, NIC12 has been reported in a separate HTS as a “weak” inhibitor of the poliovirus RdRp activity, with approximately 22% inhibition when tested at 86 µM (38), suggesting NIC12 is more potent against NoV than poliovirus RdRp.

The mechanism by which each of these compounds inhibited the NoV RdRp was examined using double reciprocal Lineweaver–Burk plots (Figure 4). Interestingly, the two larger compounds, NIC02 and NIC04 (365 and 347 daltons, respectively), inhibited the NoV RdRp by a mixed mechanism, whereas NIC10 and NIC12 (189 and 233 daltons, respectively) had a mode of action that represented uncompetitive inhibition. This suggests that NIC02 and NIC04 bind to both free enzyme and enzyme-substrate complexes, while NIC10 and NIC12 only bind to the enzyme-substrate complex and therefore likely bind to an allosteric pocket that is distinct from the substrate-binding site. Taken together these findings indicate that NIC02 and NIC04 occupy a different binding pocket/s of the NoV RdRp compared to NIC10 and NIC12.

The four compounds demonstrated variable inhibition profiles when examined across a panel of related RdRps in vitro. A broad-spectrum of inhibitory activity was observed for
NIC02, with similar IC$_{50}$ values across RdRps from different species within the *Caliciviridae* family (Figure 5). These results suggest that the NIC02 could inhibit the enzymes in a non-specific manner, e.g. protein reactivity (39). However, the lack of NIC02 inhibitory activity against an RdDp and a DdDp, as well as our subsequent validation of its inhibitory activity in available cell culture models of NoV, demonstrated this was an inhibitor of the viral RdRp. An explanation for its broad inhibitory activity therefore could be that NIC02 binds to a highly conserved RdRp motif. Using the infectious GV.1 MNV system, the antiviral activity of NIC02 was demonstrated both by reduction of plaque size (EC$_{50}$ = 4.8 µM, Figure 7A), and by a reduction in viral genome replication in treated cells (EC$_{50}$ = 2.3 µM, Table 1, Figure 7C). NIC02 also inhibited the replication of the GI.1 NoV replicon, although it was less potent compared to MNV, with an EC$_{50}$ of 30.1 µM (Figure 6A). It should be noted, however, that NIC02 was the only compound of the four lead hits to display cytotoxicity against Huh-7 cells (CC$_{50}$ = 134 µM) and RAW 264.7 cells (CC$_{50}$ = 57.1 µM).

The inhibitory profile of the remaining three hits was more restricted; NIC10 was significantly less potent against the RdRps of NoV GI.1 and SaV (56 and 11-fold, respectively), compared to GII and MNV RdRps. These findings were consistent with the cell culture observations; NIC10 did not inhibit the replication of the GI.1 replicon in Huh-7 cells, but did inhibit MNV in cell culture with an EC$_{50}$ of 34.5 µM (Table 1). Interestingly, in the RdRp assays NIC12 was more potent against GV.1 MNV when compared to all three GII enzymes, however, it demonstrated only modest activity against MNV in cell culture (EC$_{50}$ = 38.1 µM, Table 1, Figure 7). When examined for inhibitory activity against NoV GI.1, NIC12 was only 2.3-fold less potent compared to the GII RdRps (Figure 5), but had no effect on the replication of GI.1 replicon. The lack of activity of NIC12 in the viral culture systems could be explained by a lack of cell internalisation or metabolic instability; these parameters were not measured in the current study. This is further supported by the large difference in
the lipophilicity between NIC12 (CLogP = 0.4) when compared to NIC02 and NIC04 (CLogP 3.6 and 3.5, respectively), which were both active in the viral cell culture models.

Finally, NIC04 demonstrated the highest specificity towards GII enzymes, which is consistent with the high EC$_{50}$ observed with the GI.1 replicon (71.1 µM). However, NIC04 was not active against GV.1 MNV RdRp in the in vitro assays but weakly inhibited MNV replication (EC$_{50}$ = 32.8 µM).

