Norovirus antiviral discovery: host-modulators and direct-acting antivirals

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Norovirus antiviral discovery: host-modulators and direct-acting antivirals

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Thesis/Dissertation Sheet

Abstract

Human norovirus is a leading cause of acute gastroenteritis (AGE) worldwide and is estimated to be responsible for over 200,000 deaths each year. Norovirus infections are estimated to cost $60 billion in societal costs globally each year. Yet despite the substantial health and economic burden of norovirus, there is no vaccine or norovirus-specific antiviral approved for clinical use. Effective norovirus therapies are highly desired, particularly for the treatment of chronic norovirus infections, or for prophylaxis to limit outbreaks and protect high-risk groups, including the immunocompromised. Several direct-acting antivirals (DAAs) and host-targeted therapies have demonstrated inhibitory activity against noroviruses *in vitro* and *in vivo*, however none of these compounds have progressed through clinical trials. Therefore, this thesis aimed to discover new antivirals and expand the repertoire of compounds with the potential to be further developed for the treatment of norovirus infections. In the first study, RNA-sequencing was performed to provide insights into norovirus pathogenesis and to help identify new host targets that could be explored antiviral development. We found that norovirus infection dampens the transcriptional profile of several genes involved in MHC class I antigen presentation, likely for immune evasion. We also observed a significant reduction in *TLR7* expression which could represent a mechanism to avoid recognition by the host. To explore this further, we screened several Toll-like receptor (TLR) agonists, currently in clinical trials for antiviral therapy, against norovirus and found that these compounds potently inhibit infection *in vitro*. Here we discovered a new target for norovirus antivirals. Lastly using *in vitro* antiviral assays against several caliciviruses, we discovered a new nucleoside analogue (NA), called NITD008, which represents the most potent NA described in the literature to date and a strong candidate for continued development. The new anti-norovirus compounds described in this thesis could be used as scaffolds for the generation of derivatives with improved drug properties or be used in combination with other compounds for improved efficacy. Overall, this thesis involved a multi-faceted approach to antiviral discovery which has increased the available compounds in the pre-clinical pipeline for norovirus therapy.

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&

Paea He-Lotu 'Enosi Tu'ipulotu (Née Taufalele)

This thesis is dedicated to you.

Mālō 'aupito

'Ofa lahi 'atu

PUBLICATIONS DURING CANDIDATURE

Published

1. Russo AG, Kelly AG, **Enosi Tuipulotu D**, Tanaka MM and White PA. Remarkable abundance of endogenous RNA virus sequences in *Ixodes scapularis* revealed through screening of arbovirus vector genomes. *Virus Evolution* (in press).
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12. **Enosi Tuipulotu D**, Netzler NE, Lun JH, Mackenzie JM and White PA. RNA-sequencing of murine norovirus infected cells reveals transcriptional alteration of genes important to viral recognition and antigen presentation. *Frontiers in Immunology*. 2017, 8:959.

Submitted

13. **Enosi Tuipulotu D**, Fumian TM, Netzler NE, Mackenzie JM and White PA. The adenosine analogue NITD008 has potent antiviral activity against human and animal caliciviruses. *Viruses*.

Conference publications

1. Edwards RJ, Amos TG, Tang J, Cawood B, Rispin S, **Enosi Tuipulotu D** and Waters PA. Pseudodiploid pseudo-long-read whole genome sequencing and assembly of Pseudonaja textilis (eastern brown snake) and Notechis scutatus (mainland tiger snake). The Sydney Bioinformatics Research Symposium 2018.
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3. **Enosi Tuipulotu D**, Netzler NE, Mackenzie JM and White PA. TLR7 agonists display potent antiviral effects against norovirus infection via innate stimulation. The 9th Australasian Virology Society Meeting, 2017.
4. Russo AG, Eden JS, **Enosi Tuipulotu D,** Shi M, Shine R, Rollins LA, Holmes EC and White PA. New Viral Discovery in the Cane Toad: Possible Sources for Viral Biocontrol. The 9th Australasian Virology Society Meeting, 2017.
5. Lun JH, Sitabkhan A, Eden JS, **Enosi Tuipulotu D**, Netzler NE, Morrell L, Jones R, Ressler KA, Ferson MJ, Dwyer DE, Kok J, Rawlinson WD, Deere D, Crosbie ND and White PA. Comprehensive Molecular Epidemiological Study of Norovirus with Multiple Sample Types. The 9th Australasian Virology Society Meeting, 2017.
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7. **Enosi Tuipulotu D**, Mackenzie JM and White PA. Transcriptome profiling of murine norovirus infected macrophages reveals modulation of host innate immunity. The 6th International Calicivirus Conference, 2016.
8. Netzler NE, Eltahla AA, **Enosi Tuipulotu D**, Lun JH, Urakova N, Frese M, Strive T, Mackenzie JM and White PA. Broad-spectrum non-nucleoside inhibitors for Caliciviruses. The 6th International Calicivirus Conference, 2016.

9. **Enosi Tuipulotu D**, Netzler NE, Garriga D, Kelly AG, Coulibaly F and White PA. The Norovirus RNA-dependent RNA polymerase motif B is a potential new binding site for non-nucleoside inhibitors. The 8th Australasian Virology Society Meeting, 2015.
10. Netzler NE, **Enosi Tuipulotu D**, Eltahla AA, Lun JH, Urakova N, Frese M, Strive T, Kelly AG and White PA. Non-Nucleoside Polymerase Inhibitors for Caliciviruses. The 8th Australasian Virology Society Meeting, 2015.

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LIST OF ABBREVIATIONS

2CMC	2'-C-methylcytidine
AGE	Acute gastroenteritis
AP-1	Activator protein 1
bp	Base pair
BCA	Bicinochoninic acid assay
BSA	Bovine serum albumin
CC_{50}	Half maximal cytotoxicity concentration
cDNA	Complementary DNA
CI	Confidence interval
Ct	Cycle threshold
CTB	CellTiter-Blue
CTL	Cytotoxic lymphocyte
Da	Dalton
DAA	Direct acting antivirals
DALYs	Disability-adjusted life years
DAVID	Database for annotation, visualisation and integrated discovery
DEG	Differentially expressed gene
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EV71	Enterovirus 71
FBS	Foetal bovine serum
FCV	Feline calicivirus
FPKM	Fragments per kilobase of transcript per million
GEO	Gene expression omnibus
GTP	Guanosine triphosphate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus

HRP	Horseradish peroxidase
h.p.i.	Hours post infection
h.p.t.	Hours post treatment
HPV	Human papillomavirus
HSV	Herpes simplex virus
HTS	High-throughput screening
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK	Janus kinase
kb	Kilo base
KEGG	Kyoto encyclopaedia of genes and genomes
LB	Luria-Bertani
LGP2	Laboratory of Genetics and Physiology 2
MAPK	Mitogen activating protein kinase
MDA5	Melanoma differentiation associated gene 5
MHC	Major histocompatibility complex
mm	Mus musculus
MNV	Murine norovirus
MOI	Multiplicity of infection
mRNA	Messenger RNA
Mu-LV	Murine Leukaemia virus
MyD88	Myeloid differentiation factor 88
NA	Nucleoside analog
NCBI	National Centre of Biotechnology Information
neo	Neomycin phosphatase
NFκB	Nuclear factor kappa beta
NGS	Next generation sequencing
NLR	Nucleotide-binding domain, leucine-rich repeat containing receptors
NNI	Non-nucleoside inhibitor
NTP	Nucleotide triphosphate
NTZ	Nitazoxanide
n-OPG	n-octyl glucopyranoside (n-OPG)
NS	Non-structural

nt	Nucleotide
ODN	Oligodeoxynucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PI	Protease inhibitor
PRR	Pathogen recognition receptor
PSaV	Porcine sapovirus
RAG2	Recombination activating gene
RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase
RHDV	Rabbit haemorrhagic disease virus
RIG-I	Retinoic acid inducible gene
RIPA	Radioimmunoprecipitation
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
ss	Single-stranded
STAT	Single transducer and activator of transcription
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
UCSC	University of California, Santa Cruz
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
VPg	Viral protein genome-linked
VLP	Virus-like particle
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

1 General introduction: Norovirus

Text and figures included in this chapter are adapted from the following publications:

Enosi Tuipulotu D, Fumian TM, Netzler NE, Mackenzie JM and White PA. The adenosine analogue NITD008 has potent antiviral activity against human and animal caliciviruses. *Viruses* (submitted).

Netzler NE*, **Enosi Tuipulotu D*,** and White PA. Norovirus antivirals – where are we now? *Medicinal Research Reviews*. 2019, 39:3. [* joint authors]

Enosi Tuipulotu D, Netzler NE, Lun JH, Mackenzie JM, White PA. RNA-sequencing of murine norovirus infected cells reveals transcriptional alteration of genes important to viral recognition and antigen presentation. *Frontiers in Immunology*. 2017, 8:959.

Enosi Tuipulotu D, Netzler NE, Lun JH, Mackenzie JM and White PA. TLR7 agonists display potent antiviral effects against norovirus infection via innate stimulation. *Antimicrobial Agents and Chemotherapy*. 2018, 62:5.

Declaration

I certify that these publications were a direct result of my research towards this PhD, and that reproduction in this thesis does not breach copyright regulations.

Daniel Enosi Tuipulotu [Candidate]

1.1 History and epidemiology

In 1929, John Zahorsky first described an outbreak of projectile vomiting and diarrhoea as "winter vomiting disease", however the aetiological agent was unknown[1]. In 1972, Albert Kapikian made an important discovery; using electron microscopy, he visualised virus particles within stool filtrates that originated from a "winter vomiting disease" outbreak in Norwalk, Ohio several years earlier[2,3]. At this point, Kapikian had demonstrated that that clinical syndrome originally described by Zahorsky was caused by a virus, which was then termed Norwalk virus; the prototype human norovirus strain[2].

Today, human norovirus is recognised as the leading cause of acute gastroenteritis (AGE) globally and is estimated to cause 684 million cases of diarrhoea annually[4,5]. Norovirus infections are more prevalent during winter months in temperate climates[6] and are reported to cause more than 200,000 deaths each year[5], most of which are children from developing countries[7]. Persistent norovirus infections are also a cause of several illness in immunocompromised and transplant patients[8,9]. It is estimated that up to 17-18% of all immunocompromised patients are persistently infected with norovirus, however these values are not well established[8]. It is clear however, that there are a growing number of clinical reports on the association between immunosuppression from organ transplantation or general immunodeficiency and chronic norovirus infection[8]. Overall, norovirus infections have been recognised as the fourth greatest cause of disability-adjusted life years (DALYs) across the globe[10].

Norovirus has historically been responsible for six reported pandemics, which have occurred every 3-5 years when new viral strains predominate and evade herd immunity (reviewed in [11]). These pandemics have a significant social and economic burden which is reflected in the estimated $60 billion USD in annual costs associated with norovirus infections worldwide, including medical expenses, reduction in productive work days, and loss of wages resultant from forced time off

work[12,13]. These social and economic impacts justify the intensive research undertaken to identify effective antiviral and vaccine solutions for human norovirus.

1.2 Transmission and disease manifestation

Human norovirus is highly contagious and primarily transmitted faecal-orally from person-to-person or via contaminated food and water[14]. Norovirus is highly contagious and reports have suggested that the infectious dose is between 18-2,800 virions[15,16]. Thus, epidemics of norovirus are particularly common in semi-enclosed and closed settings such as nursing homes, hospitals, restaurants, cruise ships, aged- and child-care centres[17]. Prolonged shedding of norovirus beyond the symptomatic phase also contributes to continual norovirus spread, and has been reported as a mechanism for nosocomial transmission[18]. The combination of prolonged viral shedding, a low infectious dose[16] and genetic diversity[19], makes norovirus a highly transmissible pathogen.

Infection with norovirus typically results in a combination of projectile vomiting, non-bloody watery diarrhoea, and can be associated with symptoms such as nausea, chills, headaches, fever, abdominal cramps and muscle aches. However, infections are usually self-limiting with symptoms typically lasting 2-3 days[20]. More severe symptoms and dehydration can occur in young children, the elderly and the immunocompromised, which can ultimately lead to death[8,21].

1.3 Norovirus classification, virion structure and genome organisation

Human norovirus is a member of the *Caliciviridae* family that is divided into five accepted genera including: *Norovirus*, *Sapovirus*, *Vesivirus*, *Lagovirus*, and *Nebovirus*[22], with several additional genera proposed[23-26] (Figure 1.1). Human norovirus lies within the genus *Norovirus* which is divided into seven genogroups (GI-GVII)[27] (Figure 1.1) and more then 30 genotypes; the latter are assigned based on full-length capsid and polymerase protein coding sequence diversity[28,29]. Specifically, GI, II, and

IV noroviruses can infect humans[27,28], GV infect mice[30], and other genogroups infect porcine, ovine, bovine, feline and canine species[31-35] (Figure 1.1).

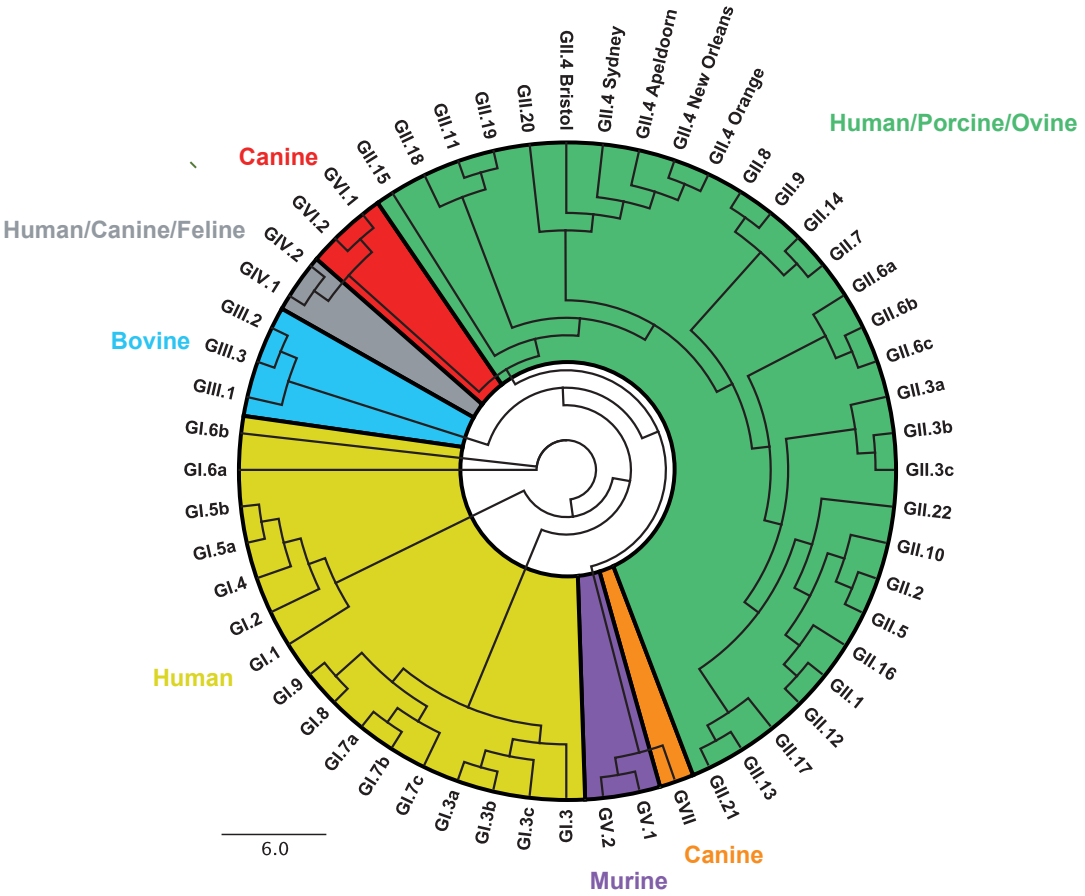


Figure 1.1. Phylogenetic diversity of noroviruses

Phylogenetic relationships between noroviruses of all seven genogroups based on an alignment on the full-length VP1 sequence. Sequences (n=56) were obtained from the NCBI database aligned using Geneious Mapper and trimmed before a neighbour-joining phylogenetic tree was constructed based on the alignment. Individual genogroups are represented by a different colour. Figure adapted from [22].

The positive-sense, single-stranded, RNA genome of norovirus is approximately 7.5-7.7 kb in length and is encapsidated within a non-enveloped icosahedron which is 27-35 nm in diameter[2,36,37]. The viral capsid has a T=3 symmetry and is composed of 90 dimers of VP1 forming 'cup-like' depressions on the surface which are a distinctive feature of the caliciviruses[38,39] (Figure 1.2). Norovirus genomic and sub-genomic RNA are flanked by a polyadenylated tail at the 3' end and covalently linked to a

viral protein genome-linked protein (VPg) at the 5' end[40] (Figure 1.2). VPg aids in both viral genome transcription and translation[40] (Table 1.1).

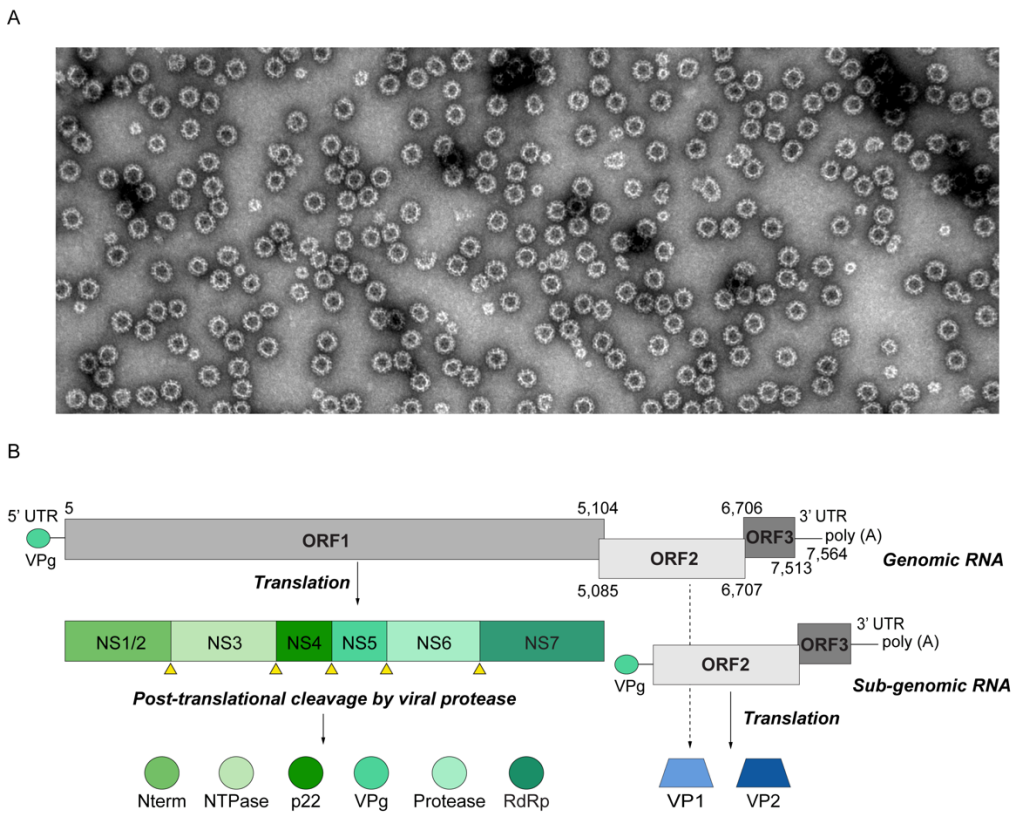


Figure 1.2. Norovirus virion structure and genome organisation

(A) Electron micrograph of human norovirus GII.4 Sydney 2012 virus-like particles (VLPs). (B) Schematic of the positive sense, single-stranded RNA genome of norovirus. The genome is comprised of three open reading frames (ORFs) that encode the non-structural proteins: p48/N-terminal (NS1/2), NTPase (NS3), p22 (NS4), VPg (NS5), protease (NS6) and RNA polymerase (NS7); and the structural proteins: VP1 and VP2. The numbers at the edges of each domain indicate nucleotide positions. Genome illustration is based on the norovirus GII.4 Sydney 2012 sequence (GenBank accession number JX459908). Panel A was provided by Dr. Grant Hansman (Heidelberg University). Panel B is taken from [41].

With respect to protein coding capacity, the norovirus genomic RNA is arranged into three open reading frames (ORFs) and the sub-genomic RNA contains two ORFs (Figure 1.2). Translation of ORF1 produces a 200 kDa polypeptide that is cleaved by the virus-encoded protease to generate seven non-structural proteins critical for viral replication (p48/N-terminal [NS1/2], a RNA helicase [p41/2-C-like, NS3], p22 [3A-like, NS4], VPg (NS5], a viral protease [Pro, 3C-like, NS6] and a viral RNA-

dependent RNA polymerase [RdRp, NS7] (Table 1.1) (reviewed in [42-44]). ORF2 translation produces the major structural protein, VP1, which assembles into the icosahedral capsid. The VP1 protein structure comprises the shell (S) and protruding (P) domains; the S domain encloses the viral RNA, while the antigenically variable P domain forms the outer surface of VP1, and is also involved in cell attachment[38,39]. Translation of ORF3 produces VP2 which is present on the interior surface of the capsid and thought to be involved in viral assembly and genome encapsidation[45].

Table 1.1. Summary of human norovirus ORF1 non-structural protein function

Protein	Proposed function(s)	Ref(s)
NS1/2	Inhibition of intracellular trafficking	46
NS3	RNA helicase and chaperoning activity NTPase activity	47,48
NS4	Membrane alteration Inhibition of actin cytoskeleton remodelling Disruption of ER to Golgi trafficking	49-51
NS5	Primer for transcription Recruitment of translational machinery	52
NS6	Cleavage of viral polyprotein	53,54
NS7	Replication of viral genome	55

1.4 Models for norovirus replication and infection

Despite the clinical significance of norovirus infection, antiviral studies have been hindered, because until recently, human norovirus could not be successfully propagated in cell culture. Recent breakthroughs have enabled human norovirus to be cultured in B cells (BJAB cell line)[56] and intestinal enteroids[57], which represent milestones in the field of norovirus biology. However, the modest replication levels generated by these new systems (≤3.5 log increase in B cells[56,58] and ≤3.8 log increase in enteroids[57]) means that they require optimisation before widespread use for antiviral screening.

The GI.1 (Norwalk virus) norovirus replicon system has been employed to assess antiviral candidates against human norovirus *in lieu* of a viral culture system. The Norwalk replicon consists of an intact ORF1, ORF3 and genomic 3' end, however ORF2 is disrupted by a neomycin gene (neo), engineered into the VP1-encoding region (Figure 1.3). Self-replicating and stably expressed in Huh-7 cells or BHK-21 cell lines[59], the Norwalk replicon has proven itself as a useful tool to screen potential antiviral compounds.

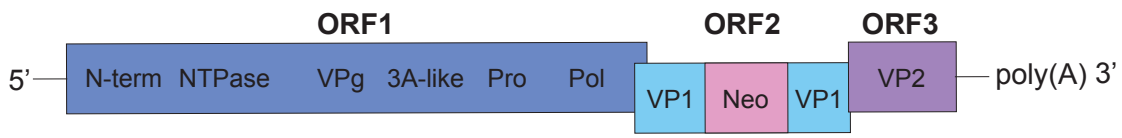


Figure 1.3. Schematic of Norwalk replicon

Representation of the Norwalk replicon capable of autonomous replication in cell culture. The replicon has a neomycin gene (neo) engineered into the VP1 coding sequencing to allow for selection *in vitro*. Figure adapted from [59].

Other models have also been used to study antiviral efficacy against norovirus (Figure 1.4). For example, researchers have used *in vitro* enzyme activity assays, such as viral polymerase assays[60] and protease assays [61] to screen compounds for antiviral activity in a high-throughput format[62]. In addition, X-ray crystallography and *in silico* modelling can be used to examine ligand and viral protein interactions to further elucidate antiviral mechanisms[63] (Figure 1.4).

Surrogate viruses from within the *Caliciviridae* family have also been exploited to screen for inhibitory activity of antiviral candidates across several calicivirus genera (Figure 1.4). These surrogates include murine norovirus (MNV; *Norovirus*), feline calicivirus (FCV; *Vesivirus*), porcine sapovirus (PSaV; *Sapovirus*), rabbit haemorrhagic disease virus (RHDV; *Lagovirus*), and Tulane virus, from the proposed genus *Recovirus*[64]. Of these, MNV has been used as the predominant human norovirus surrogate as it is classified within the same genus and can be robustly propagated in cell

culture (>4 log-fold increases in viral titre, PFU/mL)[65]. MNV has a similar genome organisation to human norovirus, with the addition of a fourth ORF that reportedly encodes a putative virulence factor-1[66]. The ability of MNV to replicate at significantly higher levels has facilitated our understanding of the fundamental properties of norovirus biology, including replication[67,68], receptor entry[69-71], pathogenesis[66,72,73], and the discovery of potential antivirals[74-78]. Additional features that make MNV desirable for antiviral screening include its ability to be manipulated though reverse genetics, whilst *in vivo* studies in mice of many genetic backgrounds are straightforward.

Antiviral studies with FCV are less common, however this virus can be utilised in an almost identical manner to MNV. FCV infection of feline kidney cell lines can generate comparable viral titres to MNV, when measured by plaque assay or $TCID_{50}$. Moreover, we have recently shown that recombinant FCV RdRp and Pro can be used for *in vitro* antiviral testing which is beneficial for large scale screening of potential antiviral compounds[286]. However, FCV has yet to be used in an animal model for antiviral screening, and thus, MNV remains the most robust, reproducible and versatile tool for norovirus antiviral studies.