Only a handful of studies have reported the development of small compound inhibitors as potential NoV DAAs, targeting two of the viral non-structural proteins; the NS6 protease and the NS7 RdRp. Using structure-based design approaches, Tiew et al. synthesised a series of peptidyl protease inhibitors (40). Derivatives were later developed from these scaffolds with inhibitory activity (EC$_{50}$s), between 0.2 and 2.8 µM as assessed using the NoV GI.1 replicon model. These compounds also had broad antiviral activity, inhibiting homologous proteases of coronaviruses and picornaviruses (41). Two classes of NIs, originally developed for the treatment of other viral infections (22-24), have also demonstrated activity against the NoV RdRp. The nucleoside analogue 2CM, developed as an inhibitor of viruses within the Flaviviridae family including HCV, has been shown to inhibit NoV replication in vitro (replicon and MNV) with EC$_{50}$ of 1.3 µM to 18 µM, respectively (23, 24). Recently, the antiviral activity of 2CM has also been demonstrated in mice infected with MNV (33). The use of 2CM in humans, however, remains in some doubt as Valopicitabine, an oral prodrug of 2CM, was halted due to gastrointestinal adverse effects in HCV infected patients. T-705 (Favipiravir) is another nucleoside analogue with broad inhibitory activity against viral RdRps, although originally developed as an influenza inhibitor (42). T-705 was found to inhibit MNV replication (22), but only at high concentrations (EC$_{50}$ of 250 µM compared to 0.2 µM for influenza).
Using available crystal structures, Mastrangelo et al. (25) targeted the active site of the NoV RdRp with an *in silico* approach to identify inhibitors from a panel commercially available compounds. Two molecules were identified as potential NoV NNIs; Suramin, a drug used in the treatment of sleeping sickness caused by the protozoan *Trypanosoma*, and its analogue NF023. These relatively large compounds inhibited the NoV RdRp at nanomolar concentrations, with IC₅₀ values of 24.6 nM and 71.5 nM for Suramin and NF023, respectively (25). The studies described above have so far been limited to the use of known viral polymerase inhibitors repositioned against a new viral target – the NoV RdRp, or alternative uses for existing drugs. Therefore, no new compounds have yet been explored as potential inhibitors of the NoV RdRp. The identification of the new scaffolds in this study provides a platform for NoV-specific antiviral developments. However, current limitations for NoV drug development associated with the lack of a human GII.4 NoV culture system, indicate a more difficult pathway to clinical use than that of other viruses that can be easily cultured *in vitro*. Further screening of the compound library revealed three NIC12–like molecules with increased potency against the NoV GII.4 RdRp compared to the original hit (Table S1). This indicates that NIC12 could represent an attractive scaffold for medicinal chemistry optimization in the search for viral polymerases inhibitors. The examination of the three remaining scaffolds, in contrast, did not reveal any compounds with increased RdRp inhibitory activity. However, the analysis was limited by either large structural modifications of the hit molecules (NIC04 and NIC10) or by the limited number of chemical analogues in the library (NIC02, Table S1) and further SAR would be useful to identify more potent derivatives.

In summary, through a HTS approach we have identified four inhibitors of the NoV polymerase with low micromolar activity. These can be explored for further design efforts to yield potent NNIs with minimal undesirable biological effects. We next intend to determine
the RdRp binding sites through mutational and crystallography studies, in order to guide structure activity relationship analyses for the development of effective NoV therapeutics.

442
Figure Legends

Figure 1. Outline of the pathway to identify NoV RdRp inhibitors in this study.

Figure 2. HTS for inhibitors of the NoV GII.4 RdRp. Compounds were screened for inhibitory activity against the NoV RdRp. (A) Control measurement for test plates in chronological order. The Z’ factor (blue spheres) and Z factor (red spheres) were determined for each plate and a value of > 0.5 was considered acceptable, which is highlighted with a dashed red line. (B) Inhibition results of the HTS. RdRp inhibition was calculated as a percentage of internal plate controls. The hit selection cut-off, which represented inhibition exceeding 3 × SD of the mean distribution, is shown as a dashed red line.