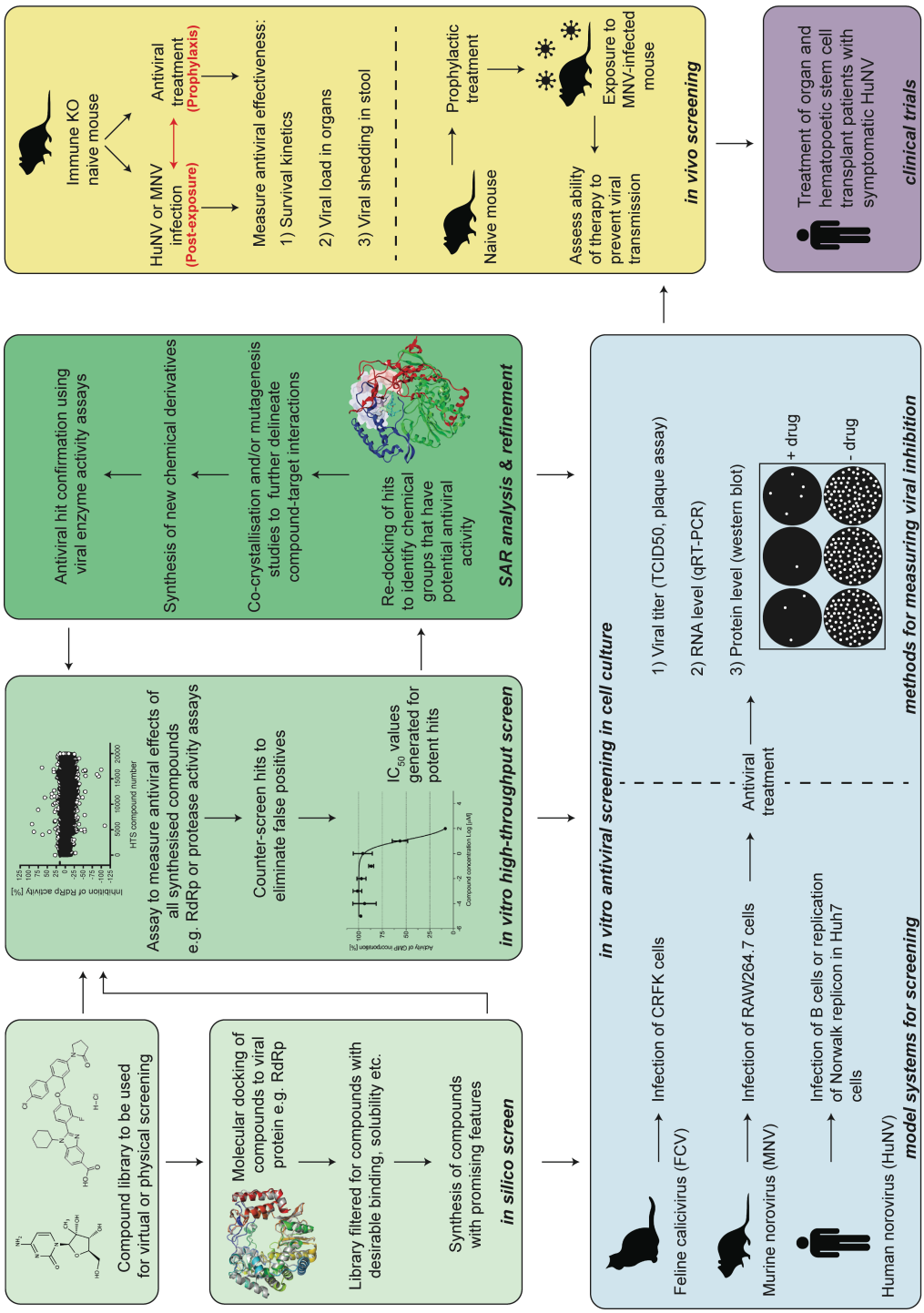


Figure 1.4. Current methods for the identification and characterisation of norovirus antivirals

A flow chart depicting the norovirus model systems as well as methods used to assess the effectiveness of norovirus antivirals. Panels in green involve a combination of *in silico* and *in vitro* methods. Panels in blue and yellow represent *in vitro* and *in vivo* methods respectively. The purple panel represents clinical testing in human patients. IC_{50}: Half maximal inhibitory concentration; $TCID_{50}$: Tissue culture infective dose; RdRp: RNA-dependent RNA polymerase; CRFK: Crandell Feline Kidney; RT-qPCR: Quantitative reverse-transcription polymerase chain reaction; SAR: Structure-activity relationship. Figure taken from [41].

1.5 Clinical management of norovirus infections

Currently, there is no approved norovirus vaccine or antiviral approved for clinical use and the standard of care for acute infection is symptomatic treatment and hydration. For persistent norovirus infections, ribavirin (RBV) and immunoglobulins can be administered, however patients that don't respond to these therapies can be given a course of nitazoxanide (NTZ). This compound was originally developed in the 1970s, and is currently an FDA-approved therapy for treating Giardia and Cryptosporidium infections[79]. NTZ has demonstrated broad-spectrum antimicrobial activity against a range of bacterial, protozoan and viral infections, including inhibition of the Norwalk replicon (half maximal effective concentration; EC_{50} of 1.6 μM) (reviewed in [80]). Several anecdotal studies and a phase II clinical trial have collectively shown that treatment with NTZ can resolve diarrhoeal symptoms and effectively clear norovirus from stool samples [81-83]. However, there is also an equal amount of evidence documenting that NTZ is ineffective against norovirus infections[84-87]. Despite this contrary evidence, NTZ is the only therapeutic option currently available when RBV, immunoglobulins and supportive care fails to cure persistently infected patients.

1.6 Emerging norovirus antivirals

There is a clear unmet need for effective norovirus antivirals, particularly for the treatment of chronically infected individuals. However, antivirals would also be useful for prophylactic treatment to reduce the burden of epidemics and protect high-risk groups from infection. Efforts thus far to develop norovirus antivirals have mainly focused on targeting viral proteins involved in replication including the polymerase (section 1.6.1) and protease. These compounds are known as direct-acting antivirals (DAAs). Moreover, antivirals that prevent cell attachment or that modulate features within the host cell (i.e. host-targeted therapies) (section 1.7) have also been screened for efficacy against norovirus. Examples of such compounds are outlined in Figure 1.5.

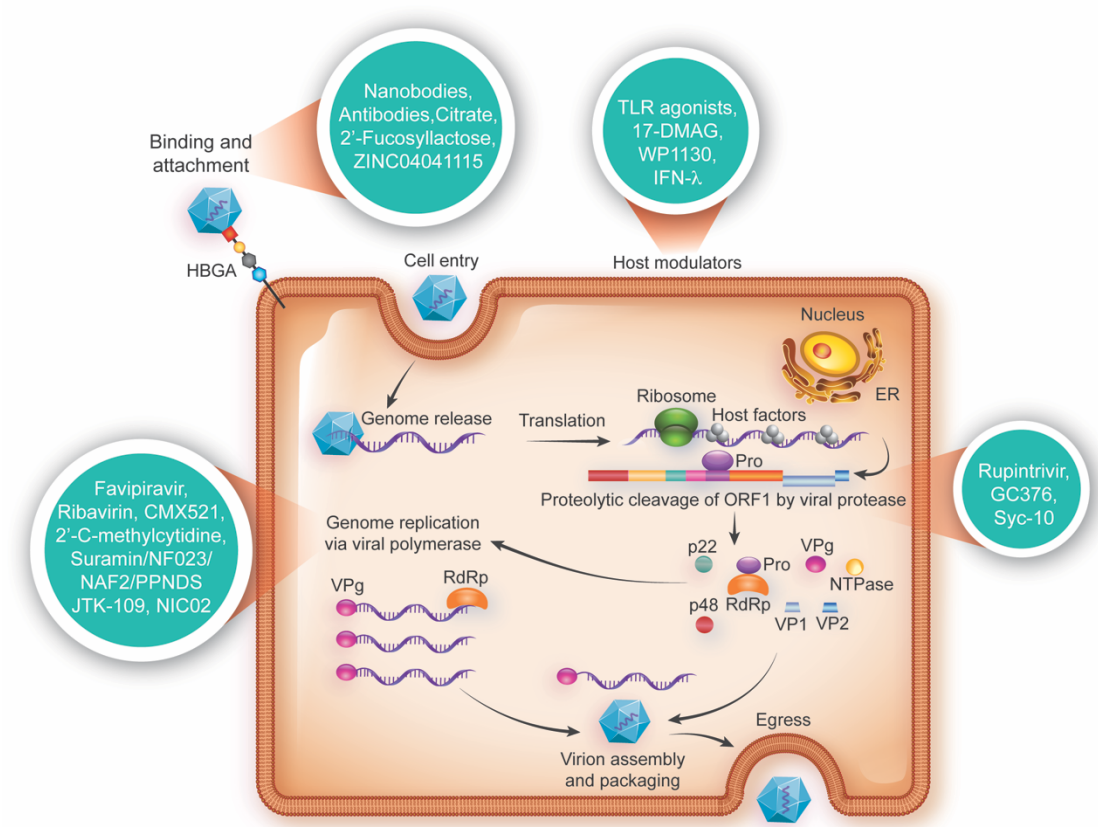


Figure 1.5. The viral replication cycle of human norovirus and antiviral targets

A schematic of the complete norovirus replication cycle is presented to illustrate the antiviral targets that are currently being explored. The stages of the replication cycle targeted for antiviral development include: host cell attachment, internalisation, genome release, viral genome replication mediated by the viral RdRp, translation of the genomic and subgenomic templates using the VPg and host cell machinery, viral protease cleavage of the viral polyprotein to yield mature viral proteins, followed by assembly, packaging and cell egress (reviewed in [88]). Examples of compounds that have shown antiviral activity against noroviruses are listed within the green circles. Figure adapted from [41].

1.6.1 DAAs of the polymerase and protease

Critical for viral replication, the norovirus RdRp (NS7) is a highly attractive antiviral target, as it largely lacks host homologs which minimises the chance of off-target adverse effects [89]. The human norovirus polymerase forms the canonical RdRp structure resembling a closed right hand, with fingers, palm and thumb domains[90] (Figure 1.6). The RdRp-targeting antivirals are divided into two major classes; the nucleoside analogues (NAs) and the non-nucleoside inhibitors (NNIs). NAs inhibit

RNA synthesis through mimicry of incoming nucleoside triphosphates (NTPs), which upon incorporation subsequently cause chain termination[91], or less commonly, increase mutations during viral genome transcription that results in lethal mutagenesis (also known as error catastrophe)[92]. NNIs generally exhibit narrow-spectrum antiviral activity, and bind allosterically to block conformational rearrangements of the viral polymerase required to form an active replication complex[93]. NAs and NNIs have been successful in the treatment of hepatitis C virus (HCV), human immunodeficiency virus (HIV), herpesvirus and hepatitis B virus (HBV) infections (reviewed in [94-98]), and candidates from both classes have been assessed for antiviral activity against norovirus.

Much like the RdRp, the norovirus protease (NS6) represents a desirable antiviral target since it plays an essential role in viral replication, through cleavage of the NS (Figure 1.6). Norovirus protease inhibitors (PIs) described in the literature are broadly divided into transition state (TS) inhibitors and TS mimics (reviewed in [99]) with a subclass of macrocyclic compounds[100-103] which are structural variants of peptide-based TS inhibitors designed to improve the membrane permeability and oral bioavailability[103]. Several PIs are documented to have low nanomolar potency across several calicivirus genera and multiple norovirus genogroups. Together DAAs of the protease and polymerase represent the largest class of antivirals that have been screened against noroviruses.

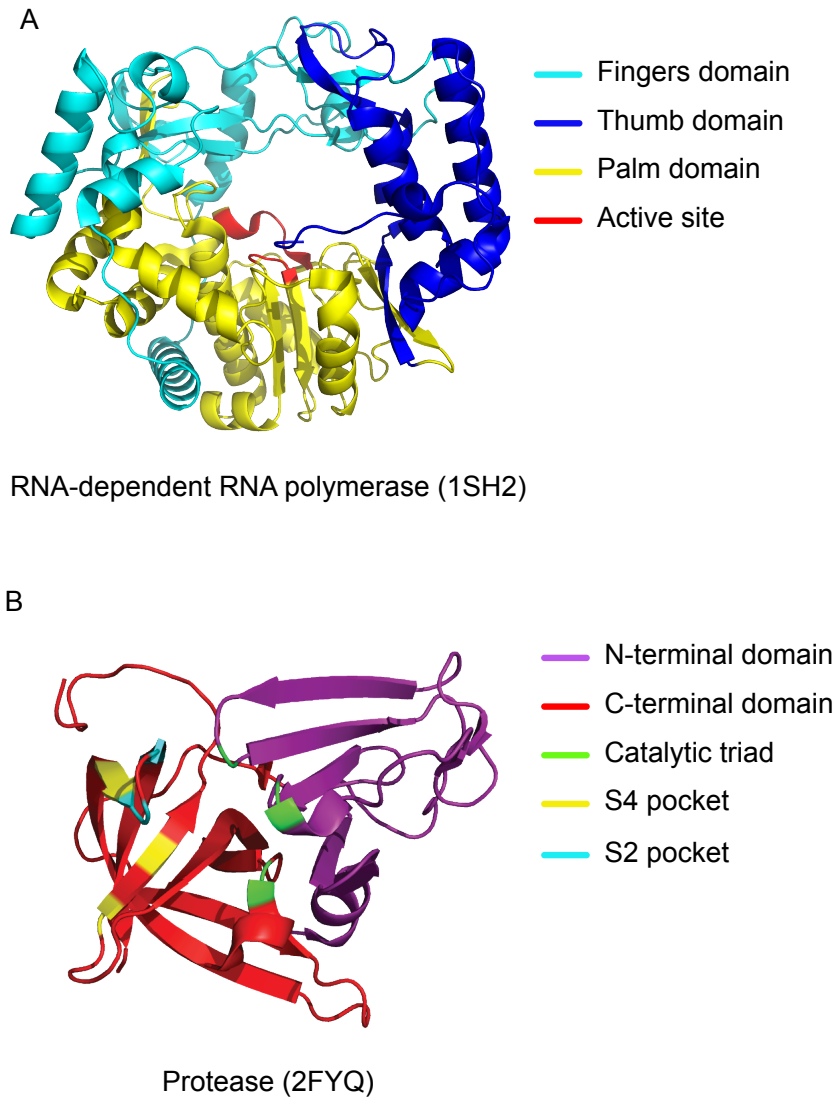


Figure 1.6. Norovirus proteins targeted by direct-acting antivirals

(A) Crystal structure of the Norwalk virus RdRp (1SH2) with the fingers (cyan), thumb (blue) and palm (yellow) domains color-coded. The active site is coloured in red and depicts the site of incorporation for NAs. (B) Crystal structure of the Norwalk virus protease (2FYQ) with N- and C-terminal chains coloured in magenta and red, respectively. Residues of the catalytic triad are coloured in green. Residues for the S2 (cyan) and S4 (yellow) pockets[145] are shown as representative binding sites for protease inhibitors. Figure adapted from [41].

1.6.2 NAs

Since NAs interact with the highly conserved RdRp active site, they generally demonstrate broad-spectrum antiviral activity compared to NNIs. Therefore, NAs developed against other RNA viruses have been examined for repurposing as human norovirus therapeutics. In terms of norovirus, 2'-C-methylcytidine (2CMC) is the most intensely studied, and was initially developed as an antiviral therapy against HCV. 2CMC is a chain terminating cytidine analogue with broad-spectrum *in vitro* activity against other flaviviruses including dengue virus (DENV), yellow fever virus (YFV) and West Nile virus (WNV)[104]. However, the development of the oral 2CMC prodrug, Valopicitabine, was halted for use against HCV following reports of undesirable gastrointestinal side effects[105].

Despite 2CMC being discontinued for clinical development, it is still widely reported as a potential norovirus antiviral. In one study, MNV plaque formation and RNA synthesis were inhibited by 2CMC, with EC_{50} values of 2.0 µM and 1.6 µM, respectively[106]. 2CMC was also found to reduce RNA synthesis of the Norwalk replicon in a dose-dependent fashion, with an EC_{50} of 18 µM[77]. In the human norovirus BJAB cell culture system, 2CMC inhibited human norovirus replication with an EC_{50} of 0.3 µM[107]. 2CMC has also shown promise as a potential norovirus antiviral in mouse model studies. Knockout mice infected with MNV and treated with 2CMC were protected from mortality, diarrhoea, and had reduced norovirus genome titres within tissues and stool (1.0-1.5 $\log_{10}$ reduction), compared to mock treated animals[77,107]. Additionally, MNV infected knockout mice treated with a high dose of 2CMC (100 mg/kg/day for 5-7 days) demonstrated reduced transmission to uninfected sentinel mice caged together, and offered prophylactic protection for up to 18 days[78]. Collectively these studies illustrate that the polymerase is an excellent antiviral target for cross-genogroup inhibition of norovirus replication.

Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is another broad-spectrum NA[108] that was shown to induce lethal mutagenesis against MNV[109], consistent with results from other

viruses[110]. T705 is approved for treatment of influenza in Japan[111] and also inhibits replication of several viruses using *in vitro* and mouse models, including; flaviviruses, arenaviruses, hantaviruses[108] and Ebola virus[112]. T705 has poor antiviral activity against MNV replication in cell culture, inhibiting virus-induced cytopathic effects with an EC_{50} of 250 μM, and RNA synthesis with an EC_{50} of 124 μM[113], with a therapeutic index of just 4.3[113]. The human norovirus replicon has also been used to examine the antiviral efficacy of T705, which exhibited a modest EC_{50} of 21 μM and a half maximal cytotoxicity concentration (CC_{50}) of >100 μM[114]. Despite clinical approval for influenza, T705 displays a level of potency that is likely too low to be pursued further as a norovirus therapeutic.

Another broad-spectrum NA examined against norovirus is RBV (1-α-d-ribofuranosyl-1,2,4-triazole-3-carboxamide). RBV has broad spectrum antiviral activity against both DNA and RNA viruses and has been used clinically to treat HCV, hepatitis E virus, Lassa fever and respiratory syncytial virus, amongst others (reviewed in [115]). A guanoside analog, RBV was first shown to inhibit the human norovirus replicon with an EC_{50} of around 40 μM for Norwalk replicon[116], however, RBV poorly inhibits MNV (EC_{50} of 63.5 μM)[117]. The modest inhibition of RBV against MNV and the Norwalk replicon, coupled with the numerous adverse effects reported with RBV treatment, suggests that this antiviral is not desirable as an anti-norovirus agent.

CMX521 is a novel NA discovered through high-throughput screening (HTS), and according to the manufacturer's press releases, has potent and pan-genotypic activity against norovirus[118]. CMX521 is reportedly in the recruitment stage of phase I clinical trials to evaluate the safety, tolerability and pharmacokinetics in ≤50 healthy adults. No peer-reviewed work has been published, however results are projected to be released later in 2018[118].

While a growing body of research has been invested into identifying antivirals that target the norovirus polymerase, to date all candidates are still in early preclinical development and the search for a compound continues in earnest.

1.7 Host targeted antivirals

A pitfall of using direct-acting antivirals is the emergence of resistance mutations which can undermine their effectiveness, although combinational therapy is a proven option in HIV and HCV therapy to circumvent this. In comparison to DAAs, antivirals that target the host generally have a higher barrier to resistance than direct-acting antivirals (reviewed in [119]) and thus represent an important antiviral class to be considered for the treatment of norovirus infections. Host-factor drugs can target individual cellular components that directly interact with the virus, or aid viral replication. Alternatively, host-factor drugs may influence multiple cellular components that culminate in antiviral defences.

Protein targets

A recently explored class of host-targeted norovirus antivirals include deubiquitinase (DUB) inhibitors[120-122]. DUB inhibitors are a class of enzymes involved in regulation of the ubiquitin proteasome system (UPS) which is commonly exploited by viruses for replication (reviewed in [123]). WP1130 is an example of a small synthetic DUB inhibitor (M_w of 384.2 g/mol) that was shown to effectively inhibit MNV and norovirus replication through induction of the unfolded protein response (UPR)[122]. However, this initial study revealed that inhibition of MNV in mice was limited to the small intestine due to poor bioavailability of WP1130[122]. To address the poor bioavailability, libraries of WP1130 variants were developed and tested for improved antiviral efficacy[120,121]. Out of 40 derivatives, compound 9 was found to be the most potent antiviral[120]. Treatment at 2.5 µM with this derivative resulted in an 84.7% reduction in replication of the Norwalk replicon and a 2.5-log reduction in MNV titre in infected mice; more than double the potency of the parental compound[120].

Another host protein that has been identified as an antiviral target is heat-shock protein 90 (Hsp90), a molecular chaperone involved in the maturation of proteins responsible for multiple biological

processes (reviewed in [124]). Using small interfering RNA (siRNA) knockdown and overexpression studies, Vashist and colleagues initially showed that Hsp90 interacts with the 5' and 3' termini of the MNV genome and plays a key role in MNV replication[125]. The authors then showed that abolishment of Hsp90 activity using 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) potently inhibited MNV replication *in vitro* (IC_{50} of 60 nM and CC_{50} of 4.5 μM) and reduced MNV titre (~ 1-log) in an *in vivo* mouse model[125]. These studies collectively demonstrated that targeting specific host proteins results in effective antiviral activity with minimal off-target effects, which are desirable features for norovirus therapeutics.

Immunomodulators

Immunomodulators are an excellent therapeutic option for virus infections due their ability to induce a powerful host response against intracellular parasites. The best example of immunomodulators are interferons (IFNs) and for over a decade, studies have shown that type I and II IFNs, as well as their receptors, provide protection against murine and human norovirus infections[59,126-133]. However, the role of type III IFNs (IFN-λ), in norovirus infection and their potential as norovirus antivirals has only recently been explored[134]. IFN-λ binds to a heterodimeric receptor comprised of IFN-λ receptor 1 (IFNLR1) and interleukin-10R2 (IL-10R2), which induces the expression of many of the same genes induced by type I and II IFNs[135-138]. However, unlike type I and II IFN, a single dose of IFN-λ (1 μg) has been shown to both prevent and clear persistent MNV infection in mice[139]. IFN-λ was shown to target non-haematopoietic cells [139] and intestinal epithelial cells (IECs) which express high levels of IFNLR1[140]. Using the MNV mouse transmission model, endogenous IFN-λ expression (induced using a transfected plasmid) was shown to block MNV transmission from mice infected with the acute CW3 MNV strain[141]. IFN-λ treatment also prevented intestinal CW3 replication, inflammation and antibody responses in mice[141], which illustrates that it has an integral role in the prevention of norovirus transmission. Taken together, the antiviral activity

displayed by IFN-λ in these studies reveal that it has potential as a therapeutic to not only cure infections, but also prevent norovirus spread if given prophylactically.

More recently, the group of immunomodulators known as toll-like receptor (TLR) agonists have been explored as norovirus antivirals. These compounds have been used for many years as vaccine adjuvants, but have also been shown to inhibit the replication of the RNA and DNA viruses, HIV and HBV respectively[142,143]. TLR agonists induce the innate immune response and stimulate IFN production (Figure 1.7), which is known to have antiviral activities against MNV and human norovirus *in vitro*[59,126-133]. The TLR4 agonist, poly-γ-glutamic acid (γ-PGA) was explored as an antiviral against norovirus and when tested against MNV *in vitro* displayed an EC_{90} value <100 nM[144]. In addition, a 50 mg/kg dosage of γ-PGA to mice prior to and following MNV infection resulted in ≥ 47% reduction in viral genomes[144]. Given that the TLR4 agonist monophospholipid A is approved for use as an adjuvant for human papillomavirus (HPV) and HBV vaccines (reviewed in [145]) and TLR7 agonists such as R-837 are already approved for the treatment of HPV-associated genital warts[146], repurposing of TLR agonists could hugely expedite their use for norovirus clinical treatment.

Antivirals that target host-factors have wide-reaching therapeutic applications not only for norovirus treatment, but also for many other viruses. In addition, the limited risk of resistance is another benefit of host-modulating compounds, especially for the genetically divergent norovirus. Moreover, host-modulators can invoke multiple antiviral pathways that collectively limit viral replication within infected cells. Thus, the clearance of persistent norovirus infection with such compounds (namely IFN-λ) show promise for the treatment of patients with chronic norovirus infection.

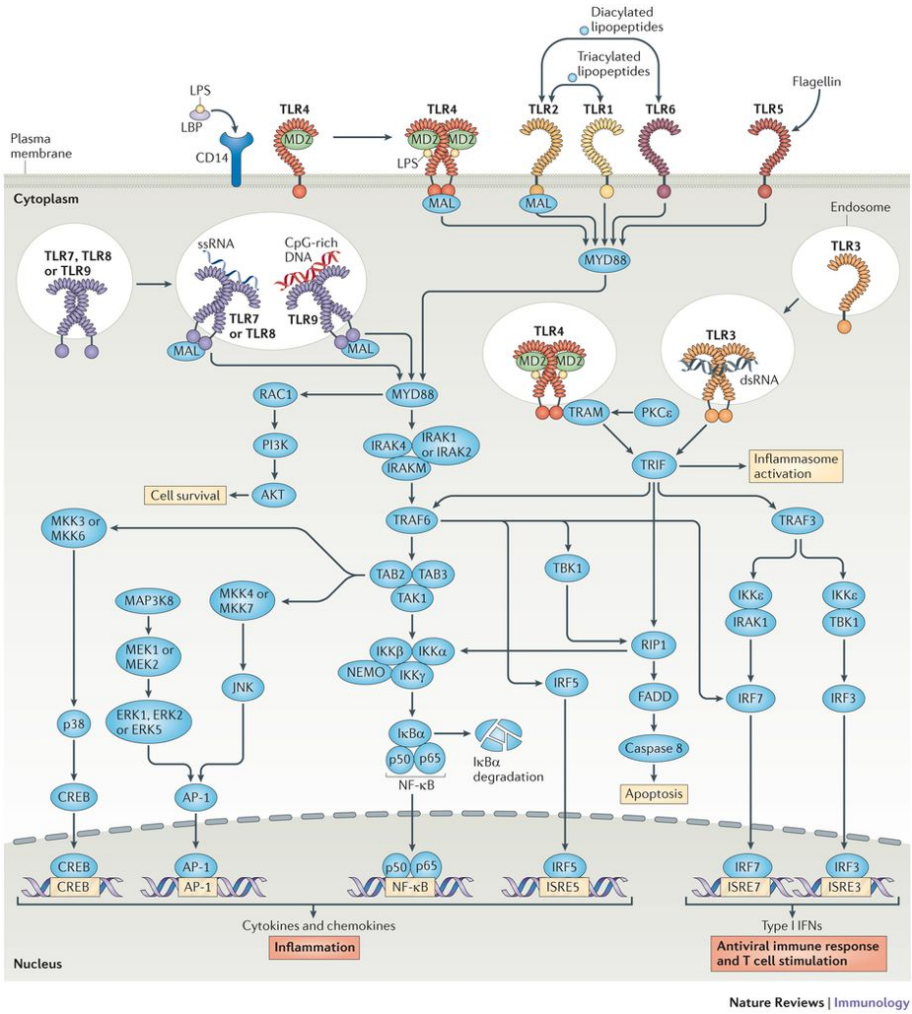


Figure 1.7. Overview of TLR signalling pathways

Schematic of the location of TLRs within a mammalian cell and associated signalling pathways. TLRs are present within the endosome membrane or plasma membrane and recognise specific ligands. Nucleic acids of viral origin are recognised by TLR3, TLR7, TLR8, and TLR9. Signalling cascades activate transcription factors that can translocate to the nucleus and increase expression of genes involved in antiviral defence and immunity. Figure taken from [147].

1.8 Aims and outline of this thesis

Human norovirus infections have a significant social and economic impact globally, causing hundreds of thousands of deaths each year. In the absence of an effective vaccine, antiviral compounds to combat norovirus infection are highly desired. However, despite intensive research for safe and effective norovirus antivirals, none have been clinically approved and most candidates are still in pre-clinical development.

Therefore, the overall aims of this thesis were to increase our understanding of the host-response to norovirus infection and to use this knowledge to identify new host targets for norovirus antivirals. Moreover, we aimed to screen antiviral compounds against said targets to assess their potential as norovirus therapeutics. Lastly, we aimed to identify new potential DAAs for the treatment of calicivirus infections (human norovirus included).

In **chapter three**, an in-depth transcriptomics analyses of the host response to MNV infection was performed to (1) better characterise the features of the innate response that are altered by infection, (2) identify biological processes of the host that may be manipulated by noroviruses, and (3) identify potential features of the host that could be targeted therapeutically. Importantly, we identified significant downregulation in the expression of several genes involved in MHC class I molecule maturation as well as genes involved in virus recognition, including TLR7, which suggest a dampening of the innate immune response to promote continued viral replication.

In **chapter four**, we targeted TLR7 using small molecule agonists to identify whether stimulation of this receptor would inhibit norovirus replication. We screened TLR7 agonists against MNV and the Norwalk replicon and show that these compounds have potent antiviral activity. Overall, this study identified a new receptor that can be targeted for norovirus antiviral development.

In **chapter five**, we investigate the antiviral activity of a viral polymerase inhibitor, NITD008, against MNV, FCV and human norovirus (Norwalk replicon). This compound is the most potent polymerase inhibitor of noroviruses described in the literature to date.

By using a multi-targeted approach, we discovered new therapeutic targets and candidates for norovirus infections. Several of these candidates are already approved for the treatment of viral infections or are in the late phases of clinical development and thus could be more easily repurposed as a therapy for norovirus.

2 Materials

Table 2.1. General reagents and kits

Materials	Source and details
Bacterial strains	
Escherichia coli BL21 (DE3)	New England Biolabs (C2527H)
Escherichia coli DH5α	New England Biolabs (C2987H)
Mammalian cell lines	
Murine macrophages (RAW264.7)	Gift from Dr. Hebert Virgin
Human hepatocytes (Huh7)	Gift from Dr. Mark Douglas
Human monocytes (THP-1)	Gift from Dr. Sheila Donnelly
Viruses and replicons	
Murine norovirus (MNV), CW1 strain	Gift from Dr. Hebert Virgin
Norwalk virus replicon	Gift from Dr. Kim Green
Antibodies	
MNV polymerase (NS7)	Gift from Dr. Jason Mackenzie
MNV major capsid protein (VP1)	Abcam (92976)
Anti-rabbit, HRP-linked	Cell Signaling Technology (7074)
Plasmids	
pET26b(+)	Novagen (69862)
pTrcHis2c	Life Technologies (V36520)
Kits	
BigDye Terminator sequencing kit	Applied Biosystems (4337458)
CellTitre-Blue cell viability assay	Promega (G8080)
Immobilon Western HRP substrate kit	Millipore (P36599A)
iTaq Universal SYBR Green Supermix	Bio-Rad (1725121)
Pierce™ BCA Protein assay kit	Life Technologies (23225)
Pur-A-Lyzer™ Maxi dialysis kit	Sigma-Aldrich (PURX12015)

Materials	Source and details
QIAamp Viral RNA kit	Qiagen (52904)
QIAprep spin plasmid miniprep kit	Qiagen (27104)
Quanti-IT™ PicoGreen dsDNA assay kit	Life Technologies (P11496)
Ribo-Zero Gold kit (human/mouse/rat)	Illumina (MGZG12324)
RNase-Free DNase set	Qiagen (79254)
RNeasy Mini RNA extraction kit	Qiagen (74104)
Superscript™ VILO™ cDNA synthesis kit	Life Technologies (11755050)
TruSeq Stranded Library kit	Illumina (20020594)
Reagents	
2-Mercaptoethanol	Sigma-Aldrich (M3148)
Agarose (UltraPure)	Life Technologies (16500500)
Agarose (low gelling temperature)	Sigma-Aldrich (A9414)
Amicon® Ultra-4 centrifugal filters	Sigma-Aldrich (UFC8030)
Ampicillin	Sigma-Aldrich (A9518)
Benzonase Nuclease (250 U/µL)	Sigma-Aldrich (E1014)
Bio-Scale™ Mini Profinity™ IMAC cartridges	BioRad (7324610)
Bovine serum albumin (BSA)	Sigma-Aldrich (A3059)
CelLytic™ B	Sigma-Aldrich (C8740)
Coomassie® Brilliant Blue R-250 dye	BioRad (1610400)
CpG oligodeoxynucleotide (ODN)	Innaxon (IAX-200-007)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (D4540)
Diothiothreitol (DTT)	Astral Scientific (C-1029)
Dulbecco's Modified Eagle Medium (DMEM)	Life Technologies (11965118)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (E8008)
Foetal Bovine Serum (FBS)	Sigma-Aldrich (12003C)
Formaldehyde	Univar (EUD2)
Geneticin (G418)	Life Technologies (10131035)
GlutaMax™ Supplement	Life Technologies (35050061)
Glycerol	Sigma-Aldrich (G5516)
HEPES	Life Technologies (15630080)
Imidazole	Sigma-Aldrich (I5513)
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Sigma-Aldrich (11411446001)

Materials	Source and details
Luria Bertani (LB) broth	Life Technologies (12795027)
Lysozyme	Sigma-Aldrich (L7651)
Methanol	Univar (A2314)
Nickel sulphate	Sigma-Aldrich (656895)
n-octyl glucopyranoside (n-OPG)	Sigma-Aldrich (O8001)
Penicillin-Streptomycin	Life Technologies (15140122)
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich (P8139)
Polycytidylic acid (poly C)	Innaxon (IAX-200-021)
Protease inhibitor cocktail	Sigma-Aldrich (P8849)
Protease/Phosphatase inhibitor (100X)	Cell Signaling Technology (5872)
Radioimmunoprecipitation assay (RIPA) buffer	Sigma-Aldrich (R0278)
Ribonucleotide triphosphates (rNTPs)	Promega (P1221)
RPMI-1640 Medium	Sigma-Aldrich (R5886)
SOC outgrowth medium	New England Biolabs (B9020S)
Sodium chloride	Univar (AJA465)
TRIzol LS reagent	Life Technologies (10296010)
Mini-PROTEAN^{TM} polyacrylamide gels	BioRad (4561034)
Antiviral compounds	
2'-C-Methylcytidine (2CMC)	Sigma-Aldrich (M4949)
Gardiquimod	Adipogen (AG-CR1-3583)
Imiquimod (R-837)	Adipogen (AG-CR1-3569)
Loxoribine	Adipogen (AG-CR1-3584)
NITD008	In Vitro Technologies (RDS60451)
Resiquimod (R-848)	Adiopgen (AG-CR1-3582)
Vesatolimod (GS-9620)	Cayman Chemical (19628)
Buffers	
Laemmli sample buffer (2x)	BioRad (1610737)
Phosphate buffered saline (PBS)	Life Technologies (14190250)
Protein extraction buffer	25 mM Tris-HCl pH 7.5, 10 mM NaCl
Protein binding buffer	25 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% (w/v) n-OPG

Materials	Source and details
Protein binding buffer	25 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% (w/v) n-OPG
Protein storage buffer	25 mM Tris-HCl, 200 mM NaCl, 20% glycerol, 1 mM DTT
Tris/Acetate/EDTA (TAE) buffer	Life Technologies (24710030)
Tris/Glycine (10X) buffer	BioRad (1610734)
Tris/Glycine/SDS-buffer (10X)	BioRad (1610732)
Tris-HCl (pH 7.5) (1M)	Life Technologies (15567027)

3 RNA-sequencing of murine norovirus infected cells reveals transcriptional alteration of genes important to viral recognition and antigen presentation

Text and figures included in this chapter are adapted from the following publication:

Enosi Tuipulotu D, Netzler NE, Lun JH, Mackenzie JM and White PA. RNA-sequencing of murine norovirus infected cells reveals transcriptional alteration of genes important to viral recognition and antigen presentation. *Frontiers in Immunology*. 2017, 8:959.

Author contributions

Conceived and designed experiments: DET, JM and PW

Performed the experiments: DET, NN and JL

- Bioinformatic analysis: DE
- MNV infections: DE and NN
- Blotting: DE
- RNA extractions and qRT-PCR: DE and JL
- Data analysis: DE

Wrote the manuscript: DET and PW

Declaration

I certify that this publication was a direct result of my research towards this PhD, and that reproduction in this thesis does not breach copyright regulations.

Daniel Enosi Tuipulotu [Candidate]

3.1 Abstract

Viruses inherently exploit normal cellular functions to promote replication and survival. One mechanism involves transcriptional control of the host, and knowledge of the genes modified and their molecular function can aid in understanding viral-host interactions. Norovirus pathogenesis, despite the recent advances in cell cultivation, remains largely uncharacterised. Several studies have utilised the related MNV to identify innate response, antigen presentation, and cellular recognition components that are activated during infection. In this study, we have used next-generation sequencing to probe the transcriptomic changes of MNV-infected mouse macrophages. Our in-depth analysis has revealed that MNV is a potent stimulator of the innate response including genes involved in IFN and cytokine production pathways. We observed that genes involved in viral recognition, namely *IFIH1*, *DDX58*, and *DHX58* were significantly upregulated with infection, whereas we observed significant downregulation of cytokine receptors (*Il17rc, Il1rl1*, *Cxcr3*, and *Cxcr5*) and TLR7. Furthermore, we identified that pathways involved in protein degradation (including genes *Psmb3*, *Psmb4*, *Psmb5*, *Psmb9*, and *Psme2*), antigen presentation, and lymphocyte activation are downregulated by MNV infection. Thus, our findings illustrate that MNV induces perturbations in the innate immune transcriptome, particularly in MHC maturation and viral recognition that can contribute to disease pathogenesis.

3.2 Introduction

Innate immunity is an essential part of the host response to limit viral replication and prevent disease manifestation. The general innate pathway has the following steps: virus detection by cellular receptors, receptor activation, recruitment of adapter proteins, intracellular signalling cascades, nuclear translocation of transcription factors, and expression of genes important for host defense and adaptive immune stimulation[148]. Viral detection is carried out by host pathogen recognition receptors (PRRs), which are activated through their interaction with components of the virus structure called PAMPs (pathogen associated molecular patterns)[149]. PRRs are divided into three groups, which include the TLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding domain, leucine-rich repeat-containing receptors (NLRs)[150]. Members of each receptor family are involved in nucleic acid detection and are expressed either within the plasma membrane or within the endosome membranes[150].

Viral activation of PRRs causes a powerful stimulation of several signalling pathways[151] involved in the type I IFN response, including the mitogen activating protein kinase (MAPK)[152], nuclear factor kappa B (NF-κB)[153], IFN regulatory factor (IRF)[154], and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways. These antiviral pathways can influence host gene expression, protein production, and post-translational modifications to generate an antiviral state within the infected cell. In this study, we aim to understand how MNV overcomes this antiviral state and continues with robust replication in the host cell.

Previous work on MNV has demonstrated that STAT1, RAG2 (recombination activating gene), type I and type II IFN receptors are used to limit MNV infection in mice[30]. Other components of innate immunity shown to play a role in MNV infection include MDA5 for viral recognition[155], IRF3 and IRF7 for antiviral transcriptional control[156], IFN stimulated gene 15 (ISG15)[157], type I, II, and III IFNs[129,139]. These innate pathways are involved in cytokine production, stimulation of the adaptive immune

system, cell proliferation, and apoptosis[158]. Other aspects of innate biology during MNV infection have also been explored, including MHC class I expression[159-161]and the antiviral properties of IFN-γ in the context of persistent infection[139].

The overall aim of the current study was to characterise the biological processes subverted by MNV, particularly those of the innate immune response, to gain insights into norovirus pathogenesis. Earlier transcriptomic analyses of MNV infection have demonstrated alterations in the immune response[162], chemokine production[163], regulation of apoptosis[72], cholesterol synthesis[164], and the cell cycle[165]. However, the availability of next-generation sequencing (NGS) in recent years has provided an unparalleled technique to measure the global transcription changes in response to viral infection. In the current study, RNA sequencing was used to probe the cellular transcriptome of mouse macrophages following longitudinal MNV infection to identify changes that occur as viral infection progresses. Since transcriptomic profiling by next-generation sequencing can result in experimental noise, we employed the use of the TLR7 agonist Loxoribine firstly as a positive control, since the effects of this compound on the host transcriptome have been well described. Secondly, since TLR7 activation and MNV infection are both known to induce IFN, we hypothesised that many genes would be similarly altered in their transcriptional profile. As such, we compared the transcriptional profiles between both experimental conditions to identify changes unique to virus infection. Such unique transcriptional changes are likely to represent viral-mediated modifications of the host transcriptional response, rather than the generalised host response to PRRs. Our findings highlight key components of the host cell response affected by MNV and provide plausible explanations into the mechanisms by which norovirus causes disease. First, we characterised induction of a robust innate response with changes detected as early as 4 hpi that continued to develop with increased viral replication. We show global downregulation of genes that encode proteins important for the control of gene transcription and protein translation, which may be involved in the host protein shut-off, a characteristic of calicivirus infection[166]. In addition, we

propose a mechanism by which MNV could regulate the expression of MHC class I molecules in a bid to limit immune recognition. We discuss the complexities by which MNV modulates transcript levels of genes in several biological pathways and their impact on our understanding of norovirus pathogenesis.

3.3 Methods

3.3.1 Cell maintenance, stimulation and virus infections

Murine macrophage RAW264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% (v/v) foetal bovine serum (Sigma-Aldrich, St. Louis, USA), 2 mM Glutamax (Thermo Fisher, Waltham, USA), 100 U/mL penicillin (Thermo Fisher) and 100 µg/mL streptomycin (Thermo Fisher). MNV-1 CW1 strain used in this study was purified from culture supernatant by ultracentrifugation, as previously described[133]. MNV infections were carried out longitudinally from 4 to 20 h at a multiplicity of infection (MOI) of 5, and infection at 12 h (MOI 5) was carried out in quadruplicate for comparisons with loxoribine treatment (1 mM). All experiments were carried out on monolayers of $1x10^7$ RAW264.7 cells and after the appropriate incubation cells were collected for sequencing. Mock infections were performed for all experiments with complete media.

3.3.2 RNA extraction and quality control

Viral and cellular RNA was extracted from infected RAW264.7 cell monolayers using TRIzol LS (Invitrogen, Carlsbad, USA), with phase separation carried out as per the manufacturer's instructions. RNA was further purified from contaminants using the RNeasy Mini Kit (Qiagen, Hilden, Germany), which included DNA removal using RNase-free DNase (Qiagen). RNA was quantified using spectrophotometry and RNA integrity was assessed on a Bioanalyzer (Agilent Technologies) prior to downstream analysis.

3.3.3 Library preparation and sequencing

RNA extracted from loxoribine treated, MNV infected and mock infected RAW264.7 cells was depleted of cytoplasmic and mitochondrial ribosomal RNA using the Ribo-Zero Gold Kit (Human/Mouse/Rat) (Illumina) and libraries were prepared using reagents and protocols supplied

in the TruSeq Stranded Library prep kit (Illumina). Briefly, RNA was chemically fragmented and then reverse transcribed using random hexamers. Thereafter, unique adapter sequences were ligated to the newly synthesised cDNA products and PCR amplified. Libraries were validated by BioAnalyzer followed by 75-bp paired-end read sequencing on the NextSeq500 platform (Illumina), carried out at the Ramaciotti Centre for Genomics at the University of New South Wales. All sequence data have been submitted to the Gene expression omnibus (GEO) data repository under series numbers GSE94821 and GSE94843.

3.3.4 Sequence analysis

Bioinformatic analysis of RNA sequencing was performed using the Cufflinks tool suite[167] on the Galaxy server at the University of Queensland, Australia[168-170]. Following quality control (removal of adapter sequences and trimming), reads were mapped to the mm10 genome (UCSC) using TopHat (v0.9) with default parameters[171]. Mapped reads were then assembled into transcripts, normalised and quantified using Cufflinks (v2.2.1.0)[167]. Assembled transcripts of all replicates, within all conditions, were merged into a single catalogued transcriptome. Thereafter, transcript abundance was compared between mock and treated or infected samples using Cuffdiff (v2.2.1.2) to identify differentially expressed genes (DEGs)[172]. Genes with a 4-fold or greater expression change with a FPKM value greater than 1 in at least one sample were considered differentially expressed (DE) for the longitudinal infection analysis. Genes with a q-value <0.05, 2-fold or greater expression change and a FPKM value of 1 in at least one sample were considered DE for the analysis of cells infected with MNV or treated with loxoribine for 12 h.

3.3.5 Gene enrichment analysis

Enrichment analysis was performed to identify the functional role that the DEGs, identified from MNV infection and loxoribine treatment, play within the host. Gene lists were analysed on the online

servers DAVID[173] and GOrilla[174] for gene ontology and/or KEGG pathway analysis, and those most significant outputs were collated.

3.3.6 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total cellular RNA (1 µg) was reverse transcribed in 10 µl reactions using the SuperScript VILO cDNA MasterMix synthesis kit (Thermo Fisher), following the manufacturer's instructions. To quantitate viral genome copies and host gene expression levels following infection, qPCR was performed. Each reaction contained 2 µL of 10-fold diluted cDNA, 10 µL 2x iTaq universal SYBR Green supermix (Bio-Rad, Hercules, USA) and 0.5 µM of both forward and reverse primers in a total volume of 20 µL (Table 3.11). Amplification was performed on a RotorGeneQ (Qiagen) with 95^{o}C denaturation for 1 min followed by 40 cycles of 95^{o}C for 5 s, 55^{o}C for 20 s and 72^{o}C for 20 s. Fold changes in mRNA abundance following MNV infection, were calculated using the ΔΔCt method[175]. Viral RNA was quantified using qPCR primers that amplify a 187 bp product within the MNV RdRp encoding region at the 3' end of ORF1, as previously described[176].

3.3.7 Protein extraction and immunoblotting

To extract proteins, MNV or mock infected cells were lysed in RIPA buffer (Sigma) supplemented with 100x protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, USA). Protein concentrations were measured using the BCA assay (Thermo Fisher). Protein (50 µg) from each sample was separated on an 10% Tris-glycine SDS-polyacrylamide gel (BioRad) and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Kenilworth, USA). Membranes were blocked with 5 % skim milk in PBS with 0.1% Tween 20 and incubated with a primary antibody against the human norovirus capsid protein (Abcam, Cambridge, UK) (ab92976) at a 1:1,000 dilution for 90 min at room temperature. Thereafter, the membrane was incubated for 1 hr with an anti-rabbit, HRP-linked secondary antibody (Santa Cruz Biotechnology) at a 1:10,000 dilution. Western blots were developed using a chemiluminescence HRP-substrate (Merck Millipore).

3.3.8 Statistical analyses

All bar and linear regression graphs were generated in in PRISM v.6.0h and error bars are plotted with the standard deviation of either triplicate or quadruplicate experiments of a condition. Correlation analysis was performed using the Pearson method on linear regression analysis.

3.4 Results

3.4.1 Productive MNV infection in RAW264.7 cells

The replication kinetics of MNV has been well studied[65,68]; however, little is known about the point at which MNV induces transcriptional changes within the host. We anticipated that cellular changes would mimic increases in viral replication, with more noticeable changes in gene expression occurring as infection progressed. RNA sequencing was used to quantify transcriptomic changes over time from 4 to 20 hpi and approximately 30 million 75-bp paired-end reads were generated for each sample (n = 12) with an average of 84% of reads mapped successfully to the mouse 10 mm (UCSC) reference genome (Table 3.1).

To have confidence that host expression changes were attributable to MNV infection, viral replication was assessed using several methods. Primarily, the RNA sequencing reads from each infection time point were mapped to the MNV CW1 strain reference sequence (Table 1.1) and nucleotide coverage at each position across the MNV genome was quantified (Figure 3.1, panel A). This analysis revealed complete coverage of the MNV genome from sequencing reads with a >2 log increase in the abundance of MNV transcripts from 4 to 20 hpi. MNV replication was also confirmed by quantification of viral protein by Western blot targeting the major capsid protein, viral protein 1 (VP1) (Figure 3.1B) and RT-qPCR quantification of viral genomes (Figure 3.1C). We observed increased signal intensity corresponding to MNV VP1 that correlates to increased viral replication (Figure 3.1B). Similarly, viral genomes increased >60-fold over the 20 h infection period when measured by RT-qPCR (Figure 3.1C). Together, these gradual increases in viral genome and protein levels, detected by RNA sequencing, quantitative RT-qPCR, and Western blot, demonstrate robust MNV replication over time.

Further to this, we also mapped reads to Abelson Mu-LV and Moloney Mu-LV genomes to rule out viral contamination. A negligible number of reads were mapped to either viral genome, with

minimal coverage; >92% of either viral genome was missing. In addition, no significant increase in reads was detected over the time course (Table 3.12).

MNV replication involves the production of both genomic and subgenomic RNA[65,177,178] both of which encode proteins that are critical to the production of infectious virions[179]. To ascertain the relative abundance of genomic and subgenomic RNA, the average nucleotide coverage spanning ORF1 and ORF2–3 were individually quantified and compared. We found a greater level of nucleotide coverage at the ORF2-3 structural region compared to non-structural ORF1 region at each infection time point (1.39-, 2.45-, 1.5-, 1.71-, and 1.52-fold increase for 4, 8, 12, 16, and 20 hpi, respectively) (Figure 3.1D). The higher proportion of reads mapped to the ORF2-3 region (average of 1.7-fold) confirms the presence of a subgenomic RNA species.

Table 3.1. Summary of sequencing reads mapped to the host and MNV genomes

		% of reads mapped to:	
Sample	Total no. of reads*	MNV	*M. musculus*
mock	57380304	0.00	91.70
4 hpi	59221498	0.05	91.50
8 hpi	61203972	3.12	88.70
12 hpi	54950136	6.21	84.70
16 hpi	56463488	11.18	80.20
20 hpi	57627090	11.81	78.60

***** trimmed and filtered

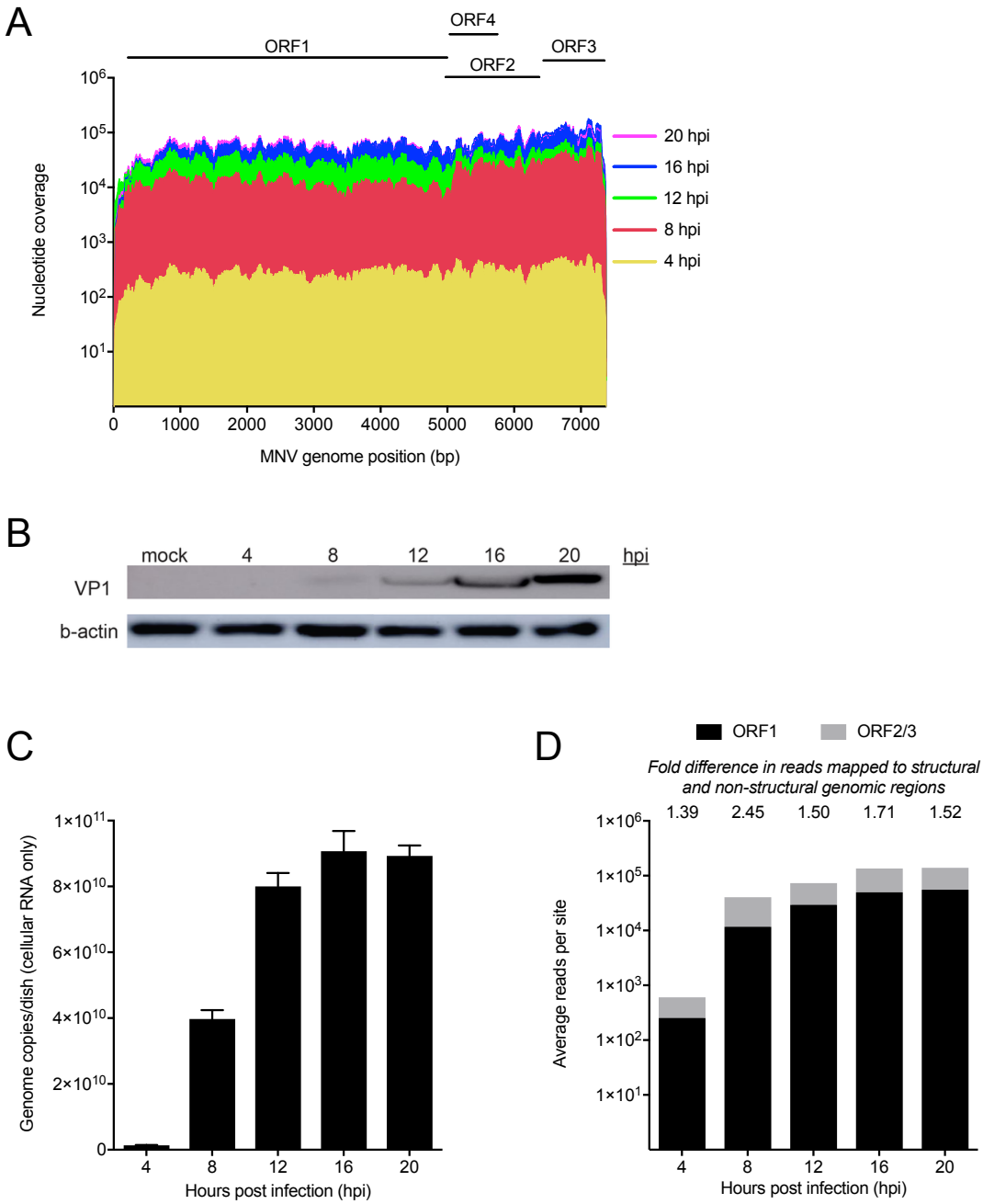


Figure 3.1. Longitudinal analysis of MNV replication

(A) Sequencing reads from each infection time point were mapped to the MNV genome and are represented in a coverage plot stratified by genome position. (B) Western blot for MNV VP1 was performed to quantify viral protein levels over time. (C) RT-qPCR for was performed quantify viral genomes over time. (D) Quantification of reads mapped to structural (grey) and non-structural regions (black) of the MNV genome. Fold differences between the average read coverage of ORF2-3 relative to ORF1 are listed above the graph.