Figure 3. Chemical structures of the NoV RdRp inhibitors in this study.

Figure 4. Differential mechanism of RdRp inhibition by the four NIC compounds. In order to determine the mode of inhibition, RdRp activity was measured at different concentrations of substrate (0.2 to 66 µM) in the presence of 0 to 20 µM of inhibitors. Double-reciprocal plots were generated for NIC02 (A), NIC04 (B), NIC10 (C) and NIC12 (D).

Figure 5. Inhibitory profiles of lead NoV inhibitors against RdRps from related caliciviruses. (A) Unrooted Neighbor-Joining tree of RdRp amino acid sequences used in this study. The evolutionary distances were computed using the number of differences method and are in the units of the number substitutions per sequence, indicated by the scale bar. (B) Amino acid percentage identity between the analysed enzymes. (C) The inhibitory activity of the identified compounds against enzymes from related viruses. Compounds were assayed in triplicate at increasing concentrations and average changes in IC50 values are shown with standard deviations for each enzyme relative to the target GII.4 Den Haag 2006b RdRp.

Figure 6. Inhibition of the Norwalk sub-genomic replicon by lead norovirus NNIs. (A) The inhibitory activity of the top four compounds was assessed using Huh-7 cells harbouring the Norwalk replicon (HG23 cells). NIC02 and NIC04 inhibited the replicon with EC50 values of 30.1 µM and 71.1 µM, respectively. NIC10 and NIC12 had limited effect on the replication of the replicon. (B) Cytotoxicity of the NIC compounds at concentrations ranging from 1 µM to 100 µM. Only NIC02 demonstrated cytotoxic effect at high concentrations (CC50 = 134 µM). All results are the average of triplicate experiments plotted with standard deviations.

Figure 7. Effect of NIC compounds on the replication of murine norovirus. The antiviral activity of the NIC compounds was examined using the infections MNV. (A) The mean plaque area for the different treatments shown as percentage of mock (DMSO) treated cells. NIC02 demonstrated the highest inhibition of MNV replication (EC50 = 4.8 µM). NIC04, NIC10 and NIC12 had similar efficacies with EC50 values of 32.8, 34.5 and 38.1 µM,
respectively. (B) Cytotoxicity profiles of the NIC compounds with uninfected RAW 264.7 cells, shown as percentage of viable cells compared to untreated wells. (C) Inhibition of MNV by NIC02 was assessed by quantitation of viral RNA levels where the antiviral activity was similar to that observed with the plaque reduction assay, with EC_{50} 2.3 μM. Results are the average of triplicate experiments plotted with standard deviations.
References:


24


Primary HTS: **19,956** compounds

Compounds screened at 10 µM
Hit: > 15.5 % inhibitory activity
Hit rate: 0.18 %

Secondary screen: **35** compounds

Compounds screened with a radioactive assay
Hit: > 40 % inhibitory activity

Dose-response curves: **12** compounds

Half-maximal inhibitory concentrations calculated
Hit: IC$_{50}$ < 10 µM

Characterisation of top inhibitors: **4** compounds
Amino acid identity [%]

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<tr>
<th>RdRp strain</th>
<th>GII.4 2006b</th>
<th>GII.4 2009</th>
<th>GII.7</th>
<th>GI.1</th>
<th>GV.1</th>
<th>SaV GII</th>
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Fold change in IC₅₀
Table 1. Activity of the identified inhibitors using the NoV RdRp and cell culture models

<table>
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<tr>
<th>Compound</th>
<th>CLogP</th>
<th>GII.4 RdRp IC₅₀ (µM)</th>
<th>GII.1 replicon EC₅₀ (µM)</th>
<th>GV.1 MNV EC₅₀ (µM)</th>
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<td>NIC02</td>
<td>3.6</td>
<td>5.0 (3.6-6.9)</td>
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<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>
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<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>
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<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>
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</table>

IC₅₀ values were determined by *in vitro* radioactive GTP incorporation RdRp assays, shown with 95% CI. EC₅₀ values were determined using cell-based replicon and infectious NoV model systems, shown with 95% CI.