3.4.2 MNV infection induces pronounced innate host expression changes with time

To determine if gene expression changes would intensify as MNV replication proceeds and to measure the intensity of the host response to MNV infection, we analysed the global expression changes over time (Figure 3.2A) (Table 3.4), in addition to a more focused analysis of innate gene expression (Figure 3.2B). A simple enumeration of all genes altered within the mouse macrophages revealed a marked increase in the number of DEGs from 4 to 20 hpi (23- and 78-fold for upregulated and downregulated genes, respectively) (Figure 3.2A) that correlated with increased MNV replication (Figure 3.1). Furthermore, a heatmap of 280 innate immune-related genes (Figure 3.2B) exhibits that increased gene expression occurred as early as 4 hpi, whereas decreases in gene expression were less prominent at this time point and were generally detected in the later stages of the infection time course (12–20 hpi) (Figure 3.2B). Our longitudinal analysis demonstrated substantial increases in transcript abundance of innate genes, especially at 20 hpi for *Cxcl2* (174.8-fold), *Cxcl3* (30.9-fold), *Cxcl10* (16.5-fold), and *Ccl7* (7.0-fold) encoding chemokines[180,181]; *Il1a* (78.8-fold) and *Il1b* (48.3-fold) encoding interleukins [182,183]; as well as signalling molecules involved in induction of the IFN and genes induced by IFN (Figure 3.2B). Conversely, several genes involved in viral recognition and intracellular signalling were significantly downregulated over time including *TLR13* (5.3-fold), *TLR7* (3.4- fold) which encode TLRs [184,185]; *Il17rc* (4.2-fold), *Il1rl1* (2-fold), *Cxcr3* (13.6-fold), and *Cxcr5* (3.9-fold) encoding cytokine and chemokine receptors[186-188]; and *Trim14* (2.3-fold), *Trim2* (2.6-fold), *Trim68* (3.1-fold), *Trim47* (2.1-fold), and *Trim7* (3.0-fold) encoding members of the tripartite motif (Trim) family, which are important adaptor molecules of viral recognition receptors involved in activation and initiation of downstream intracellular signalling (Figure 3.2B)[189]. Overall, we see that genes involved in the IFN response were highly upregulated with infection, while genes that encode proteins important for viral recognition displayed decreases in transcript abundance as infection progressed.

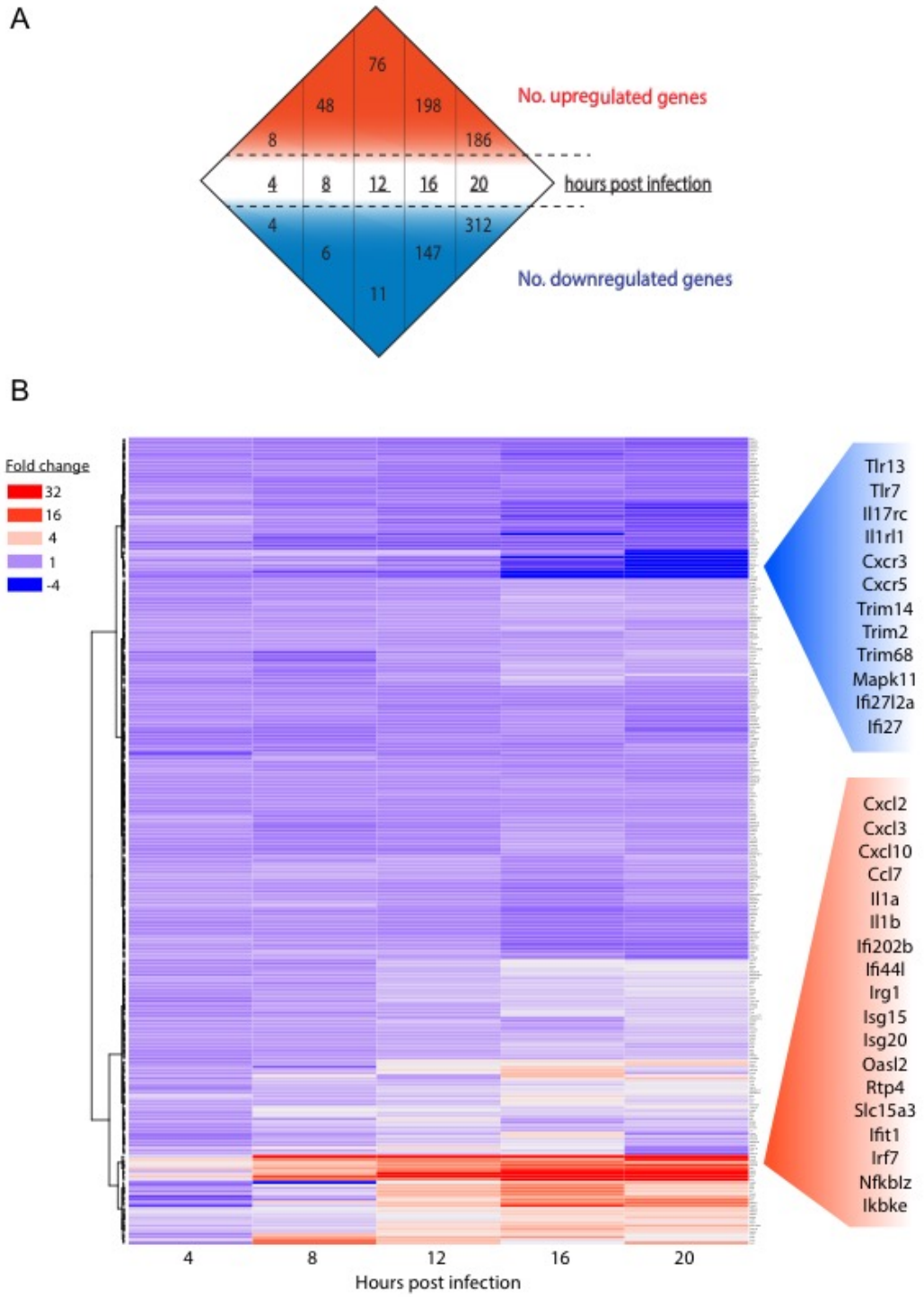
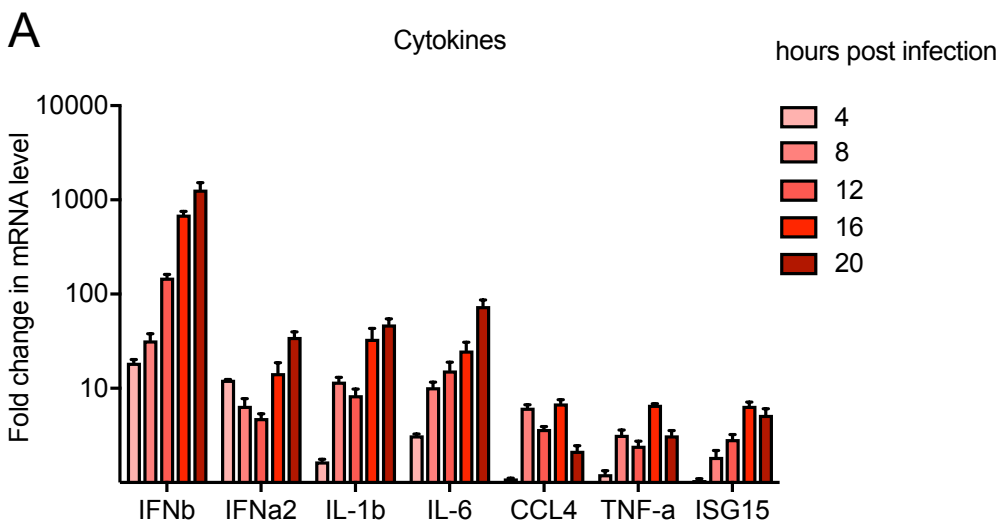


Figure 3.2. Host response of RAW264.7 cells infected with MNV over time

(A) Differentially expressed genes (fourfold or more change and FPKM value >1 in at least one sample) for each infection time are summarised numerically. Values above (red) and below (blue) the hashed lines represent upregulated and downregulated genes, respectively. (B) A total of 280 genes involved in the innate response were probed over time and are displayed in a heat map. Each panel represents a particular gene, and the colour depicts the fold change at each time point. Red and blue side panels represent upregulated and downregulated genes, respectively, that show the greatest level of change with MNV infection.

3.4.3 qPCR validates MNV-induced innate gene expression changes

To validate transcriptomic changes detected from RNA sequencing during MNV infection over 20 h, 20 genes that encode key innate molecules of the antiviral response were probed for expression changes using RT-qPCR (Figure 3.3A–C). A steady increase in the level of gene expression was observed over time for genes encoding cytokines (Figure 3.3A), transcription factors and signalling molecules (Figure 3.3B) and PRRs, apart from *TLR3* and *TLR7* (Figure 3.3C). The most significant changes occurred in genes encoding cytokines (Figure 3.3A), with upward of 1,000- fold difference in expression recorded for *IFN-β1* at 20 hpi with *IFNα2*, *IL-1b*, and *IL-6* displaying >10-fold increase in transcript abundance at 20 hpi (Figure 3.3A). Increased expression changes for genes encoding transcription factors and PRRs were less prominent, although a gradual intensification of expression occurred over time for all genes analysed (Figure 3.3A–C). A strong correlation existed between gene expression levels determined by RT-qPCR and NGS transcriptomic analysis ($r = 0.88$, p-value < 0.0001, Pearson coefficient) across all five time points of the longitudinal MNV infection, as demonstrated by linear regression analysis (Figure 3.3D).



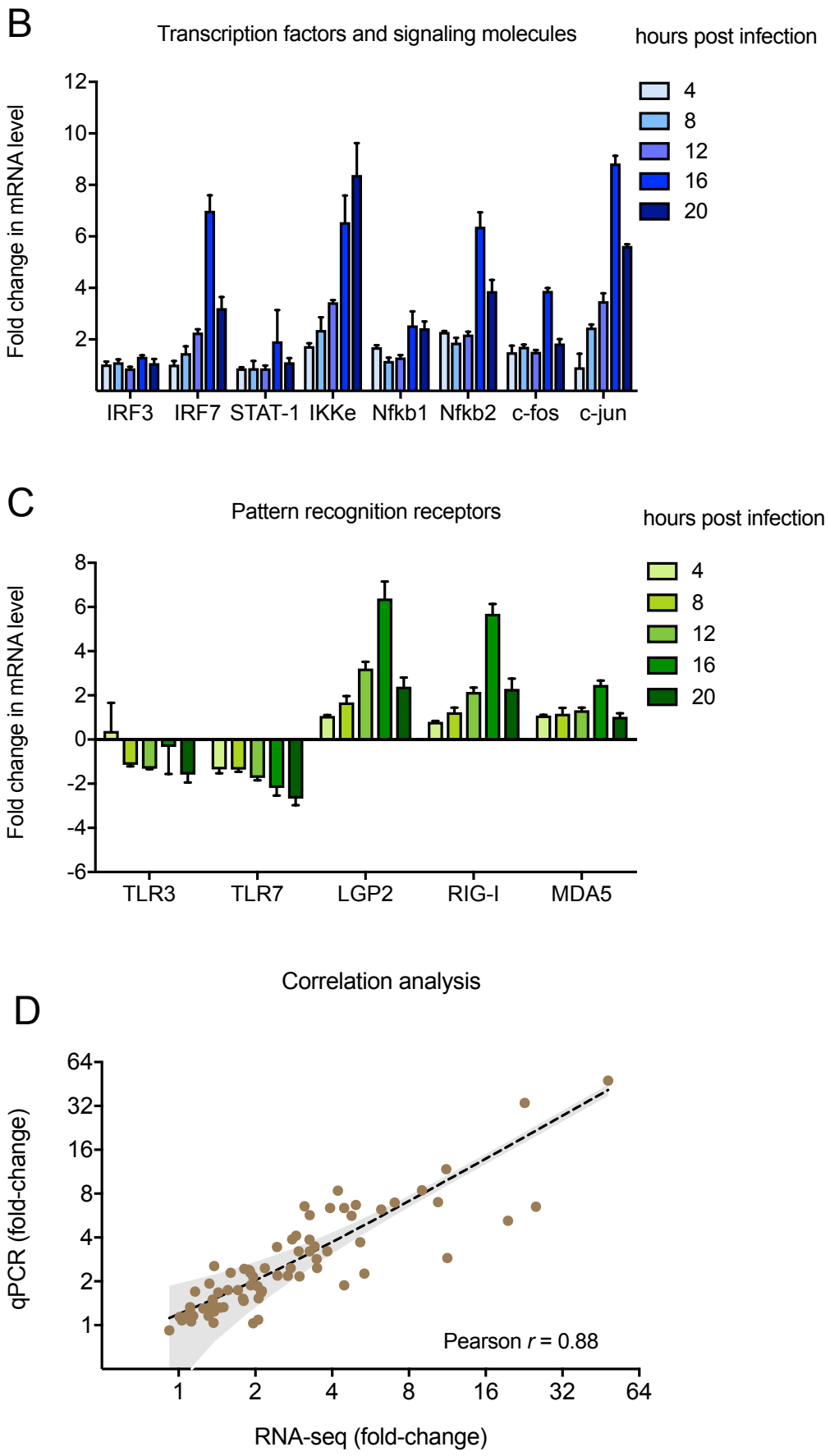

B
Transcription factors and signaling molecules
hours post infection
4
8
12
16
20
Fold change in mRNA level
12
10
8
6
4
2
IRF3
IRF7
STAT-1
IKKe
Nfkb1
Nfkb2
c-fos
c-jun
C
Pattern recognition receptors
hours post infection
4
8
12
16
20
Fold change in mRNA level
8
6
4
2
0
-2
-4
-6
TLR3
TLR7
LGP2
RIG-I
MDA5
Correlation analysis
D
qPCR (fold-change)
64
32
16
8
4
2
1
Pearson r = 0.88
1
2
4
8
16
32
64
RNA-seq (fold-change)

Figure 3.3. Validation of NGS analyses

Reverse transcription-quantitative polymerase chain reaction was performed to validate the use of NGS to quantify transcriptomic changes in murine norovirus (MNV)-infected cells. A total of 20 genes encoding (A) cytokines, (B) transcription factors, and (C) pathogen recognition receptors were screened for changes in expression with MNV infection over time. Fold changes (relative to mock) were calculated using the ΔΔCt method. (D) Changes in transcript abundance obtained from RNA-sequencing and qPCR were plotted for correlation analysis. The inverse fold changes for downregulated genes were used to generate a graph with positive correlation. Grey shading represents the 95% confidence interval for linear regression analysis (dotted black line, r = 0.88, p-value < 0.0001, Pearson correlation coefficient).

3.4.4 MNV infection and loxoribine treatment induce distinct expression profiles

Following the initial time-course screen, we aimed to obtain a more detailed picture of host response changes induced by MNV infection, specifically at 12 hpi when infection is robust (Figure 3.4)[67]. RNA sequencing of RAW264.7 cells infected with MNV or treated with loxoribine was performed and subsequent analysis yielded information on >11,000 genes, and the expression profiles for both conditions, relative to mock, are presented in Figure 3.4. The transcriptome of macrophages infected with MNV had 476 genes significantly DE based on a q-value < 0.05 (FDR adjusted p-value), FPKM value >1 in at least one of the experimental conditions and a twofold or greater change in expression (Figure 3.4A) (Table 3.5). Comparatively, loxoribine treatment yielded a greater increase in expression changes and a higher number of DEGs (n = 1,956) (Figure 3.4B) (Table 3.6). Interestingly, both datasets contained significantly more downregulated genes than upregulated (Figure 3.4A-B). For MNV specifically, 345 genes were downregulated and 131 genes were upregulated (Figure 3.4A) and the 25 genes with the greatest degree of change following infection for 12 h are summarised in Table 3.2 and Table 3.3. A volcano plot distribution of altered expression induced by either MNV infection or loxoribine treatment is presented in Figure 3.4C-D. This illustrates the predominance of downregulated genes in both datasets, and a greater fold change in expression is noted for loxoribine treatment.

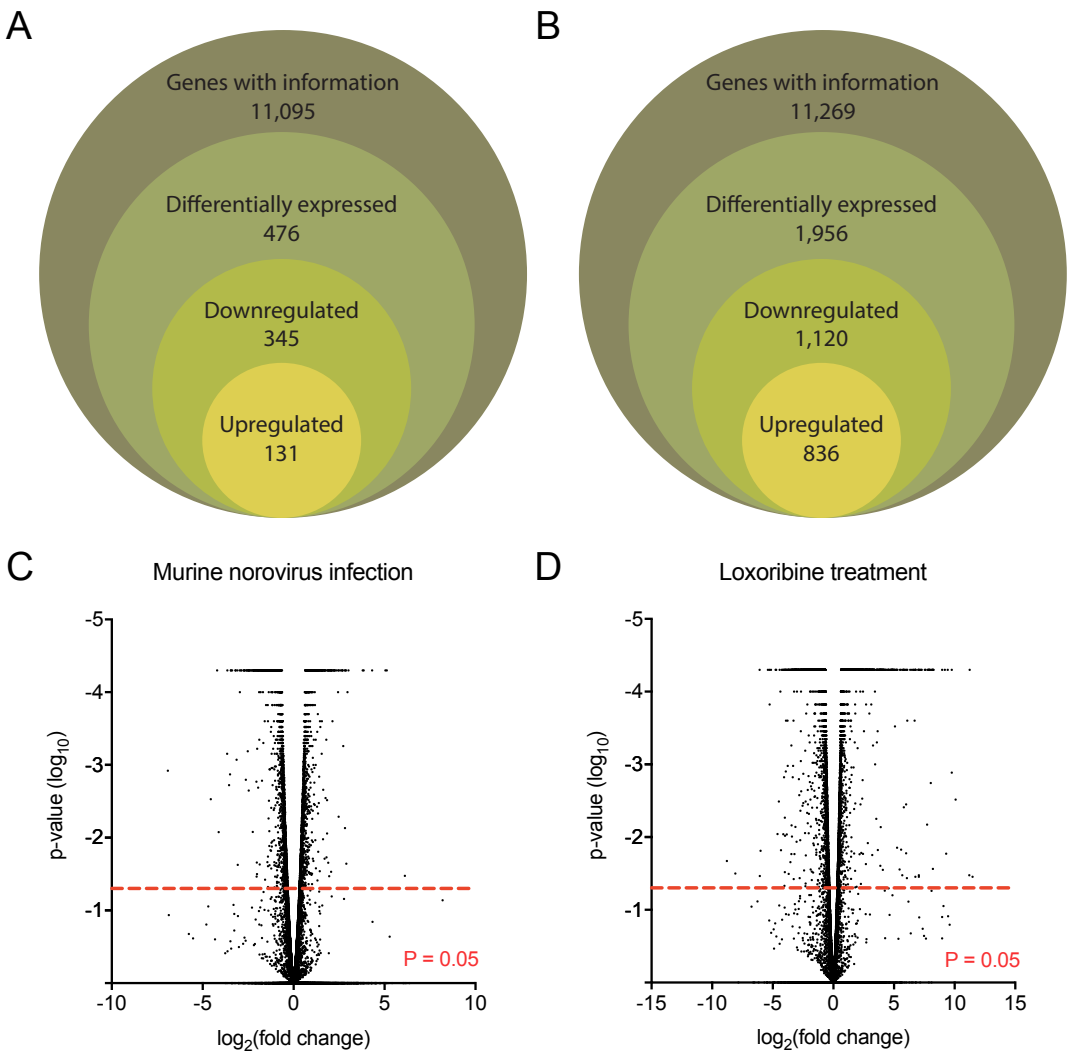


Figure 3.4. Gene expression profiling of MNV-infected and loxoribine-treated RAW264.7 cells

The global gene expression changes in RAW264.7 cells following 12 h of (A) MNV infection or (B) loxoribine treatment. Genes were further classified as differentially expressed based on the following stringencies: q value < 0.05, twofold or more change in transcript abundance and an FPKM value greater than 1 in at least one sample (mock or infection/treatment). The global distribution of gene expression in both MNV infection (C) and loxoribine treatment (D) is shown as a volcano plot with expression fold change plotted on the x-axis and significance on the y-axis. Genes significantly expressed are above the hashed red line (p-value < 0.05).

Table 3.2. Top 25 upregulated genes 12 hpi

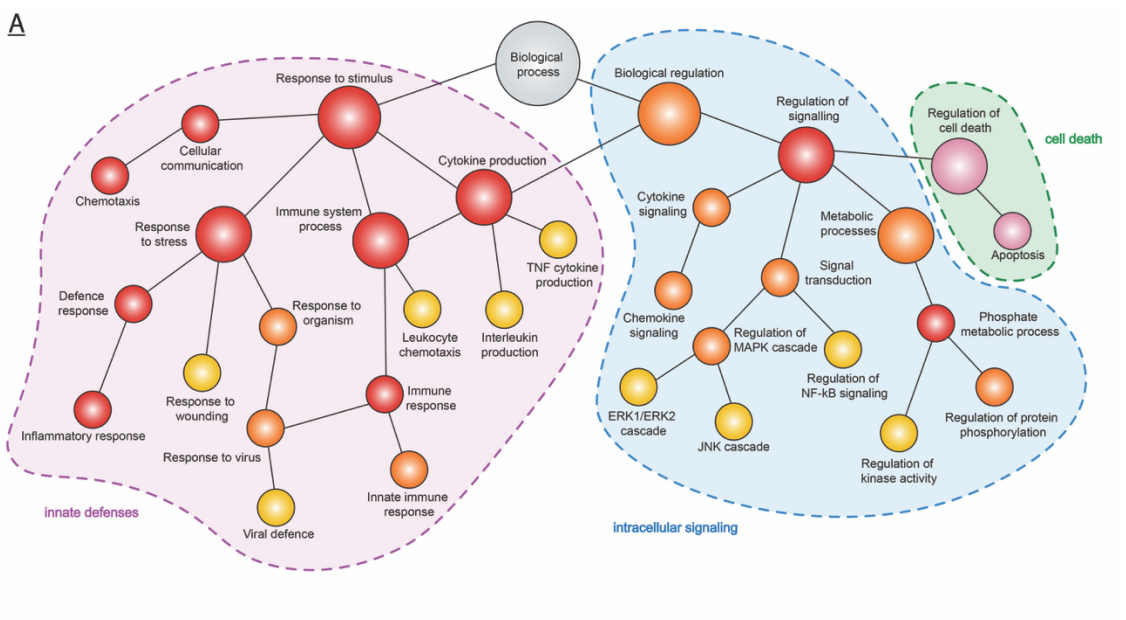
Gene ID	Description	Fold change
Lamc2	Laminin, gamma 2	34.77
Egr1	Early growth response 1	33.22
Cxcl2	Chemokine (C-X-C motif) ligand 2	19.94
Flrt3	Fibronectin leucine rich transmembrane protein 3	14.47
Plk2	Polo-like kinase 2	13.90
Egr2	Early growth response 2	8.08
Il1a	Interleukin 1 beta	7.78
Ccrl2	Chemokine (C-C motif) receptor-like 2	7.29
Kdm6b	KDM1 lysine (K)-specific demethylase 6B	7.00
Arc	Activity regulated cytoskeletal-associated protein	6.45
Mir155	microRNA 155	6.43
Irg1	Immunoresponsive gene 1	6.36
Phlda1	Pleckstrin homology like domain, family A, member 1	6.02
Rtp4	Receptor transporter protein 4	5.56
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	5.55
Ccl7	Chemokine (C-C motif) ligand 7	5.46
Oas2	2'-5' oligoadenylate synthetase 2	5.22
Olr1	Oxidised low density lipoprotein (lectin-like) receptor 1	4.94
Il7r	Interleukin 7 receptor	4.77
Arid5a	AT rich interactive domain 5A (MRF1-like)	4.68
Ifit1	IFN-induced protein with tetratricopeptide repeats 1	4.67
Gem	GTP binding protein overexpressed in skeletal muscle	4.63
Ppp1r15a	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	4.39
Tnfaip3	Tumour necrosis factor, alpha-induced protein 3	4.35
Myc	Myelocytomatosis oncogene	4.33

Table 3.3. Top 25 downregulated genes 12 hpi

Gene ID	Description	Fold change
Hist1h2br	Histone cluster 1, H2br	-120.20
H2-Q10	Histocompatibility 2, Q region locus 10	-23.55
Hist1h2ad	Histone cluster 1, H2aD	-18.48
Dancr	Differentiation antagonising non-protein coding RNA	-17.41
Ppia	Peptidylprolyl isomerase A	-12.62
Rps23	Ribosomal protein S23	-12.51
Snord89	Small nucleolar RNA, C/D box 89	-11.66
Snord22	Small nucleolar RNA, C/D box 22	-11.26
Hist1h3h	Histone cluster 1, H23h	-10.92
Cacng8	Calcium channel, voltage-dependent, gamma subunit 8	-10.88
Hist1h4n	Histone cluster 1, H4n	-10.37
Snora78	Small nucleolar RNA, H/ACA box 78	-9.17
Rps15a-ps4	Ribosomal protein 15a, pseudogene 4	-9.13
Rpl9	Ribosomal protein L9	-8.80
Hist2h2aa2	Histone cluster 2, H2aa2	-8.51
Dnaja1	DnaJ heat shock protein family (Hsp40) member A1	-7.79
Snora74a	Small nucleolar RNA, H/ACA box 74A	-7.75
Mir682	MicroRNA 682	-7.48
Rps10	Ribosomal protein S10	-7.26
Rps14	Ribosomal protein S14	-6.69
Oaz1	Ornithine decarboxylase antizyme 1	-6.65
Rpl36	Ribosomal protein L36	-6.51
Hist1h2bp	Histone cluster 1, H2bp	-6.38
Rps28	Ribosomal protein 28	-6.19
Tufm	Tu translation elongation factor, mitochondrial	-6.03

3.4.5 A robust innate response is mounted following MNV infection (12 h)

To identify the function of DEGs induced by MNV 12 hpi (Figure 3.4A), ontology analyses were performed using GOrilla (Figure 3.5A-B) (Table 3.7). Although despite the higher number of downregulated DEGs (n = 345) compared to upregulated DEGs (n = 131) (Figure 3.4A), fewer GOterms (p-value < 0.001) were represented by the downregulated DEGs (n = 90) relative to the upregulated DEGs (n = 392). Of the upregulated DEGs (n = 131), several biological functions were significantly overrepresented including the response to stimulus (GO:0050896) (n = 65/131), immune system process (GO:0002376) (n = 41/131), regulation of cytokine production (GO:0001817) (n = 22/131), regulation of apoptotic process (GO:0042981) (n = 25/131), and regulation of signalling (GO:0023051) (n = 42/131) (Figure 3.5A). Downregulated DEGs (345) were represented by GOterms related to ribonucleoprotein assembly (GO:0022618) (n = 11/344), nucleosome assembly (GO:0006334) (n = 22/344), and translation (GO:0006412) (n = 35/344), which were some of the most significant (Figure 3.5B) (Table 3.7).



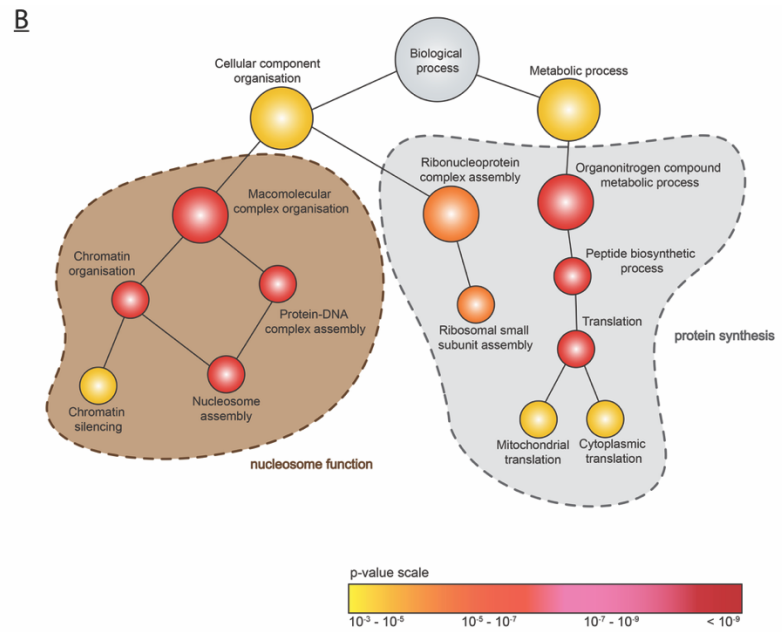


Figure 3.5. Enrichment analysis of DEGs following MNV infection

A hierarchical tree diagram of derived GOterms overrepresented by (A) upregulated and (B) downregulated DEGs (132 and 344 genes, respectively) is presented. Each circle node represents a biological process, and the colours depict the statistical significance of each process represented by MNV-induced DEGs.

Gene enrichment analysis provided useful information on the function of DEGs, and to further our primary GOrilla analysis (Figure 3.5) (n = 476), we also performed KEGG pathway enrichment using DAVID to identify cellular pathways modified by MNV infection (Figure 3.6) (Table 3.8). Consistent with our GOrilla analysis, genes upregulated by MNV infection (n = 131) (Figure 3.4A) had predominant involvement in immune signalling pathways including viral detection by TLRs (n = 9/131), NLRs (n = 5/131), RLRs (n = 5/131), MAPK signalling (n = 9/131), and cytokine–cytokine receptor interactions (n = 12/131) (Figure 3.6A). Analysis of downregulated genes (n = 345) (Figure 3.4A) revealed pathways involved in the function of the ribosome (n = 28/345), proteasome (n = 6/345), and oxidative phosphorylation (n = 22/345) as well as pathways known to be involved in several human pathologies including Alzheimer's disease (n = 19/345), Huntington's disease (n = 19/345), Parkinson's disease (n = 18/345), and systemic lupus erythematosus (n = 16/345) (Figure 3.6B).

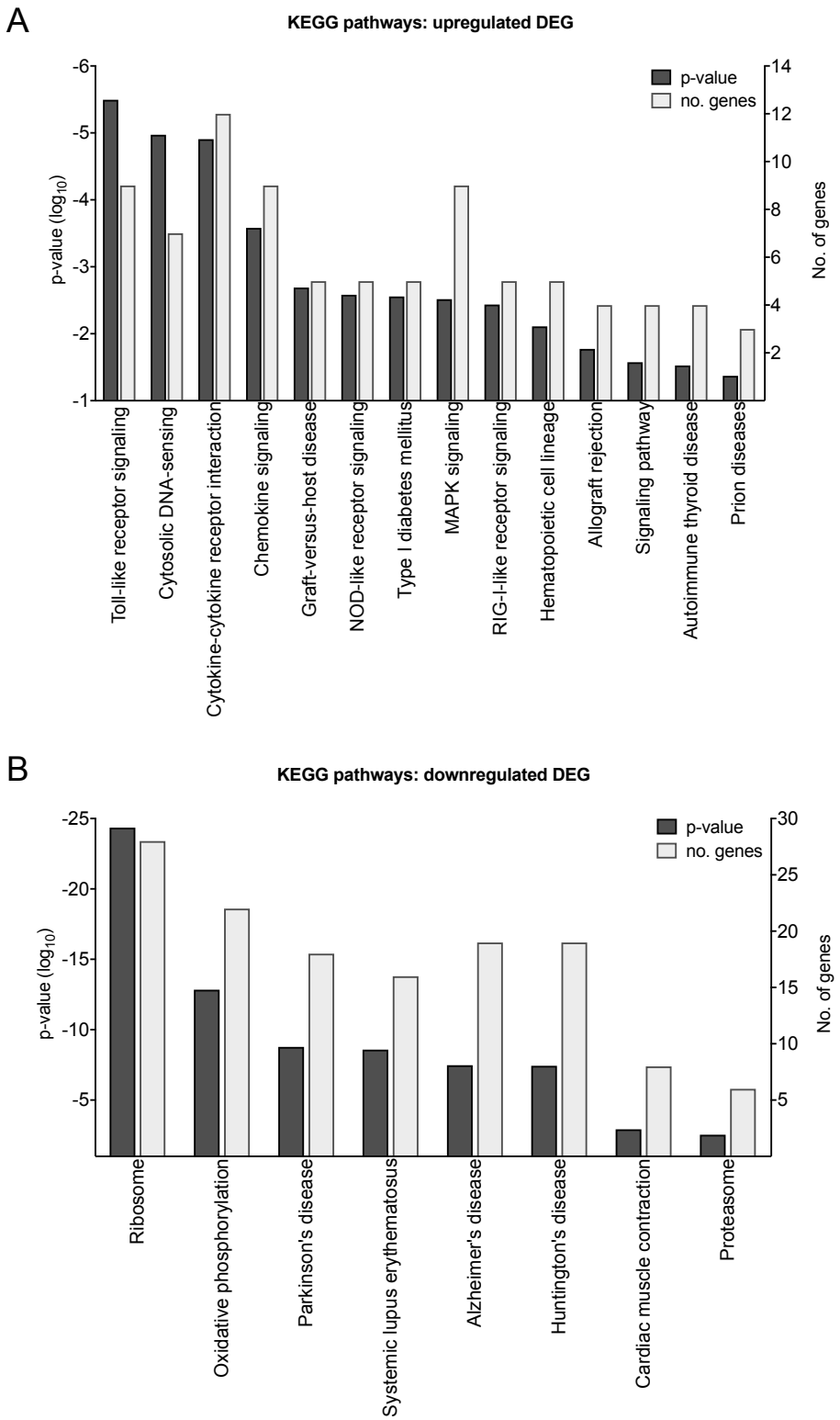


Figure 3.6. KEGG pathway analysis of MNV-induced DEGs

KEGG pathways overrepresented by DEG in response to MNV infection were determined by submitting gene lists to the online bioinformatics database server, DAVID. (A) Upregulated and (B) downregulated gene lists were submitted individually to identify biological pathways altered by MNV and are listed the x-axes, p-value significance on the left y-axes and number of genes represented for each category on the right y-axes.

An essential feature of the innate response are the transmembrane receptors, such as TLRs and RLRs, that play an integral role in the detection of viruses and initiation of intracellular pathways[190]. Members of the RLR family, including *DHX58* (LGP2) (1.89-fold), *DDX58* (RIG-I) (2.85-fold), and *IFIH1* (MDA5) (2.07-fold), had significantly increased gene expression at 12 hpi (Table 3.5) (Figure 3.3C). Conversely, *TLR8* encoding a receptor for ssRNA recognition[191] and *TLR9* encoding the receptor for CpG sequences in DNA [192] were the only TLR members with a reportable increase in gene expression at 12 hpi (1.84- and 1.95-fold, respectively). Furthermore, both qPCR and RNA-sequencing read analysis of longitudinal infection (4–20 hpi) show that *TLR7*, which also encodes a receptor important for recognition of ssRNA, had modest downregulation (approximately threefold) as infection progressed (Figure 3.2B and Figure 3.3C). Overall, we show that all RLR genes are upregulated with MNV infection; however, we report mixed expression of genes that encode TLRs.

3.4.6 MNV-specific effects on the host response

It is known that MNV infection results in IFN production which can induce transcriptional changes in genes that are involved in innate antiviral defense[193-196]. As such, many of the transcript changes that occur with infection are resultant from IFN production and associated signalling pathways. To eliminate the transcriptional changes associated with the general innate responses to pathogens, we performed differential expression analysis with the TLR7 agonist, loxoribine which similarly induces IFN production. Briefly, we compared DEGs found in both the loxoribine treatment and MNV infection datasets to identify transcriptional changes unique to virus infection (Figure 3.7). Forty-three DEGs were upregulated (Figure 3.7A) and 69 DEGs were downregulated (Figure 3.7B) (Table 3.9) solely by MNV infection. These revised gene lists (Table 3.9) were submitted to DAVID for enrichment analysis to identify their biological functions (GOterms) (Table 3.10). The most significant GOterms (p-value < 0.05) are depicted in Figure 3.7A for upregulated (n = 17) and Figure 3.7B for downregulated (n = 39) DEGs. Upregulated MNV-specific DEGs were enriched for functions including regulation of exocytosis (GO:0017157), regulation of cellular localisation (GO:0060341),

regulation of secretion (GO: 0051046), and GOterms related to the immune and defense responses. Conversely, GOterms generated from the MNV-specific downregulated DEGs were representative of protease activity (GO:0000502), protein transport (GO:0015031), and protein localisation (GO:0034613). These biological functions align with GOterms also represented by the downregulated MNV- specific DEGs, involved in antigen presentation (GO:0019882) and immune cell activation (GO:0050863, GO:0002694, GO:0032944, and GO:0050670).

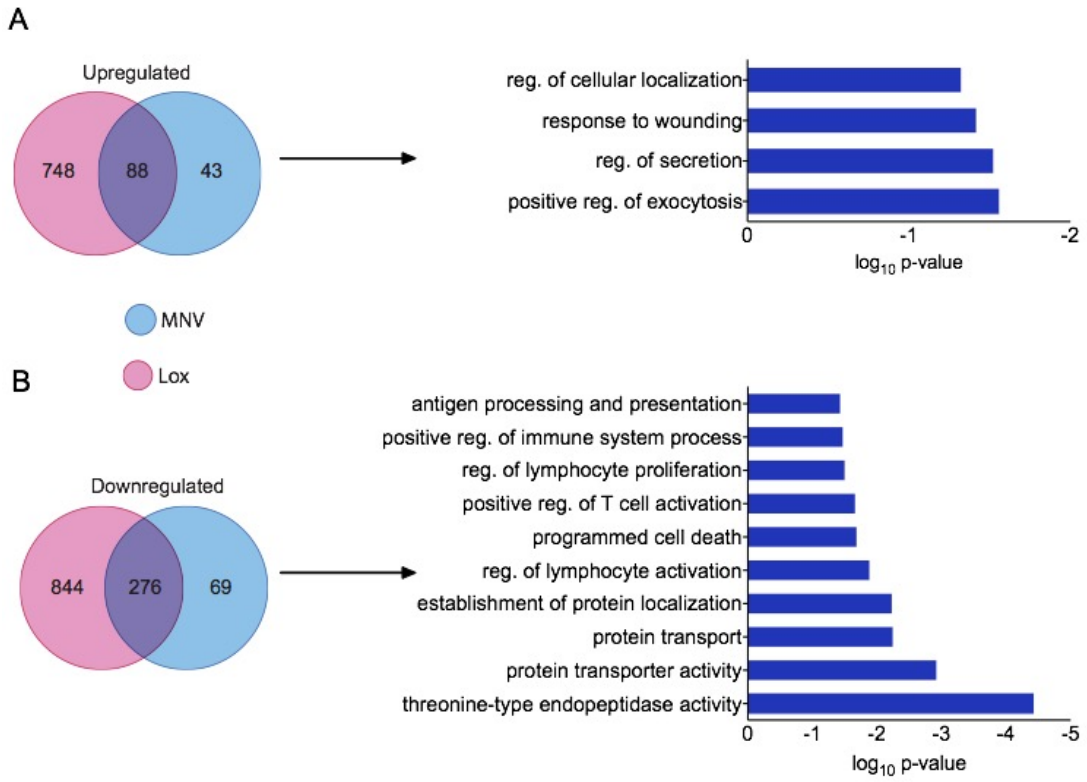


Figure 3.7. Comparative analysis of MNV- and loxoribine-induced DEGs

Upregulated and downregulated DEGs from both MNV and loxoribine datasets were compared and are represented as Venn diagrams in panels (A, B), respectively. DEGs induced only by MNV were further characterised by enrichment analysis using DAVID and GOterms (significance p-value < 0.05) and are represented as bar graphs.

3.4.7 MNV affects genes involved in immune recognition

Closer examination of the 69 downregulated MNV-specific DEGs (Figure 3.7B) (Table 3.9) revealed genes involved in several steps of MHC class I molecule maturation, including proteolysis and vesicular trafficking (Table 3.10). Several examples include genes that encode beta catalytic subunits of the 26S proteasome including *Psmb3* (2.3-fold), *Psmb4* (2.5-fold), *Psmb5* (2.4-fold), and *Psmb9* (twofold) (Table 3.9) that are important for the degradation of viral proteins to generate antigenic peptides[197,198]. *Psme2*, which was downregulated 2.5-fold, encodes the proteasome activator 28 subunit (Table 3.9), an IFN-γ inducible protein that is involved in antigen processing by the immunoproteasome (i-proteasome)[199-202]. Expression of *Ap1s1* was also decreased (2.2-fold) and encodes a subunit of adaptor protein 1 (Table 3.9), a complex that mediates vesicular transport between the endoplasmic reticulum and the Golgi[203,204]. Furthermore, we observed a 24-fold reduction in the abundance of *H2-Q10* (Table 3.9) mRNA, which encodes a secreted MHC class 1b molecule (Qa-10) involved in immune cell activation[205-207].

3.5 Discussion

Viruses need to overcome the innate response for continued replication, survival, and transmission. Knowledge of viral manipulation of the host can elucidate how immune evasion occurs, indicating potential pathways that contribute to pathogenesis. Previous studies investigating MNV infection have focused on individual receptors, signalling molecules, transcription factors or subsets of cytokines involved in innate immunity[129,154-158], showing them to be important for MNV clearance. In the present study, we conducted a non-biased, broad transcriptomic analysis of the host response within MNV-infected macrophage cells and compared this with the response from TLR7 induction by loxoribine. This differential analysis allowed us to identify the subset of genes, whose expression is altered by MNV infection alone. We demonstrate that MNV perturbs the transcriptional profile of IFN signalling, viral recognition, cytokine stimulation, protein degradation, antigen presentation, and lymphocyte activation pathways (Figure 3.8).

MNV replicates within RAW264.7 macrophages to generate high titres of infectious virions (~1×10^7 PFU/mL) [65], and the effectiveness of this system has made MNV a useful tool to understand the interplay between human norovirus and host biology. To confirm MNV replication over time (4–20 h), increases in genome levels were measured by qPCR (~60-fold) and NGS read mapping (>2 log-fold) (Figure 3.1). Several studies have previously shown the presence and increased quantity of the subgenomic species within MNV-infected cells, compared to full-length genomes[65,208]; however, the proportions of the different genomic species have not been characterised. Using NGS, we detected an average of 1.7-fold higher nucleotide coverage spanning ORF2-3 when compared to ORF1 (across all infection time points) (Figure 3.1E), which provided reliable confirmation of the existence of the MNV subgenomic RNA species. The increased abundance of the subgenomic RNA species likely plays an integral role in MNV replication and infection by providing more template for capsid production. We also aimed to rule out the presence and replication of Abelson Mu-LV and Moloney Mu-LV, since both viruses have previously been shown to contaminate the RAW264.7 cell line[209]. In contrast to

the complete genome coverage of MNV (Figure 3.1E), there was no full-length genome coverage detected for Mu-LVs; over 92% of the genome was missing. Furthermore, the absence of increments in the small number of reads that did map to each genome over time (Table 3.12) indicated no viral replication. These findings confirm the absence of both viruses and provide confidence that our analysis is based solely on MNV-induced cellular changes.

One of our main goals was to evaluate the transcript changes of innate immune genes (n = 280) in response to MNV infection (Figure 3.2B). Our longitudinal analyses illustrated that many of these innate genes had increased expression changes as infection advanced. Specifically, we found that genes encoding cytokines, interleukins, cellular transporters, and transcription factors were some of the most highly upregulated in early infection (Figure 3.2B). Conversely, downregulation of innate genes was also observed later in infection from 12 hpi onward. Several of these downregulated genes encoded TLRs, interleukin, and chemokine receptors (Figure 3.2B and Figure 3.8), which are important for pathogen recognition and stimulation of the immune response[148,149]. Together these findings indicate that there is immediate viral recognition by the host and an early induction of the antiviral response. However, these data also suggest that MNV employs a strategy to reduce the available innate receptors, including those encoded by *TLR13*, *TLR7*, *Il17rc*, *Il1rl1*, *Cxcr3*, and *Cxcr5* (Figure 3.2B and Figure 3.8)[187-189,210], thereby reducing viral recognition and innate stimulation. We hypothesise that MNV induces these changes later in infection to dampen the host defences for continued replication and cell-to-cell spread.

One limitation of our study is the use of the mm10 reference genome, which is based on a mouse strain that differs from the origin of the RAW264.7 cell line. Previous work has revealed that this discrepancy may induce bias in read mapping and thus affect transcript quantitation[211]. To confirm that our analyses were accurate, we validated transcript abundance by RT-qPCR of genes (n = 20) commonly involved in the innate response to viral infection[212-215] (Figure 3.3A–C). The robust

correlation between both datasets (Figure 3.3D) demonstrated that our conclusions about the host response to MNV infection were not based on sequencing or read mapping bias. This qPCR analyses also allowed us to further investigate expression changes of PRRs with infection. We observed an increase (more than twofold) in expression of the RLR genes *IFIH1* (MDA5), *DDX58* (RIG-I) and *DHX58* (LGP2) (Figure 3.3C and Figure 3.8), which corroborates previous work by McCartney et al. who showed MNV is recognised by the MDA5 receptor[155]. These findings suggest that the RLRs play a predominant role in MNV recognition and are likely responsible for induction of the antiviral response seen in early infection (Figure 3.2B).

An important objective of our study was to interrogate the biological function of DEGs identified following MNV infection (12 h) to better understand the viral-host interaction. GOterm and KEGG pathway enrichment analysis (Figure 3.5 and Figure 3.6) demonstrated that upon MNV infection, the host reacts by increasing the transcription of genes involved in the innate immune response, particularly those related to viral defense (Figure 3.5A). This is not a surprise as most of the PRRs in the cytoplasm and endosome will encounter and engage with viral PAMPs (early in infection) to stimulate downstream signalling. However, what is apparent from our study is that MNV decreases the expression of the PRRs as the infection cycle continues (Figure 3.2B and
Figure 3.3C) and more importantly transcriptionally controls host cell translation machinery (Figure 3.5B and Figure 3.8) and pathways involved in antigen presentation (Figure 3.7 and Figure 3.8). Combined, these data imply a molecular mechanism to reduce the ability of an infected cell to present MNV antigens and thus provide ample opportunity for MNV to replicate and disseminate to neighbouring cells.

To gain better resolution of the host biology modulated directly by MNV, we carried out a differential analysis of the transcriptomes generated by loxoribine (TLR7 agonist) treatment (Figure 3.4B) (Table 3.6) and MNV infection (Figure 3.7). Since MNV infection and TLR7 activation induce an

IFN response[156,216], the resulting cellular changes likely alter the expression of many of the same genes. We detected a large proportion of genes common to both conditions (Figure 3.7), which emphasise that many gene expression changes observed in the initial analysis (Table 3.5) are induced by IFN production[217,218]. Herein, we focused on the MNV-induced genes (Figure 3.7) with a view to characterise aspects of the host response modulated directly by the virus itself.

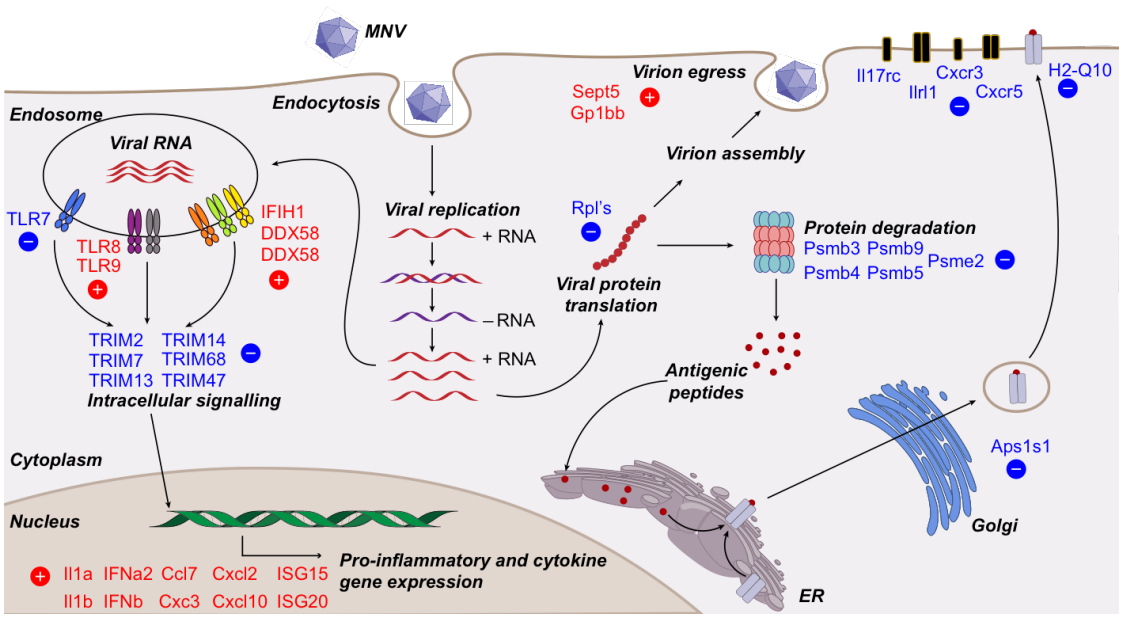


Figure 3.8. Working model of MNV-induced changes in the infected host

A summary of transcriptional changes characterised by RNA sequencing of MNV-infected cells is presented diagrammatically. Genes coloured in red and blue are upregulated and downregulated, respectively.

One compelling finding from this comparative analysis was the downregulation of several Psmb genes (*Psmb3, Psmb4, Psmb5, and Psmb9*) which encode components of the 26S proteasome catalytic core[219] (Figure 3.8). *Psbm9* encodes LMP2, one of the three proteins that replace the constitutive 26S proteasome catalytic subunits following immune stimulation to form the i-proteasome[200]. The i-proteasome plays an essential role in the degradation of viral proteins for MHC class I antigen presentation and is induced by inflammatory mediators including IFN-γ[202,220]. In addition, the downregulation of Psme2, which encodes an activator of the i-proteasome, implies

that MNV modulates the i-proteasome in a multifaceted manner to limit protein degradation for antigen presentation (Figure 3.8). Simply put, despite the generation of a robust innate immune response with MNV infection, the downregulation of genes involved in i-proteasome regulation hints toward immunological modulation by MNV to prevent MHC class I maturation. Further to this, we report the downregulation of *Ap1s1* (2.2-fold), encoding a protein, which has been shown to have a role in the trafficking and processing at the trans-Golgi network, a key step in the pathway taken by MHC class I molecules prior to integration into the plasma membrane (Figure 3.8). Previous work has demonstrated that RAW264.7 cells infected with MNV-1 show no increase in surface expression of MHC class I molecules up to 16 hpi[161], which is consistent with our hypothesis that MHC class I maturation is interfered through the downregulation of genes involved in proteasome function. Taken together, we can speculate that defects in protein trafficking and proteasome function (Figure 3.7B and Figure 3.8) would be major drivers in MNV pathogenesis as an infected cell would have a limited capacity to communicate (via cytokines) and respond (via antiviral effectors such as ISGs) to the infection itself.

In summary, we believe that the described changes at the transcript level demonstrate the intricate nature by which MNV can regulate viral recognition (Figure 3.8) and it is reasonable to infer that such changes enable MNV to evade or dampen the immunological onset of the host response.

3.6 Conclusion

We present a summary of the host gene expression changes induced by MNV infection in mouse macrophage cells. We show that a robust innate immune response is induced by MNV, which coincides with the disease manifestation known to accompany infection. Moreover, we have discovered that several elements of the host biology important to innate stimulation and immune recognition are directly affected by MNV infection. Overall, this study provides a novel source of global expression changes following MNV infection in an *in vitro* setting. However, given the richness

of NGS, our findings represent only a small proportion of the possible analyses with significant potential for further bioinformatic mining. Our findings will likely benefit subsequent research into host–pathogen interactions, not just for MNV, but human norovirus in general.

3.7 Supporting information

Table 3.4. Differentially expressed genes following MNV infection over time

Table 3.5. Significantly expressed genes (MNV 12 hpi)

Table 3.6. Significantly expressed genes (Lox 12 hpt)

Table 3.7. Enrichment terms generated from GOrilla (MNV 12 hpi)

Table 3.8. Enrichment terms generated from DAVID (MNV 12 hpi)

Table 3.9. Significantly expressed genes (MNV 12 hpi vs. Lox 12 hpt)

Table 3.10. Enrichment terms generated from DAVID (MNV 12 hpi vs. Lox 12 hpt)

Table 3.11. qPCR primers used to determine gene expression changes with MNV infection

Table 3.12. Read mapping to Abelson and Moloney Mu-LV

All supporting information is available from the below link:

https://www.frontiersin.org/article/10.3389/fimmu.2017.00959/full#supplementary-material

4 TLR7 agonists display potent antiviral effects against norovirus infection via innate stimulation

Text and figures included in this chapter are adapted from the following publication:

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Author contributions

Conceived and designed experiments: DET, JM and PW

Performed the experiments: DET, NN and JL

- Bioinformatic analysis: DE
- Antiviral assays: DE and NN
- Blotting: DE
- RNA extractions and qRT-PCR: DE and JL
- Data analysis: DE

Wrote the manuscript: DET and PW

Declaration

I certify that this publication was a direct result of my research towards this PhD, and that reproduction in this thesis does not breach copyright regulations.

Daniel Enosi Tuipulotu [Candidate]

4.1 Abstract

Norovirus infections are a significant health and economic burden globally, accounting for hundreds of millions of cases of acute gastroenteritis every year. In the absence of an approved norovirus vaccine, there is an urgent need to develop antivirals to treat chronic infections and provide prophylactic therapy to limit viral spread during epidemics and pandemics. TLR agonists have been explored widely for their antiviral potential, and several are progressing through clinical trials for the treatment of HIV and HBV and as adjuvants for norovirus VLP vaccines. However, norovirus therapies in development are largely DAAs with fewer compounds that target the host. Our aim was to assess the antiviral potential of TLR7 agonist immunomodulators on norovirus infection using the MNV and human Norwalk replicon models. TLR7 agonists R-848, Gardiquimod, GS-9620, R-837, and loxoribine were screened using a plaque reduction assay, and each displayed inhibition of MNV replication (EC_{50} values of 23.5 nM, 134.4 nM, 0.59 μM, 1.5 μM, and 79.4 μM, respectively). RNA sequencing of TLR7-stimulated cells revealed a predominant upregulation of innate immune response genes and ISGs that are known to drive an antiviral state. Furthermore, the combination of R-848 and the NA 2CMC elicited a synergistic antiviral effect against MNV, demonstrating that combinational therapy of host modulators and DAAs might be used to reduce drug cytotoxicity. In summary, we have identified that TLR7 agonists display potent inhibition of norovirus replication and are a therapeutic option to combat norovirus infections.

4.2 Introduction

Human norovirus is recognised as a leading cause of viral gastroenteritis globally with an estimated 699 million norovirus infections and 212,000 attributed deaths annually[5], mostly young children in developing countries[7]. Norovirus infections cause diarrhoea, vomiting, abdominal cramps, fever and nausea with symptoms typically lasting three days[20]. The combination of prolonged viral shedding, a low infectious dose[16] and genetic diversity[19], makes norovirus a highly transmissible pathogen which commonly causes epidemics of gastroenteritis and less frequently pandemics, costing the world economy $60 billion annually[12]. Without an approved vaccine there is a clear unmet need for the development of norovirus antivirals, particularly as prophylactic agents for use during outbreaks and for the treatment of chronic norovirus infections.

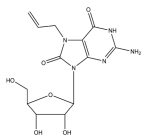
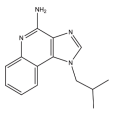
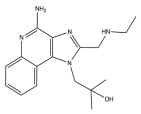
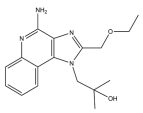
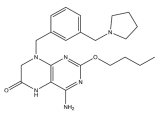
The innate immune response is an important antiviral system that plays an integral role in the recognition and elimination of intracellular pathogens. One key aspect of mammalian innate recognition involves the TLRs which lie within plasma, endosomal and lysosomal membranes and initiate intracellular signalling pathways that culminate in the activation of transcription factors[221]. Several important examples of these factors include NF-κB, activator protein-1 (AP-1), IRF3 and IRF7 which can alter the expression of genes involved in antiviral defence[222-224].

The use of TLR agonists as therapeutics is a rapidly growing area of research, with several compounds in the clinical and pre-clinical phases of development. For example, the TLR7 agonist imiquimod (R-837) is approved as a topical treatment for genital and perianal warts associated with HPV infection[146,225] and has been used to treat drug-resistant genital herpes simplex virus (HSV) infections[226]. Vesatolimod (GS-9620) is another TLR7 agonist that has shown promise as a potent inhibitor of HIV *in vitro*[143] and HBV in chimpanzees[142]. GS-9620 is currently in phase I and II clinical trials for treatment of HIV and HBV infections, respectively[227,228]. A benefit of using TLR7 agonists is

that they pose a low risk for the development of antiviral resistance which is commonly reported with DAAs[229]. These agonists bind to TLR7 causing homo- or hetero- dimerisation and subsequent recruitment of the adaptor protein, MyD88 (Myeloid differentiation primary response protein 88) to TIR (toll and interleukin-1 receptor) domains of TIR-domain containing proteins [224,230]. This results in the formation of a protein complex containing TRAF (TNF receptor associated factor) proteins 3 and 6, IRAK (interleukin 1 receptor associated kinase) proteins 1 and 4, IKKα and IRF7, amongst others, which ultimately results in the phosphorylation of IRF7[224,230]. Translocation of p-IRF7 into the nucleus induces robust expression of IFN and inflammatory cytokine genes[224,230].

The development of antivirals for the treatment of norovirus infections predominantly consists of DAAs that target the viral polymerase and the protease[231]. In addition, there is an increase in research towards targeting features of the host to limit norovirus replication[120-122,125] however, the antiviral effects of TLR7 agonists have not been reported against norovirus. We hypothesised that TLR7 agonists would be capable of inhibiting norovirus replication since the innate immune response plays an essential role in the elimination of norovirus infections[129,130,232]. We explored the antiviral effects of the TLR7 agonists Loxoribine, R-837, Gardiquimod, GS-9620 and R-848 (Table 4.1), using both MNV infectious cell culture and the Norwalk replicon systems. We show that several of these agonists potently inhibit MNV infection and can be used to generate conditioned media (CM) capable of inhibiting the human Norwalk replicon. Moreover, we have measured the host transcriptional changes induced by Loxoribine to show that TLR7 agonists induce strong expression of genes involved in the innate immune response that contribute to the antiviral effects observed. Overall, we show that TLR7 agonists could represent a new class of antivirals that could be used to limit the burden of norovirus-associated disease.

Table 4.1. Chemical structures and classification of compounds used in this study

Name	Structure	Drug class	Target receptor	Molecular weight (g/mol)
Loxoribine		Guanosine analog	TLR7	339.3
Imiquimod (R-837)		Imidazoquinoline	TLR7	240.3
Gardiquimod		Imidazoquinoline	TLR7	313.4
Resiquimod (R-848)		Imidazoquinoline	TLR7/8	314.4
Vesatolimod (GS-9620)		Pteridine	TLR7	410.5

4.3 Methods

4.3.1 Cells, viruses and compounds

HG23 cells (Huh-7 origin) bearing the Norwalk virus replicon[232] and murine macrophage RAW264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin-streptomycin (Life Technologies) and 2 mM Glutamax (Life Technologies). The THP-1 human monocyte cell line was maintained RPMI-1640 (Sigma-Aldrich) with identical supplements. HG23 cells were also supplemented with 10 mM HEPES (Life Technologies) and 1 mg/mL Geneticin (Life Technologies). MNV CW1 strain stocks prepared as previously described[155]. All experimental incubations were performed at 37°C with 5% CO_2. The small molecule TLR7 agonists Resiquimod (R-848), Vesatolimod (GS-9620), Gardiquimod, Imiquimod (R-837) and Loxoribine were purchased from Adipogen Life Sciences (San Diego, CA, USA) (Table 4.1). 2'-C methylcytidine (2CMC) and Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. Compounds were reconstituted in 100% DMSO and stored at -20°C in single use aliquots.

4.3.2 Generation of TLR7 agonist-induced conditioned media (CM)

To prepare CM of murine origin, $2.5x10^6$ RAW264.7 cells were seeded into 10 cm culture dishes and incubated overnight. Cells were then treated with 5 μM of R-848 and incubated for 12 h. Thereafter, media was removed and cell monolayers washed with PBS to remove residual compound. Fresh serum-free media was then added and cells incubated for an additional 24 h. To prepare CM of human origin, $8x10^6$ THP-1 cells were treated with PMA at a concentration of 100 nM in 10 cm culture dishes and incubated for three days to differentiate into macrophage-like cells. Media was removed and monolayers were washed with complete RPMI-1640 media to remove excess PMA. Following a rest period of three days, the cells were treated with R-848 at 1 or 10 μM for 24 h in serum-free media. All CM was filtered before storage at -80°C.

4.3.3 Cytotoxicity assays

To assess cytotoxic effects, 20,000 RAW264.7 cells or 5,000 HG23 cells were seeded per well into 96-well plates and treated with each compound (0.05 – 50 μM) or 50% v/v CM for 48 or 72 h. Following incubation, cytotoxicity was determined using the CellTitre-Blue Viability Assay (Promega, Madison, WI, USA) as per the manufacturer's instructions and fluorescence was measured on a FluoStar Optima microplate reader (BMG Labtech, Ortenberg, Germany).

4.3.4 Antiviral assays

For all experiments, DMSO (vehicle only, 0.5% v/v) was used as a negative control and 2CMC (10 μM) as a positive control. All antiviral activity attributed to TLR7 agonists or CM was calculated relative to the negative control. To generate EC_{50} values, each TLR7 agonist was tested over a range of eight concentrations (R-848: 0.1 nM-10 μM; Gardiquimod and GS-9620: 10 nM-20 μM; Imiquimod: 50 nM-20μM; Loxoribine: 1 μM-1 mM) in antiviral assays.

Inhibition of MNV infectivity: To test the antiviral effects of TLR7 agonists or CM against MNV infection, plaque reduction assays were performed as previously described[67,233]. Semi-solid overlays contained TLR7 agonist, CM (50% v/v), or combinations of R-848 (0-40 nM) and the nucleoside analogue 2CMC (0-3 μM). Plaques were enumerated to determine the EC_{50} values or % inhibition of MNV infection. The % inhibition of MNV infection resultant from the combination dosing of R-848 and 2CMC were assessed for synergistic using SynergyFinder[234] with the zero-interaction potency (ZIP) model[235] which generates synergy scores from a dose-response matrix.

R-848 inhibition of MNV replication: RAW264.7 cells (20,000) were seeded into wells of a 96-well plate and incubated overnight. The next day, monolayers were infected with MNV at an MOI 0.05 for 1 h followed by the addition of R-848 (0.5 μM) in a final volume of 150 μL and incubated from 0 to 48 h (12 h intervals). In addition, R-848 was tested at a range of concentrations (1 nM – 10 μM)

and incubated for 48 h. For both experiments, total RNA was extracted using the RNeasy mini kit (QIAGEN, Hilden, Germany) and MNV genomes were quantified using quantitative real-time polymerase chain reaction (RT-qPCR), as previously described[176].

Inhibition of MNV protein synthesis: The effect of R-848 on viral protein production was examined by western blot detection of the MNV NS7 polymerase. Briefly $1x10^6$ cells were seeded into wells of a 12-well plate and incubated overnight. Media was removed and monolayers infected with MNV (MOI 0.05) for 1h, followed by the addition of R-848 (0.5 μM) in a final volume of 1 mL and cells were incubated for 48 h. Infected cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease/phosphatase inhibitor (Cell Signaling Technology, Danvers, MA, USA). Extracted protein (50 μg) was separated by SDS-PAGE (10% w/v) and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) skim milk before incubation with rabbit polyclonal sera raised against MNV NS7 (1:8,000) (Life Technologies) in 5% BSA overnight at 4°C. Membranes were washed and probed with goat anti-rabbit HRP in blocking solution (Santa Cruz, Dallas, TX, USA) at room temperature for 2 h. Protein detection was performed using Immobilon Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

Effect of R-848 on polymerase activity: Recombinant MNV RdRp containing a C-terminal hexahistidine tag was expressed in *E. coli* and purified by nickel affinity chromatography, as previously described[236]. A fluorescent polymerase assay was performed using a homopolymeric poly(C) template[237] to measure R-848 inhibition of polymerase activity at 10, 50 and 100 μM.

Antiviral effects of R-848 and R-848-induced CM on the Norwalk replicon: The antiviral effects of R-848 were tested against the GI.1 Norwalk replicon at eight concentrations between 1-100 μM or alternatively with a 50% (v/v) solution of CM generated by R-848 stimulation of THP-1 cells at 1 or 10 μM. HG23 cells were seeded (5,000 cells/well) in 96-well plates and incubated for 24 hr followed

by the addition of R-848 or CM at the appropriate concentration. After 48 and/or 72 h, total RNA was isolated using TRIzol and the RNeasy kit (QIAGEN) and norovirus genomes quantified using methods described elsewhere[176,238]. The percentage inhibition of Norwalk replication was calculated relative to the mock DMSO control using ΔΔCt method[175] with *β-actin* as the reference gene.

4.3.5 RT-qPCR gene expression profiling of cells following TLR7 stimulation

RAW264.7 cells ($2x10^6$) were treated with Loxoribine (1 mM) or R-848 (0.5 μM) for 48 h. After incubation, RNA was extracted using Trizol LS (Life Technologies), treated with RNase-free DNase (Qiagen) and then reverse transcribed using Superscript VILO (Life Technologies). To quantitate the relative expression changes of innate genes *Irf7*, *Stat1*, *Il1b*, *Il6*, *IL12a*, *Oas2* and *Mx1*, RT-qPCR was performed using iTaq Universal SYBR Green Supermix (BioRad) with 2 μL of cDNA (diluted 10-fold) and 0.5 μM of each primer (Table 4.3). The following cycling conditions were used: 95°C denaturation for 5 min followed by 40 cycles of 95°C for 5 s, 55°C for 20 s and 72°C for 20 s. Gene expression changes in TLR7 agonist treated cells were compared to mock treatment and normalised to Gapdh using the ΔΔCt method[175]. In a similar analysis, $5x10^4$ Huh7 and HG23 cells were treated with 1 or 10 μM of R-848 for 48 h. The method described above was used to measure the fold changes of innate genes *Ifnb*, *Isg15* and *Il6* with normalisation to *β-actin*.

4.3.6 Transcriptomic profiling of Loxoribine treated cells

RNA-sequencing reads obtained from RAW264.7 cells treated for 12 h with Loxoribine (1 mM) or mock treated were analysed to probe the cellular pathways altered by TLR7 agonists. Expression data is available on the NCBI Short Reads Archive with accessions SRX2556753-56 and SRX2556761-64. Sequencing reads were trimmed with Trimmomatic (v0.32)[239], mapped to the mm10 (UCSC) reference genome using TopHat (v2.0.14)[240], and transcript counts performed with HTSeq (v0.5)[241]. Expression analysis was performed using EdgeR (v3.18.1)[242] and genes were considered differentially expressed based on the following parameters: logCPM greater than or equal to 1,

logFC greater than or equal to 1 or less than or equal to 1, and the FDR-adjusted p-value <0.01. Differentially expressed genes were submitted to DAVID for enrichment analysis[173,243] and redundant ontology terms were removed using REVIGO[244]. In addition, ISGs (n=76) known to be involved in viral infection[195,245] were queried for expression changes with TLR7 agonist treatment. To confirm the accuracy of our RNA-sequencing analysis, the transcript abundance of 12 genes (*Il1b*, *Il6*, *Oas2*, *Ddx58*, *Cd40*, *Ifih1*, *Stat1*, *Dhx58*, *Tnfa*, *Ccl4*, *Cxcl11*, *Cxcl10*) were also queried using RT-qPCR and a correlation analysis was performed against RNA-sequencing data as previously described[246].

4.3.7 Recombinant protein expression and purification

The murine norovirus (CW1 strain) RdRp CDS was previously cloned into the pET26b+ expression construct under the control of a T7 promoter to yield a recombinant polymerase with a C-terminal hexahistidine tag. For expression, this vector was transformed into chemically competent *E. coli* BL21 (DE3)(NEB) as per the manufacturer's instructions and a single colony was used to generate a 10 mL culture which was grown overnight at 37^{o}C in LB containing 100 µg/mL of kanamycin. The following day, cultures were diluted into 50 mL of identical media and incubated for 2-4 h at 37^{o}C until the OD_{600} reached 0.8. To induce expression of the RdRp, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and incubated for 16 h at 25^{o}C. Cells were harvested by centrifugation at 5,000 x g for 10 min at 4^{o}C and then washed with phosphate-buffered saline (PBS). Bacterial pellets were stored at -20^{o}C until use.

For RdRp purification, bacterial pellets were lysed in protein extraction buffer (Table 2.1) supplemented with CelLytic^{TM} B lysis reagent (Sigma) 100 mg/mL lysozyme (Sigma), 50 U/mL of Benzonase nuclease (Sigma), 2 mM $MgCl_2$, protease inhibitor cocktail (1x final concentration) and incubated with gentle shaking for 30 min at room temperature. Following lysis, the concentration of NaCl was adjusted to 500 mM to inactive the Benzonase nuclease. Lysates were clarified by

centrifugation at 16,000 x g for 30 min at 4°C and the supernatant was passed through a 1 mL Ni^{2+} charged Bio-Scale™ Mini Profinity™ IMAC column (BioRad) pre-equilibrated with protein binding buffer (Table 2.1). The column was washed with binding buffer containing 5 mM imidazole to remove impurities and the bound RdRp was subsequently eluted by increasing the imidazole concentration in the binding buffer to 300 mM. RdRp purity was assessed by sodium dodecyl sulphate (SDS) PAGE and then concentrated using a Amicon® Ultra-4 centrifugal filter (Sigma) to a final volume of 3 mL. Concentrated protein was dialysed in 1 L of protein storage buffer (Table 2.1) in a Pur-A-Lyzer Maxi (Sigma) unit. After 3 h, storage buffer was refreshed and samples allowed to dialyse overnight. The following morning the storage buffer was again refreshed for a final 3 h incubation. All dialysis incubations were performed with constant stirring at 4°C. Dialysed protein was quantified using a bicinochoninic acid assay (BCA) kit (Life Technologies) and aliquoted before storage at -80°C.

4.3.8 Quantitative RdRp assays

Recombinant MNV RdRp containing a C-terminal hexahistidine tag was expressed in *E. coli* and purified by nickel affinity chromatography as above (section 4.3.7) and in our previous publications[236]. A fluorescent polymerase assay was performed using a homopolymeric poly(C) template[237] to measure R-848 inhibition of polymerase activity at 10, 50 and 100 µM. In this assay, the recombinant RdRp converts ssRNA into dsRNA which can be quantified using the commercially available fluorescent dye PicoGreen (Invitrogen). For a single reaction, 400 ng of MNV RdRp (5 µL) was combined with R-848 (5 µL) and incubated at room temperature for 10 min. Thereafter, 25 µL of the RNA template/reaction buffer (containing 250 ng of poly(C) RNA, 2.5 mM $MnCl_2$, 5 mM DTT, 0.23 mM rGTP and 20 mM Tris-HCl) was added to the RdRp/inhibitor mixture for a final volume 35 µL (R-848 at desired concentration) and incubated for 1 h at 30 °C to allow polymerisation. Reactions were then terminated by the addition of EDTA (10 µL) to a final concentration of 5 mM and the entire mixture (45 µL) was then transferred to black 96-well plates. Thereafter, 165 µL of 1:680

diluted PicoGreen dye was added to each well and incubated for 5 mins protected from light. Fluorescence was measured at standard wavelengths (excitation 480 nm, emission 520 nm) and compared between mock and treatment conditions to calculate the level of polymerase inhibition.

4.3.9 Statistical analysis

All statistical calculations were performed using Graphpad Prism software (v7.0b). Data was analysed using with either one- or two-way ANOVA or using an unpaired t-test with Welch's correction. All error bars depict standard errors of the mean (SEM) and the following shorthand was used to indicate the level of significance: not-significant (n.s) = $p>0.05$, * = $p\leq0.05$, ** = $p\leq0.01$ and *** = $p\leq0.001$.

4.4 Results

4.4.1 TLR7 agonists inhibit MNV infectivity.

The antiviral effects of TLR7 agonists against HPV, HSV, HIV, and HBV have recently been described; however, the antiviral potentials of these compounds against calicivirus infections have yet to be explored. We therefore investigated whether a range of these molecules inhibited norovirus replication using the infectious MNV cell culture and human norovirus replicon models. Five TLR7 agonists were chosen and initially tested for their antiviral effects, including R-848, Gardiquimod, GS-9620, R-837, and the prototype agonist, loxoribine (Table 4.1). The dose-response curves of all agonists were examined using a MNV plaque reduction assay, and the inhibitory activity of each compound was calculated after 48 h relative to a mock control (DMSO treatment) (Figure 4.1A; see also Figure 4.9). We observed that all agonists displayed dose-response inhibition of MNV infection with the following EC_{50} values: 23.5 nM (R-848), 134.4 nM (Gardiquimod), 590.0 nM (GS-9620), 1.5 μM (R-837) (Fig. 1A), and 79.4 μM (loxoribine) (Figure 4.9). R-848 was the most potent TLR7 agonist, while a 50-fold increase in concentration was required for effective inhibition of MNV infection by loxoribine (Figure 4.1A).

Each TLR7 agonist (except Loxoribine due to poor potency) was then tested for their cytotoxic effects on RAW264.7 cells at a concentration range of 0.05 - 50 μM (Figure 4.1B). At 25 μM, ≥60% cells remained viable for all compounds tested however, Gardiquimod and GS-9620 treatment at 50 μM resulted in significant cell death (>50%) (Figure 4.1B). There was a >2 log-fold difference between EC_{50} and CC_{50} values for R-848 and Gardiquimod suggesting these molecules have good therapeutic potential (Figure 4.1). Therefore, we calculated the therapeutic index of each TLR7 agonist against MNV in RAW264.7 cells using the calculation CC_{50}/EC_{50}. Therapeutic indices were approximately 2,127:1, 134:1, 33:1 and 41:1 for R-848, Gardiquimod, R-837 and GS-9620,

respectively. Overall, we found that TLR7 agonists inhibit MNV infection in a dose-dependent manner at low concentrations with minimal effects on cellular cytotoxicity.

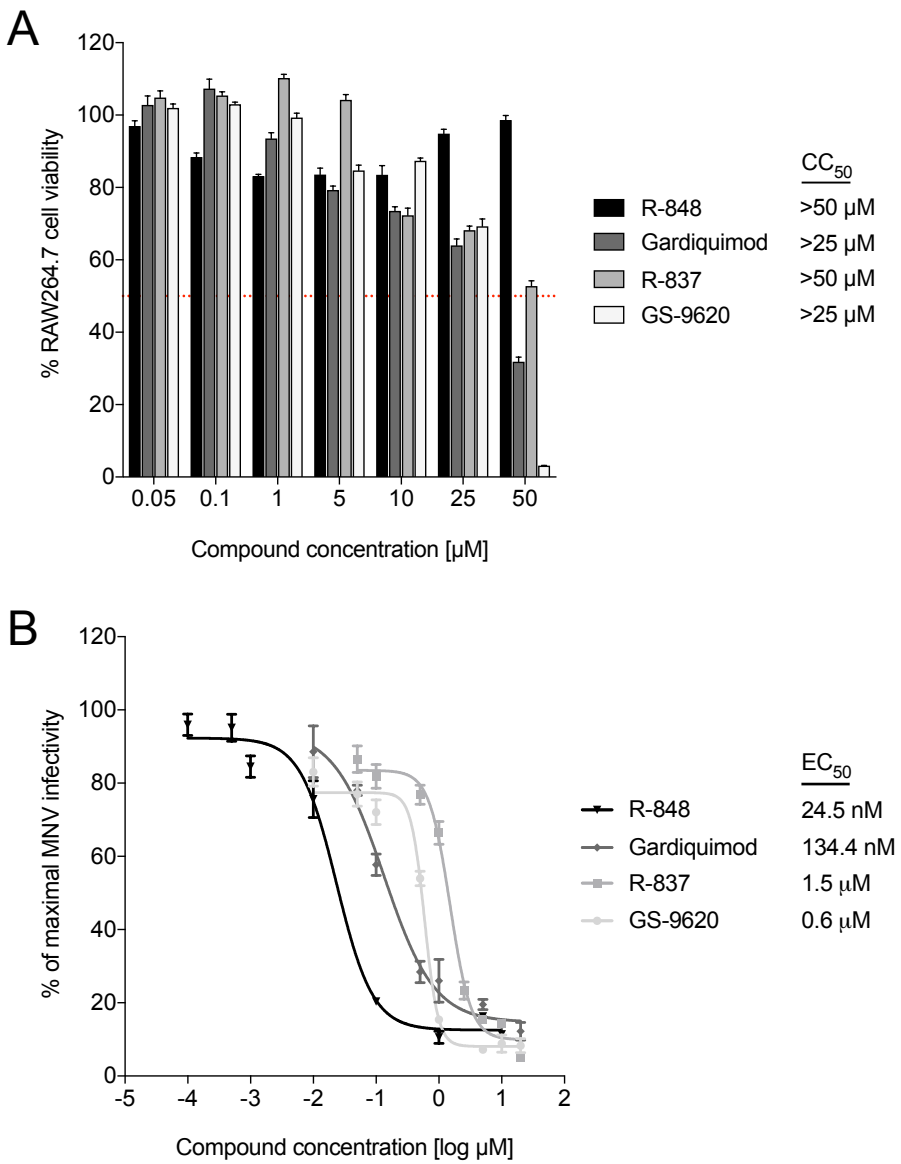


Figure 4.1. TLR7 agonists exhibit antiviral activity against MNV with minimal toxic effects

(A) The cytotoxic effects of TLR7 agonist treatment of RAW264.7 cells for 48 h was assessed at concentrations between 50 nM-50 µM using CellTitre-Blue. (B) The antiviral effects of four TLR7 agonists were assessed against MNV in cell culture using a plaque reduction assay. Each compound was tested over eight concentrations (0.1 nM-10 µM for R-848, 50 nM-20 µM for imiquimod, 10 nM-20 µM for Gardiquimod and GS-9620) for 48 h. Plaques were enumerated and compared to those obtained in uninhibited DMSO controls to calculate the EC_{50} of each compound. Plotted data represents the mean ± SEM for two (panel B) or three (panel A) independent experiments performed with triplicate reactions.

4.4.2 R-848 is a potent inhibitor of MNV replication and viral protein synthesis

Since TLR7 agonists can stimulate a broad host response, it is likely that more than one mechanism of action is involved in the antiviral activity against the MNV. To delineate the antiviral mechanism of TLR7 agonists against MNV in more detail, we examined the effect of the most potent TLR7 agonist tested, R-848, on viral genome replication and viral protein synthesis (Figure 4.2). First, we measured inhibition of viral replication by R-848 (0.5 μM), using RT-qPCR to quantify MNV genomes at 12 h intervals over 48 h. When compared to untreated controls, R-848 inhibited MNV replication by 58.2% (SEM ±6.1%), 67.2% (±2.5%), 74.5% (±0.7%) and 67.6% (±1.8%) following 12, 24, 36 and 48 h incubation respectively (Figure 4.2A). MNV-infected cells were also treated with R-848 at concentrations ranging from 5 nM – 10 μM and incubated for 48 h to gain a second EC_{50} value by quantifying RNA levels. This revealed an EC_{50} value of 42.5 nM (Figure 4.2B) compared to 23.5 nM obtained using the plaque reduction assay (Figure 4.1A).

In addition to viral replication and infectivity, we analysed the cytoplasmic levels of the NS7 viral protein to confirm whether its abundance was reduced by TLR7 agonist treatment. Densitometry analysis of NS7 levels detected by western blot showed that viral protein synthesis was attenuated 100.0% with 2CMC treatment (10 μM) and 84.0% with R-848 treatment (0.5 μM) for 48 h (Figure 4.2C). Together we show that R-848 treatment, potently decreased MNV replication and protein production; two features essential of the norovirus replication cycle.

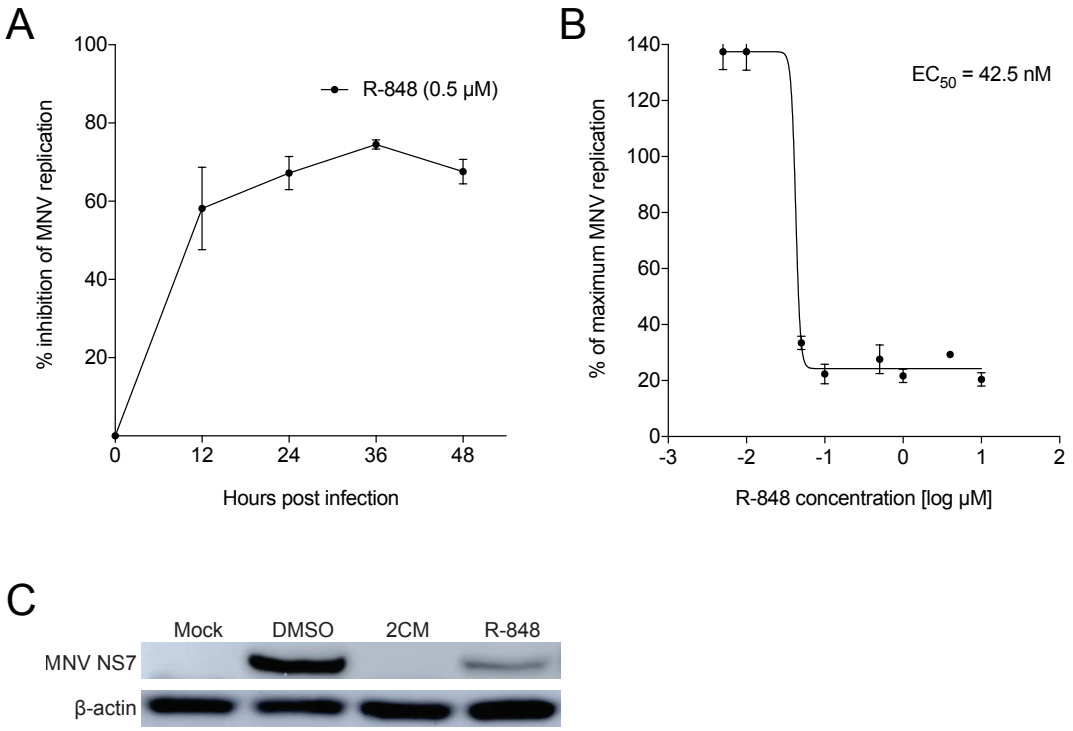


Figure 4.2. R-848 potently inhibits MNV genome replication and protein synthesis

(A) The inhibitory effect of R-848 treatment (0.5 μM) on MNV replication over time was performed by quantification of viral genomes at 12 h intervals by RT-qPCR. (B) The inhibitory effect of R-848 treatment (5 nM-10 μM) on MNV replication was also quantified by RT-qPCR over eight concentrations. (C) To illustrate the inhibition of MNV protein synthesis by R-848 treatment (0.5 μM), the levels of the NS7 polymerase were measured by Western blot of MNV-infected cell lysates. The NA 2CMC (10 μM) was used as a positive control and *β-actin* was probed for normalisation. For all experiments, RAW264.7 infections were performed with an MOI of 0.05, with 48 h of R-848 treatment. The percentage inhibition was calculated relative to mock control (0.05% vol/vol DMSO). Plotted data represent the mean ± SEM for two independent experiments each performed with triplicate or quadruplicate reactions.

4.4.3 R-848 does not inhibit MNV RdRp activity

Previous work has illustrated that the TLR7 agonist Gardiquimod inhibits the HIV reverse transcriptase *in vitro* at low micro-molar concentrations[247]. Since we observed a decrease in MNV genome replication following R-848 treatment (Figure 4.2A-B), we aimed to determine if it was attributable to inhibition of the MNV RdRp using a previously described RdRp transcription assay with polyC RNA as the template[176]. When tested *in vitro*, the MNV RdRp was not inhibited by R-848 and retained 91.2% (SEM ± 4.61%), 95.9% (± 6.75%) and 101.7% (± 3.99%) activity at R-848 concentrations of 10, 50 and 100 μM respectively (Figure 4.3A).

4.4.4 R-848 stimulated RAW264.7 CM has antiviral properties against MNV

To demonstrate whether TLR7 agonists induce the production of antiviral molecules that inhibit norovirus, we tested CM (50% v/v) from R-848 stimulated RAW264.7 cells for inhibition of MNV infection using a plaque assay (Figure 4.3B). Cytotoxicity of the RAW264.7 CM was also measured at 50% (v/v). At this concentration, we observed that the CM displayed >50% inhibition of MNV plaque formation without cytotoxicity (Figure 4.3B). These results suggested that R-848 stimulation of the host cell involves the generation of antiviral molecules that are responsible for inhibition of MNV infection and replication.

4.4.5 R-848 and 2CMC combination displays a synergistic antiviral effect against MNV

To explore whether R-848 and the NA 2CMC exhibit a synergistic antiviral effect against MNV, we measured plaque reduction with a range of drug combinations over several concentrations (Figure 4.4A). Inhibition at each combination dose was analysed with SynergyFinder to obtain a ZIP synergy score of 9.673 (Figure 4.4A). This value indicates a moderately synergistic interaction between R-848 and 2CMC. Moreover, all drug combinations resulted in little to no toxicity (Figure 4.4B).

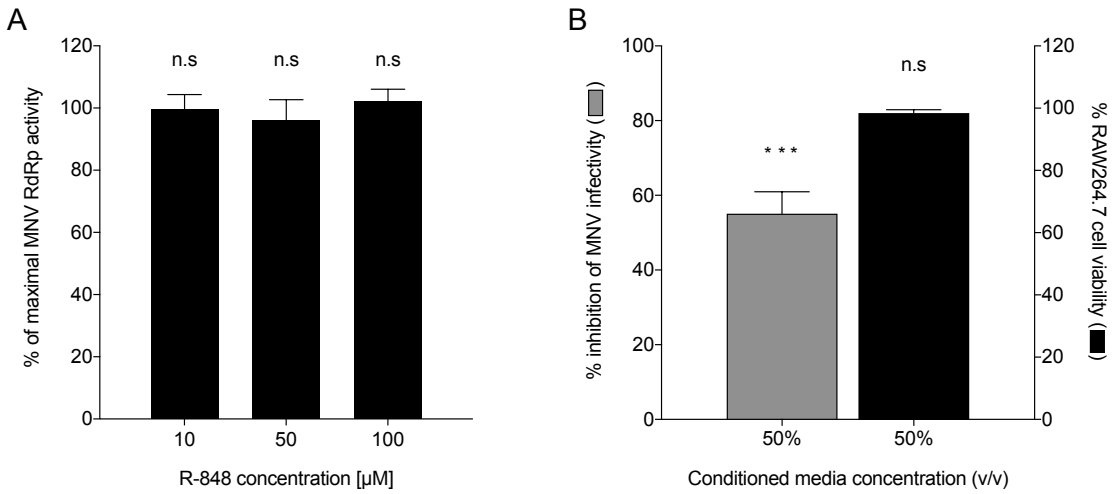


Figure 4.3. R-848 fails to inhibit the viral polymerase but induces cellular secretion of antiviral molecules

(A) The effect of R-848 on the *in vitro* transcriptional activity of the MNV RdRp was measured using a quantitative fluorescent assay. R-848 was tested at 10, 50, and 100 μM, and transcriptional activity was compared to that in mock-treated samples (DMSO). No effect on RdRp activity was observed with the addition of R-848. (B) To determine if R-848-treated cells produce soluble antiviral molecules, CM from R-848-treated cells was screened for anti-MNV activity using the plaque reduction assay. CM used at a final concentration of 50% (vol/vol) displayed 50% inhibition of MNV with an absence of cytotoxic effects. Data were analysed using an unpaired t test. n.s (not significant), $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. All data shown are the means $\pm$ SEM from three independent experiments with either triplicate reactions.

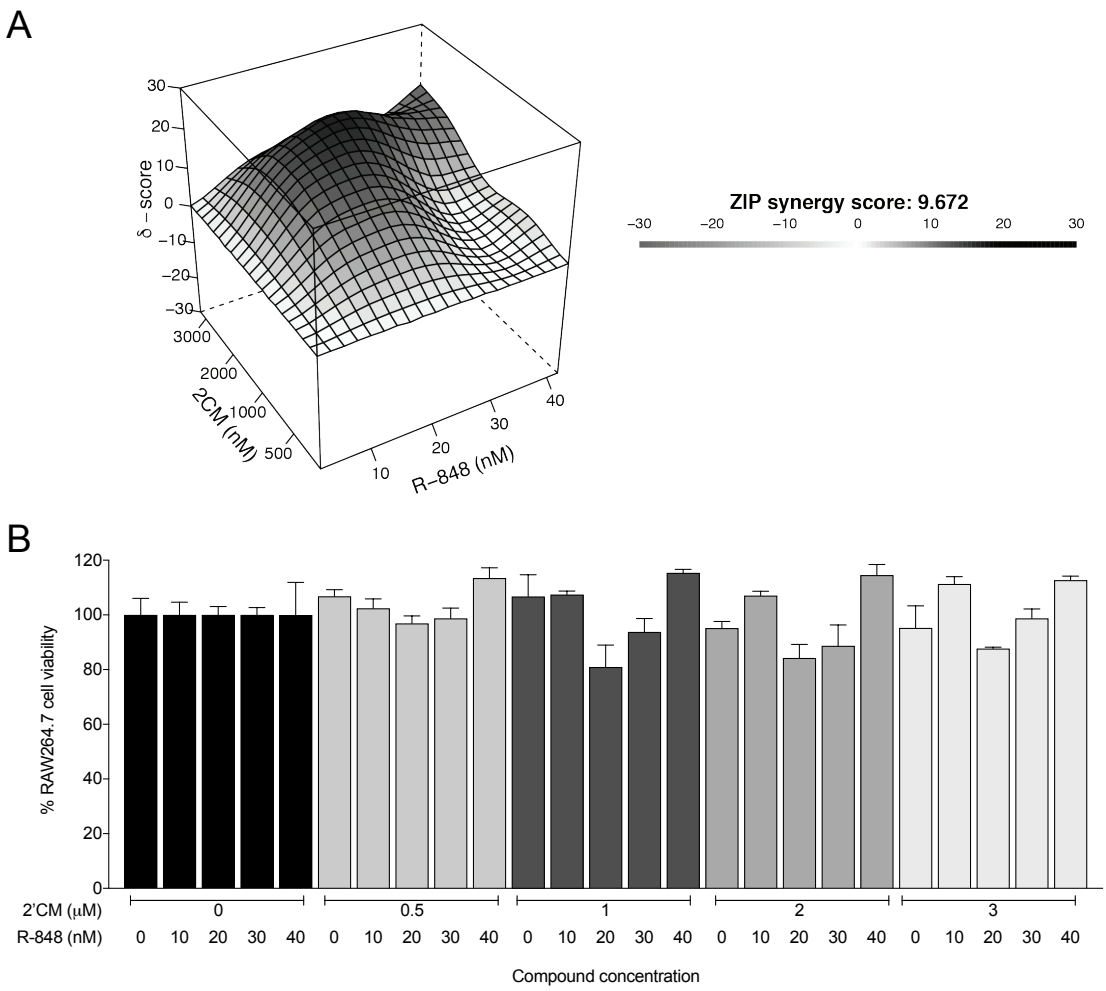


Figure 4.4. R-848 is synergistic with the nucleoside analogue 2CMC

(A) The combined inhibitory effects of the TLR7 agonist R-848 (0 to 40 nM) and the nucleotide inhibitor 2CMC (0 to 3 μM) were tested over a range of combinations against MNV in cell culture using the plaque reduction assay. A dose-response matrix was generated and analysed for synergism using SynergyFinder. The ZIP mode synergy score is presented as the average of all -scores across the dose-response landscape, and a δ-score of >0 indicates synergism. 2CMC and R-848 display a synergistic antiviral effect against MNV. (B) Assessment of cytotoxicity at each drug combination of R-848 and 2CMC.

4.4.6 TLR7 agonists upregulate the expression of ISGs and innate genes important for the control of viral infection

We next examined the transcriptome of TLR7 stimulated RAW264.7 cells to underpin the gene expression changes that induce these antiviral secretions. Sequencing data from our previous study of Loxoribine treated RAW264.7 cells[246], was analysed to characterise the ontology of differentially expressed genes (DEGs) (n=1,891/12,113) compared to untreated cells (Table 4.2). The five most significant ontological clusters overrepresented by DEGs included: immune system process (n=85/1,891), innate immune response (n=70/1,891), cellular response to lipopolysaccharide (n=40/1891), positive regulation of IKK/NFkB signalling (n=33/1,891) and negative regulation of viral genome replication (n=16/1,891) (Figure 4.5). To validate our RNA-sequencing analysis, we compared the expression changes of 12 genes involved in the innate immune response by RT-qPCR (Figure 4.10). Linear regression analysis confirmed that a good correlation existed between the values obtained with both techniques (Pearson r = 0.94 and p-value <0.001) and thus validated our analysis.

In addition, we also explored the expression changes of a subset of 76 ISGs known to have a pronounced role in the control of viral infection[195,245]. Of the 76 ISGs queried, most were significantly upregulated (n=74/76) (FDR adjusted p-value<0.01) and a select group also had >10-fold increase in expression (n=30/74) following Loxoribine treatment (Figure 4.6A). To determine whether RAW264.7 cell treatment (48 h) with Loxoribine (1 mM) or R-848 (0.5 μM) demonstrated consistent patterns of gene expression, we performed RT-qPCR of seven innate genes. We show that all seven genes displayed upregulation in gene expression with both agonists (Figure 4.6B). Together these findings demonstrated that TLR7 agonist stimulation of RAW264.7 macrophages induced powerful expression of innate genes; a key feature of the antiviral environment that limits MNV replication.

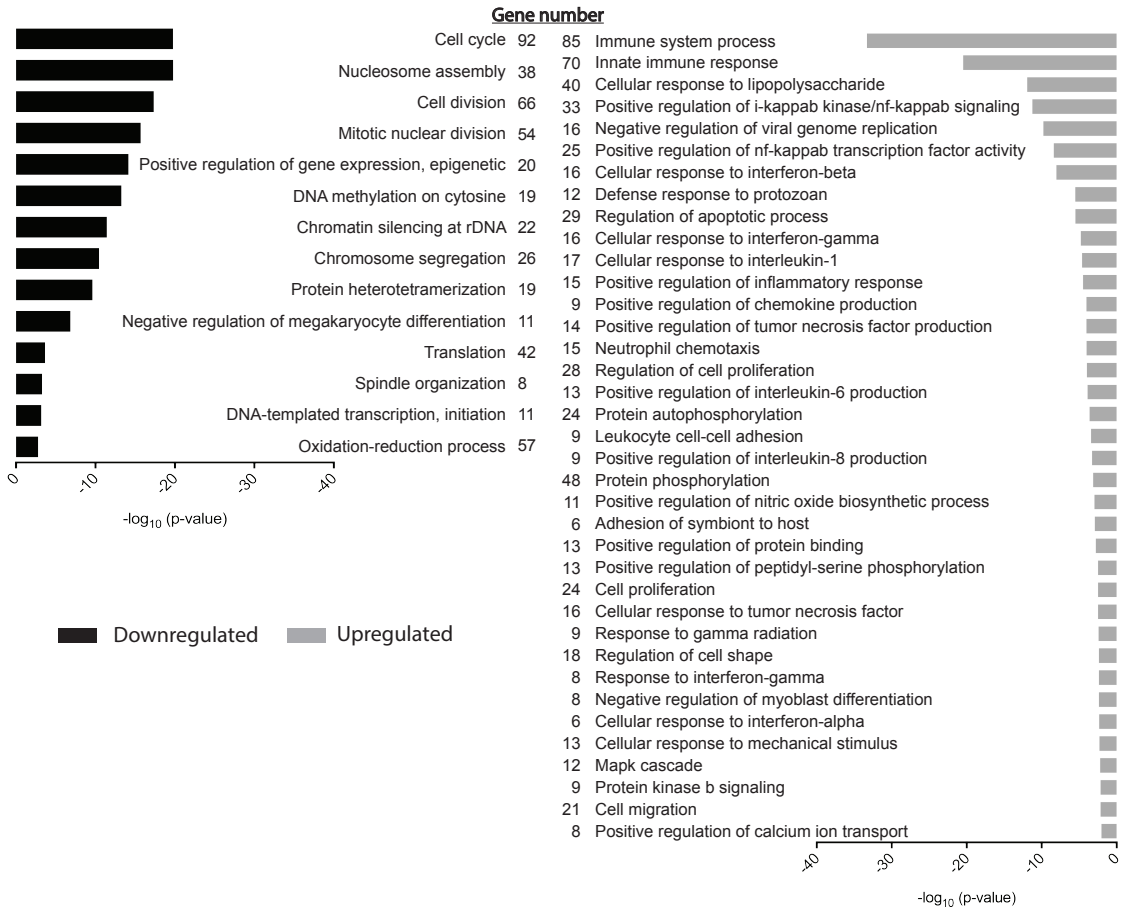


Figure 4.5. Gene ontology of DEGs from TLR7 agonist-stimulated RAW264.7 cells

Gene ontology analysis was performed on genes found to be differentially expressed in the loxoribine transcriptome data set (n = 1,891). Up- and downregulated genes were analysed separately using DAVID (grey and black bars, respectively), and the ontological terms that relate to either gene set were further refined by the removal of redundant terms using REVIGO. Ontological terms that remained are plotted based on their level of statistical significance.

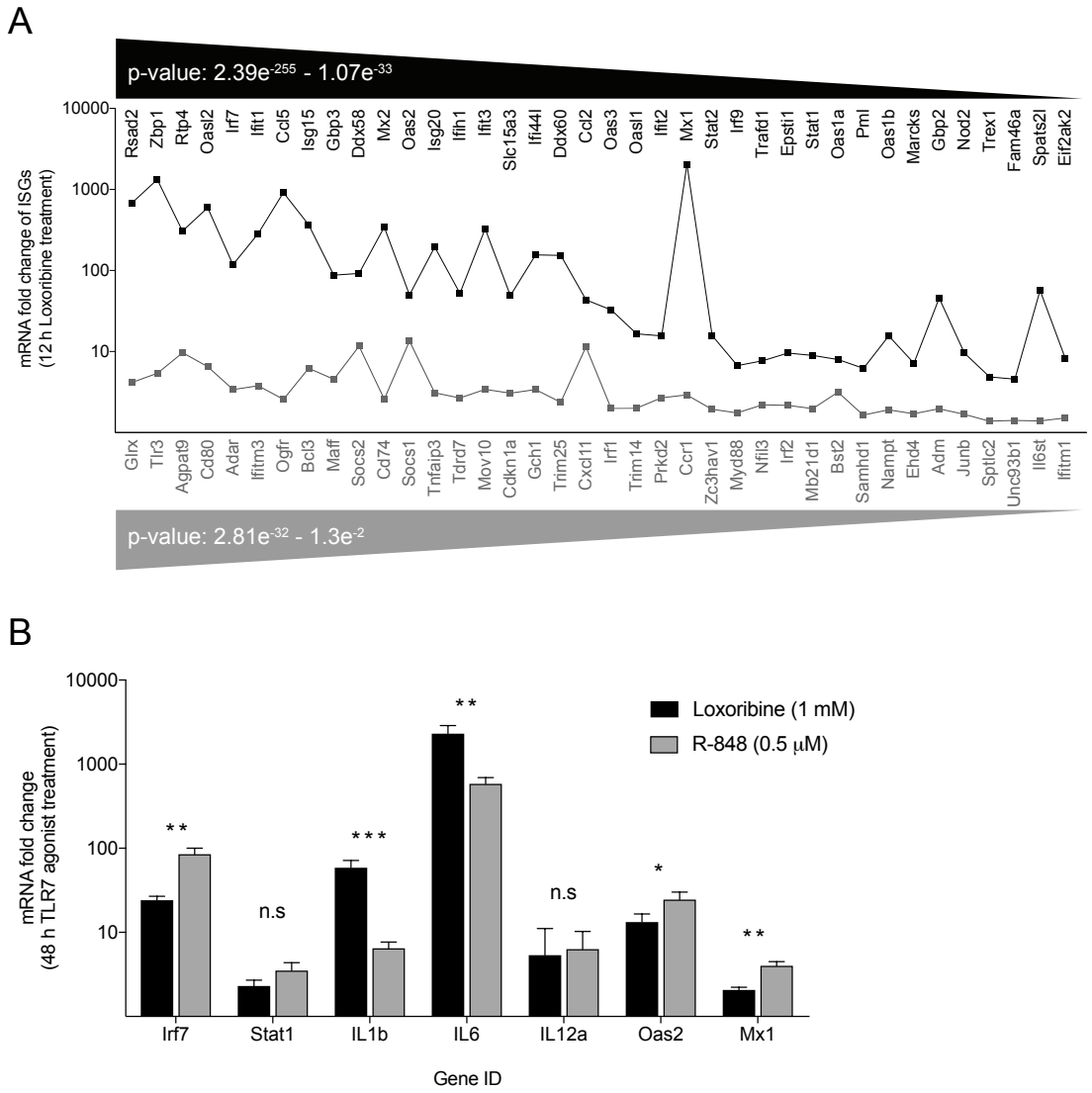


Figure 4.6. TLR7 agonists upregulate the expression of ISGs and innate genes important to viral defense

(A) Transcriptomic analyses were performed on an RNA-sequencing data set generated from RAW264.7 cells treated with the TLR7 agonist loxoribine (1 mM) for 12 h. ISGs associated with viral infection were queried for expression changes (n = 76), and a large proportion of these genes were significantly upregulated (n = 74/76). ISGs in black have a higher level of statistical significance than do ISGs in grey. (B) Transcriptional profiling of RAW264.7 cells treated with R-848 (0.5 μM) or loxoribine (1 mM) for 48 h was performed by quantifying expression changes in genes that encode transcription factors (*Irf7* and *Stat1*), chemokines (*Il1b*, *Il6*, and *Il12a*), and two prominent IFN-mediated antiviral molecules (*Oas2* and *Mx1*). Expression levels for each gene were calculated relative to mock treatment using the $\Delta\Delta C_T$ method and compared between experimental conditions. To determine whether the gene expression changes following loxoribine and R-848 treatment were significantly different from one another, ΔC_T values of both agonist treatments were analysed using an unpaired t test with Welch's correction. Results represent the means ± SEM from two independent experiments, performed in triplicate. n.s (not significant), $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

4.4.7 R-848 treatment does not inhibit the Norwalk replicon

To determine whether TLR7 agonists also inhibit human norovirus replication we measured the effect of R-848 against the human GI.1 Norwalk replicon. HG23s were treated with increasing concentrations of R-848 (ranging from 1 to 100 μM) for 72 h. Following treatment, RNA was extracted and RT-qPCR used to quantify norovirus replicon RNA levels. R-848 exhibited inhibition of the Norwalk replicon in a dose-dependent manner (Figure 4.7A), albeit with limited potency compared to MNV (Figure 4.2A-B). We observed a 54% (SEM ± 4.1%) reduction in replicon RNA with R-848 treatment at 100 μM (Figure 4.7A), however, increased cytotoxic effects were also observed at this concentration with 72.2% (SEM ± 2.9%) of cells remaining viable (Figure 4.7A). To address the limited potency of R-848 against the Norwalk replicon we determined whether R-848 stimulated innate immunity in HG23s and the parental Huh7 cell line, as seen with RAW264.7 macrophages. HG23s and Huh7 cells were treated with 1 and 10 μM of R-848 for 48 h and changes in expression of three critical innate response genes (*Il6*, I*sg15* and I*fnb*) were quantified using RT-qPCR (Figure 4.7B) and compared to mock treatment. Following treatment with R-848, none of the genes displayed >3-fold increase in transcript abundance in Huh7 or HG23 cells (Figure 4.7B).

4.4.8 R-848 stimulated THP-1 CM displays antiviral activity against the Norwalk replicon

To explore whether CM generated from R-848 stimulated THP-1 cells resulted in inhibition of the Norwalk replicon we tested its antiviral effects at a concentration of 50% (v/v) using RT-qPCR. Following 48 h incubation, THP-1 CM generated from R-848 stimulation at 1 μM and 10 μM resulted in 17.1% (SEM ± 6.4%) and 36.3% (SEM ± 2.6%) inhibition of the replicon respectively (Figure 4.8). A similar dose-dependent trend was observed at 72 h of CM incubation with 45.7% (SEM ± 1.1%) (R-848 1 μM) and 59.9% (SEM ± 2.2%) (R-848 10 μM) inhibition of replicon replication (Figure 4.8). In addition, the level of cytotoxicity was minimal and >80% viability was observed with each treatment condition (Figure 4.8).

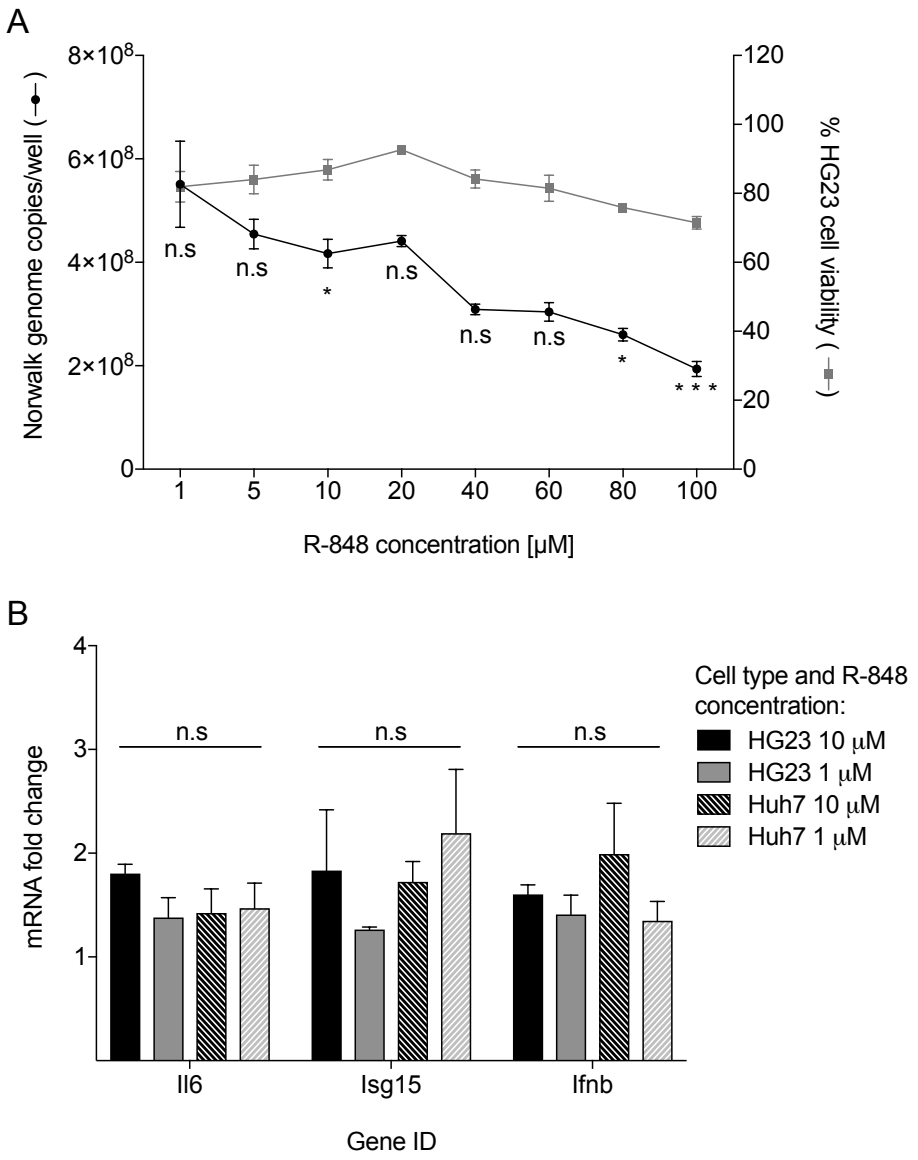


Figure 4.7. Direct R-848 treatment is ineffective against the Norwalk replicon and fails to stimulate innate gene expression

(A) HG23s were treated with eight concentrations of R-848 over a period of 72 h followed by RNA extraction and quantification of replicon RNA by RT-qPCR. The number of Norwalk genomes detected under each condition was normalised based on the abundance of the *β-actin* gene transcript. In addition, R-848-treated HG23 cells were also assessed for cytotoxic effects. One-way analysis of variance (ANOVA) was used to measure the significance of differences between viral genomes measured in the DMSO control relative to each of the R-848 doses. (B) To quantify the level of innate gene expression following TLR7 stimulation, both HG23 and the parental Huh7 cell lines were treated with R-848 at 1 μM and 10 μM. Fold changes for *Il6*, *Isg15*, and *Ifnb* were measured by RT-qPCR ($\Delta\Delta C_T$ method) and calculated relative to mock control (DMSO). ΔC_T values were analysed for significant differences between the various treatment concentrations for each cell type by using two-way ANOVA. All plotted data represent the means ± SEM from two independent experiments performed in triplicate. n.s (not significant), $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

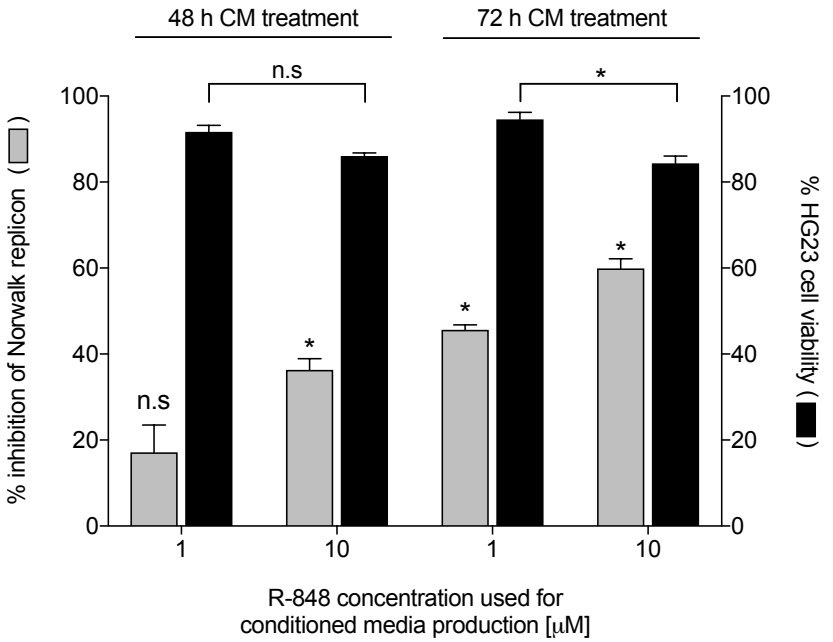


Figure 4.8. CM generated from R-848-stimulated THP-1 cells inhibits the Norwalk replicon

THP-1 cells were treated for 12 h with R-848 at 1 μM or 10 μM to generate CM, which was then tested at a concentration of 50% (vol/vol) against the Norwalk replicon for either 48 or 72 h. Norwalk replicon RNA was quantified by RT-qPCR and normalised to the *β-actin* gene transcript using the $\Delta\Delta C_T$ method. The percent inhibition of replication was calculated relative to mock control (DMSO). ΔC_T values for each CM treatment were compared to the values for the mock treatment, and differences were analysed for significance using an unpaired t test with Welch's correction. The cytotoxic effects of R-848-generated CM were also assessed for each treatment condition. One-way ANOVA statistical analysis was used to measure any significant difference in cytotoxicity between the R-848 CM dosages. All plotted data represent the means ± SEM from two independent experiments performed in triplicate. n.s. (not significant), $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

4.5 Discussion

Small molecule agonists of TLRs are currently used in cancer immunotherapy and as adjuvants for vaccine delivery[248,249]. In recent years, however, there has been increased focus on using TLR agonists as a therapeutic option to treat viral infections[250]. One important example has been the use of TLR7 and TLR9 agonists in combination with antiretroviral therapy as a part of the "kick-and-kill" eradication strategy for HIV infected patients[228,251,252]. This therapy is designed to increase the replication of latent HIV reservoirs and promote greater immune recognition of replicating HIV to facilitate viral elimination by a combination of the host response and the antiretroviral drug component[253,254]. Given the success with HIV treatment, and given the lack of antiviral agents for norovirus infections, we investigated the effects of five TLR7 agonists (Table 4.1) against the MNV *in vitro* cell culture model, and the most potent agonist, R-848, against the Norwalk replicon. Our rationale was that TLR7 agonists could activate the innate immune response resulting in inhibition of MNV and human Norwalk replication. Our data shows that TLR7 agonists have the potential to be a therapeutic option to reduce the global burden of norovirus infections.

Agonists R-837, R-848, Gardiquimod, and GS-9620 inhibited MNV replication with EC_{50} values in the range of 23.5 nM – 1.5 μM (Figure 4.1A). The potent inhibition displayed by these compounds is akin to the nanomolar and low micromolar inhibition observed against HIV and HCV infections treated with TLR7 agonists *in vitro* [143,255]. We also performed cytotoxicity analyses since the pharmacokinetic profile of any antiviral compound is an important factor in clinical trial progression. Significant cellular toxicity (>50%) was only observed at concentrations several-fold higher than the respective EC_{50} value of each TLR7 agonist (Figure 4.1B). However, cytotoxicity is only one measure of compound safety and adverse events of these compounds should be explored further, for example in the MNV mouse model. Despite this, the therapeutic index of each TLR7 agonists against MNV in the RAW264.7 cell line was 2,127, 134, 33 and 41:1 for R-848, Gardiquimod, R-837 and GS-

9620, respectively. This trend fits well with the current published safety profile of GS-9620[227,256] and thus demonstrates that these compounds have the features desirable for a norovirus antiviral.

Previous studies on the effects of TLR agonists against RNA virus infections have demonstrated that treatment results in the production of pro-inflammatory cytokines that drive the inhibition of viral replication[142,143,255,257]. Given this knowledge, we aimed to ascertain which stages of the MNV replication cycle were inhibited by TLR7 agonist treatment. When MNV-infected cells were treated with R-848 (0.5 μM) we observed greater than 50% inhibition of the expanding viral population as early as 12 hpi (Figure 4.2A). The EC_{50} of R-848 was 42.5 nM when calculated using RNA genome levels (Figure 4.2B), close to the value obtained using plaque reduction assays (23.5 nM) (Figure 4.1A) (Figure 4.2B) which demonstrates consistency between assays. Moreover, we quantified the level of MNV RdRp (NS7) using western blot and showed that viral protein synthesis is attenuated following R-848 treatment (0.5 μM) after 48 h (Figure 4.2C). Although we cannot conclusively infer that TLR7 agonists directly block MNV protein production, reduction in both RNA and protein levels are complementary and confirm that TLR7 stimulation inhibits norovirus replication.

Clinical approval of any drug therapy can be hampered by adverse reactions within patients, including toxicity. Immunomodulators, for example, can induce systemic inflammation and side-effects that may prevent clinical trial progression[258]. However, one way to reduce the toxicity of antivirals, and to reduce the development of resistance, is to combine drugs that target multiple facets of the host or viral replication that result in a combined or synergistic effect [259]. The dual action of a TLR7 agonist and a nucleoside analogue could be an effective way to overcome off-target or toxic effects. First, we concluded that inhibition MNV replication is a not a result of direct RdRp inhibition (Figure 4.3A) and thus any combination with an RdRp inhibitor would not result in antagonism. Thereafter, we showed that the combination of 2CMC (nucleoside analogue) and R-848 had a synergistic inhibitory effect against MNV with an average ZIP score of 9.672 over the dose-

response matrix of varied combinations of these two drugs (Figure 4.4). These findings suggest that TLR7 agonists could be used in combination with novel norovirus NAs[176,238,260,261] to reduce the dosage of each constituent compound and thereby limit cytotoxic effects.

Previous work has shown that CM from TLR7 agonist stimulated cells has a potent antiviral activity against HBV[262], HCV[255] and HIV[143]. These studies allude to the production of soluble molecules following TLR7 stimulation that display more potent inhibition of viral replication than direct TLR7 agonist treatment. We studied the effects of CM generated by R-848 stimulation of RAW264.7 cells and measured a significant level of inhibition (>50%) of MNV plaque formation when tested at a concentration of 50% (v/v) (Figure 4.3B). In line with previous studies of TLR7 stimulation of RAW264.7 cells[263], these data illustrate that the marked increase in gene expression induced with TLR7 agonist treatment results in the production of soluble antiviral molecules that generate an environment capable of norovirus inhibition. Furthermore, we show that TLR7 agonists can be used not only directly, but in an indirect manner (via CM) to limit norovirus replication.

We next sought to focus on the host changes that drive the antiviral effects observed. Transcriptomic analysis was performed to characterise the gene expression changes significantly altered by TLR7 agonist treatment in murine macrophages. Overall, we show that upregulated genes are predominantly involved in the host innate immune response and viral defence (Figure 4.5). Further to this, a small subset of key innate genes (n=12) were also queried by RT-qPCR and when compared to our RNA-sequencing counts, a high level of correlation existed providing support for these conclusions (Figure 4.10). We then quantified the expression changes of a select group of 76 ISGs, including but not limited to: *Isg15*, *Rsad2*, *Oas2*, *Mb21d1*, *Irf1*, *Irf7*, *Dhx58* and *Ifih1*, all which have a role in viral interference and clearance [195,245,264]. Of the ISGs examined, 74 were significantly upregulated and 14 of these had a >100-fold increase in transcript abundance (Figure 4.6A). Many of these highly expressed genes are involved in the type I IFN response and encode: chemokines

(*Ccl5*), transcription factors (*Irf7*), receptor and signalling molecules (*Rtp4*), members of the *Ifit* (IFN induced protein with tetratricopeptide) family (*Ifit1*, *Ifit3*), exonucleases (*Isg20*), nucleic acid sensors (*Zbp1*, *Ddx60*), GTPases (*Mx1*, *Mx2*), proteins involved in ISGylation (*Isg15*), RNase L activation (*Oasl2*), those with a multitude of described roles in the innate immune response (Viperin) and those with no described mechanism (*Ifi44l*). Given the extensive antiviral role that ISGs have been shown to exhibit, it is likely that these gene expression changes are a major contributing factor to the inhibition of norovirus displayed by TLR7 agonists.

Since our transcriptomic analysis was based on Loxoribine, but most of the cell-based experiments were performed with the more potent R-848, we compared expression changes of key innate genes (n= 7) by RT-qPCR. We illustrate that R-848 and Loxoribine display a consistent trend in immune upregulation (Figure 4.6B), which shows that TLR7 stimulation with different agonists results in the same antiviral effect.

Host-modulating compounds have previously been shown to inhibit the human Norwalk replicon and include de-ubiquitinase inhibitors, vitamin A and recombinant IFN[121,122,232,265]. We show that direct treatment of Norwalk replicon bearing cells with R-848 resulted in partial inhibition of replication, with ~50% inhibition at 100 μM (Figure 4.7A). However, this is >1000 fold higher than the EC_{50} exerted against MNV infection (Figure 4.1A and Figure 4.2B). To explore the lack of potency of TLR agonists in the replicon system, mRNA abundance of three key innate genes was quantified following 48 h treatment with R-848 (1 and 10 μM) in both HG23s and the parental cell line, Huh7 (Figure 4.7B). When the expression changes induced by R-848 in RAW264.7 cells (Figure 4.6B) are compared to those in the Huh7 and HG23 cell lines (Figure 4.7B), there is a clear difference in the intensity of cellular innate stimulation. Genes *Il6*, *Isg15*, and *Ifnb* were upregulated in the range of 78.2 to 705.9-fold in RAW264.7 cells (Figure 4.6B), whereas no greater than a 3-fold increase were measured in Huh7 and HG23 cells (Figure 4.7B). These findings may reflect the low-level expression

of TLRs in hepatic tissue[266] and/or the lack of cytokine production in response to TLR agonist stimulation observed in some cell lines of hepatocyte origin[267,268].

However, the lack of potency displayed by R-848 against the human Norwalk replicon is perhaps not a true representation of the antiviral effects that occur within a host. Generation of the Norwalk replicon involved serial passaging transfected cells in the presence of an antibiotic agent to select clones with the most robust replication[232]. It is possible that certain antiviral pathways are repressed or absent in the Huh-7 cell line and this in turn facilitated replication of the replicon and impaired inhibition of replicon replication by R-848. Additionally, the established replication of the Norwalk replicon could evoke shutdown of antiviral pathways which result in ineffective inhibition of replication with direct R-848 treatment. These are questions that should be addressed in subsequent studies. However, norovirus is thought to interact with many different intestinal cell types during infection which will have modest to high levels of TLR7 expression (B-cells, macrophages, epithelial cells) and thus, have the potential to be stimulated to induce an antiviral state capable of inhibiting norovirus replication. To test this hypothesis, THP-1 CM was tested at a 50% (v/v) concentration against the human Norwalk replicon for either 48 or 72 h (Figure 4.8). We observed significant dose-dependent inhibition of replication at both time points. These data show that the lack of an antiviral effect with R-848 treatment of HG23 cells is likely a result of minimal TLR7 expression. We show R-848 generated CM from other immune cells can stimulate the innate response of HG23s and inhibit the replicon by bypassing TLR7. Nonetheless, an environment capable of preventing norovirus replication can be induced by R-848, where the agonist cannot work directly.

There are several limitations of this study. Firstly, we only explored the antiviral effects of TLR7 agonists post-infection. Although this is the most appropriate assay since antivirals are generally administered after infection, the potential role for TLR7 agonists as a prophylactic therapy could

have been explored by treating cells prior to infection. Secondly, we did not directly explore the expression of TLR7 in Huh7 cells or attempt to overexpress TLR7 to reconstitute antiviral activity against the replicon which could have been used as a line of evidence for the lack of antiviral activity displayed by R-848 against the Norwalk replicon. TLR8 is non-functional in mice and as such its contribution to antiviral effects against MNV were not explored. Conversely, TLR8 is functional in human cells and it is possible that R-848 stimulation of THP-1 differentiated macrophages induces activation either TLR7, TLR8 or both. However, we did not determine whether TLR8 contributed to the production of antiviral molecules within human macrophage conditioned media, although this should be explored in subsequent studies.

In conclusion, our results indicate that immunomodulatory compounds such as TLR7 agonists have the potential to be a future therapeutic option for the treatment of norovirus infections. The most potent of agonists examined displayed effective viral inhibition in the low nanomolar range with minimal cytotoxicity and thus represent good candidates for antiviral therapy. We also show that TLR7 agonists could be used in combination with DAAs in a multi-targeted approach; a treatment strategy widely successful at curing HCV infections. We postulate that TLR7 agonists could enhance the immune response to minimise the effects of acute viral infections, but may also be useful for viral clearance of chronic norovirus sufferers and therefore could significantly reduce the burden of this disease.

4.6 Supporting information

Figure 4.9. The TLR7 agonist Loxoribine displays antiviral activity against MNV infection

Figure 4.10. Correlation analysis of gene expression changes following Loxoribine treatment (12 h) measured by qRT- PCR and RNA-sequencing

Table 4.2. DEG's in Loxoribine treated RAW264.7 cells

Table 4.3. qPCR primers used in this study (mouse and human)

All supporting information is available from the below link:

https://aac.asm.org/content/aac/suppl/2018/04/17/AAC.0241717.DCSupplemental/zac005187146s1.pdf

5 The adenosine analogue NITD008 has potent antiviral activity against human and animal caliciviruses

Text and figures included in this chapter are adapted from the following publication:

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Author contributions

Conceived and designed experiments: DET, JM and PW

Performed the experiments: DET, TF and NN

- MNV antiviral assays: DE
- FCV antiviral assays: TF
- Replicon antiviral assays: DE
- Data analysis: DE and NN

Wrote the manuscript: DET and PW

Declaration

I certify that this publication was a direct result of my research towards this PhD, and that reproduction in this thesis does not breach copyright regulations.

Daniel Enosi Tuipulotu [Candidate]

5.1 Abstract

The widespread nature of calicivirus infections globally has a substantial impact on the health and well-being of humans and animals alike. Currently, there are only vaccines approved for feline and rabbit caliciviruses, however some of these vaccines have poor efficacy. There is now a growing effort towards the development of broad-spectrum antivirals for the treatment of calicivirus infections. In this study, we evaluated the antiviral activity of the adenosine analogue NITD008 *in vitro* using three calicivirus model systems namely; FCV, MNV and the human norovirus replicon. NITD008 inhibited the replication of both FCV and MNV in a dose-dependent manner with an EC_{50} of 0.94 μM and 0.91 μM respectively. Moreover, NITD008 inhibited the Norwalk replicon with an EC_{50} of 0.21 μM and effectively cleared the replicon from cells with treatment at 5 μM. Significantly, this concentration completely prevented replicon rebound. In comparison to the most well-studied NA 2'-C-methylcytidine, NITD008 displayed greater potency against MNV (0.91 μM vs 1.6 μM), FCV (0.94 μM vs 2.6 μM) and the Norwalk replicon (0.21 μM vs 1.3 μM) with minimal toxicity (CC_{50} of 15.7 μM and >120 μM for the RAW264.7 and CRFK cell lines respectively). Moreover, the pan-*Caliciviridae* activity demonstrated in this study illustrates that NITD008 should be interrogated further to assess the antiviral activity of this compound *in vivo*. Overall, we have identified the most potent NA against caliciviruses described to-date, which represents a new therapeutic option to combat calicivirus infections.

5.2 Introduction

The *Caliciviridae* family is a group of positive-sense single stranded RNA viruses that are divided into five genera including *Vesivirus*, *Nebovirus*, *Lagovirus*, *Sapovirus* and *Norovirus*[22]. Ubiquitous in nature, caliciviruses can infect a wide range of animal hosts which usually manifest as either upper respiratory infections, AGE, encephalitis or haemorrhagic disease[269]. In comparison, calicivirus infections within humans result solely in AGE which is caused by members of the *Norovirus* and *Sapovirus* genera[270]. Human noroviruses are recognised as a predominant cause of AGE across all ages[271] and are estimated to cause around 700 million infections annually which result in over 200,000 deaths worldwide[5]. Furthermore, human noroviruses have a marked effect on the global economy, costing over $60 billion USD and associated mostly with medical care, hospitalisations and loss of productive days at work[12]. Thus, the pathogenic nature and high prevalence of caliciviruses in human and animals warrants the development of antiviral strategies against this family of viruses.

Currently, there are no approved calicivirus-specific antivirals and vaccination is only available for a subset of viruses including FCV[272], rabbit haemorrhagic disease virus (RHDV)-1 and RHDV-2[273], whilst human norovirus vaccines are in Phase II clinical trials[274-277]. Current animal calicivirus vaccines have biological and logistical shortfalls that limit their efficacy. For example, the RHDV-1 vaccine provides poor cross-protection against RHDV-2 and the RHDV-2 vaccine has had limited testing and is currently available only in the UK[273,278-280]. Similarly, approved FCV vaccines (including strains F9, 255, 431 and G1) do not provide complete protection against the antigenically distinct virulent systemic FCV (VS-FCV)[272,281,282]. In the absence of efficacious calicivirus vaccines, broad-spectrum antivirals are highly desired to cure calicivirus infections in animals and humans.

Antiviral compounds have thus far mostly been screened against caliciviruses using *in vitro* enzyme and *in vitro* cell based models, notably MNV, the human G1 replicon[232] and to a lesser extent FCV,

RHDV and rabbit calicivirus (RCV)[283-286]. These compounds are broadly divided into host-targeting agents and DAAs, with the latter targeting virus-encoded proteins essential for infection and replication, including the viral protease and RdRp[287]. Inhibitors of the RdRp are further divided into nucleoside analogues (NAs) and non-nucleoside inhibitors (NNIs)[269]. NNIs bind to allosteric sites on the RdRp which prevent conformational changes required for polymerase activity. Alternatively, NAs are incorporated into the newly synthesised viral genome during polymerisation which results in chain termination and inhibition of viral replication. The RdRp is an attractive antiviral target for several reasons: (i) there is no host cell homologue which limits off-target effects, (ii) the highly conserved nature of the active site confers broad-spectrum antiviral activity to NAs and (iii) many NAs have a high barrier to resistance, which is also related to the conserved nature of the active site where nucleotide incorporation occurs during polymerisation[288].

Currently, the antiviral which shows the greatest promise for the treatment of calicivirus infections is 2'-C-methycytidine (2CMC), a NA which was first developed for the treatment of flaviviruses[289], but has since been extensively examined as an antiviral against caliciviruses *in vitro* and *in vivo*[75,260,286,290-293]. Other NAs have also been screened against members of the *Caliciviridae*, including ribavirin and favipiravir (T-705), although both displayed poor activity against the human norovirus replicon with EC_{50} values of 40 μM and 21 μM, respectively[290,292]. NAs thus represent an important therapeutic option for calicivirus infections as they display broad-spectrum activity; 2CMC for example has been shown to be effective against the human norovirus replicon, MNV and FCV[75,260,286,290-293].

The adenosine analogue, NITD008, is a promising antiviral compound which has not been tested against any calicivirus. NITD008 was originally designed as an antiviral for dengue virus (DENV)[294], but was also found to inhibit the replication of several additional flaviviruses including, but not limited to: Zika virus (ZIKV), HCV, WNV and YFV[294-297]. Moreover, recent studies have shown that

NITD008 effectively inhibited replication of enterovirus 71 (EV71)[298,299], which belongs to the *Picornaviridae* family. Given the success of NITD008 against the abovementioned viruses we hypothesised that this compound would also inhibit members of the *Caliciviridae* family.

In this study, we evaluated the *in vitro* antiviral effects of NITD008 against a trio of caliciviruses (FCV, MNV and human norovirus) to provide insight on the applicability of this compound as a therapeutic for calicivirus infections. Importantly, we found that the potency of NITD008 exceeds that of any published NA tested against caliciviruses and is non-toxic *in vitro*. Moreover, we show that NITD008 displays inhibition against viruses that belong to two genera of the *Caliciviridae* family (*Norovirus* and *Vesivirus*) demonstrating the broad-spectrum properties desirable for a calicivirus therapeutic.

5.3 Methods

5.3.1 Drugs, cell lines, viruses

2'-C-methylcytidine (2CMC; Sigma-Aldrich, St. Louis, MO, USA) and NITD008 (In Vitro Technologies, VIC, Australia) were dissolved in 100% (v/v) DMSO. CRFK (ATCC CCL-94), RAW264.7 and the Norwalk replicon-bearing Huh7 (HG23) cell lines were maintained as previously described[261,286,300]. FCV (F-9 strain, ATCC VR-782) and MNV-1 (CW1 strain) were used for viral infections.

5.3.2 Toxicity analysis

Compound-induced toxicity of mammalian cells was assessed using CellTitre Blue (CTB) reagent (Promega, Madison, WI, USA), as previously described[63,74,261,286,300]. For consistency, the duration for both toxicity and inhibition assays were matched. The CC_{50} values were determined from at least eight concentrations using GraphPad Prism v.7.

5.3.3 Inhibition of viral infectivity

The effect of NITD008 on FCV and MNV infection was assessed by plaque reduction assays, as previously described[63,74,261,286,300]. EC_{50} values were determined from at least eight concentrations of antiviral using GraphPad Prism v.7. For drug combination assays, five concentrations of NITD008 (0-1 μM) and 2CMC (0-3 μM) were tested together against MNV in a plaque reduction assay. A dose-response matrix of all combinations was then analysed for synergy or antagonism using the Loewe Additivity model on SynergyFinder[234,301].

5.3.4 Inhibition of viral replication

The effect of NITD008 on replication of FCV, MNV and the Norwalk replicon was also assessed by measuring viral RNA levels by reverse transcription quantitative PCR (RT-qPCR). For FCV, CRFK ($2x10^5$) cells were infected at a multiplicity of infection (MOI) of 0.0005, whilst for MNV, RAW264.7 ($5x10^5$) cells were infected at a MOI of 0.01. After 1 h infection, unbound virus was replaced with media containing drug treatment and incubated for 24 h (FCV) or 48 h (MNV). For the Norwalk replicon, HG23 cells ($1x10^5$) were incubated for 24 h in G418-free media before drug treatment for

a further 48 h. The QIAmp viral RNA kit (Qiagen) and the RNAeasy mini kits (Qiagen) were used for RNA extraction for virus and replicon assays, respectively. RT-qPCR was used to determine viral or replicon RNA levels as previously described [293,300]. Targets for RT-qPCR, primer sequences, cycling conditions and plasmids for standard curve generation have been previously described[286,293,300].

5.3.5 Clearance-rebound studies

Clearance and rebound assays were performed by combining previously described methods[293,302] which are outlined in Fig. 4A. These assays provide insight into the ability of a compound to inhibit replicon/virus replication to an extent that prevents replication from reoccurring once treatment has completed. For the clearance phase, HG23 monolayers were treated with complete media (without G418) containing either DMSO (vehicle only – 0.1% v/v) or selected concentrations of NITD008 (1, 5 and 10 μM) and passaged every four days over a 12-day period. At each passage, HG23 cells were (i) harvested for RNA extraction and RT-qPCR to quantify Norwalk replicon RNA levels and (ii) seeded into new flasks with media containing the identical compound concentration for further incubation. For the rebound phase, $2.5x10^5$ HG23 cells from the second and third passages were seeded into 6-well plates with complete media (with 1.25 mg/mL G418) in the absence of any drug treatment. After seven days, cells were fixed and stained with 0.5% (w/v) crystal violet for visualisation of G418-resistant colonies containing the Norwalk replicon.

5.4 Results

5.4.1 NITD008 is inhibitory against animal calicivirus infection *in vitro*

The antiviral effects of NITD008 were evaluated against FCV and MNV *in vitro* using plaque reduction assays and quantification of viral genomes by RT-qPCR. NITD008 markedly inhibited the replication of both viruses with minimal cellular toxicity (Figure 5.1). Specifically, the EC_{50} of NITD008 was 0.91 μM (95% CI: 0.84-0.99) and 0.94 μM (95% CI: 0.88-1.02) for MNV and FCV, respectively (Figure 5.1A-B). Furthermore, the CC_{50} value of NITD008 was 15.7 μM (95% CI: 14.23-17.72) for RAW264.7 cells, whereas no toxicity was observed in the CRFK cell line at concentrations up to 120 μM (Figure 5.1C-D). The therapeutic index (TI = CC_{50}/EC_{50}) for NITD008 was therefore estimated to be 17.2 and ≥127.6 for MNV and FCV, respectively. In addition, we also confirmed the antiviral effects of NITD008 by quantification of viral genomes following treatment at 1 μM and 5 μM. We observed a dose-responsive inhibition of FCV and MNV replication with >90% reduction in genome count at 5 μM (Figure 5.1E-F). Importantly, we observed that 5 μM NITD008 is more potent than 10 μM 2CMC against both viruses (Figure 5.1E-F).

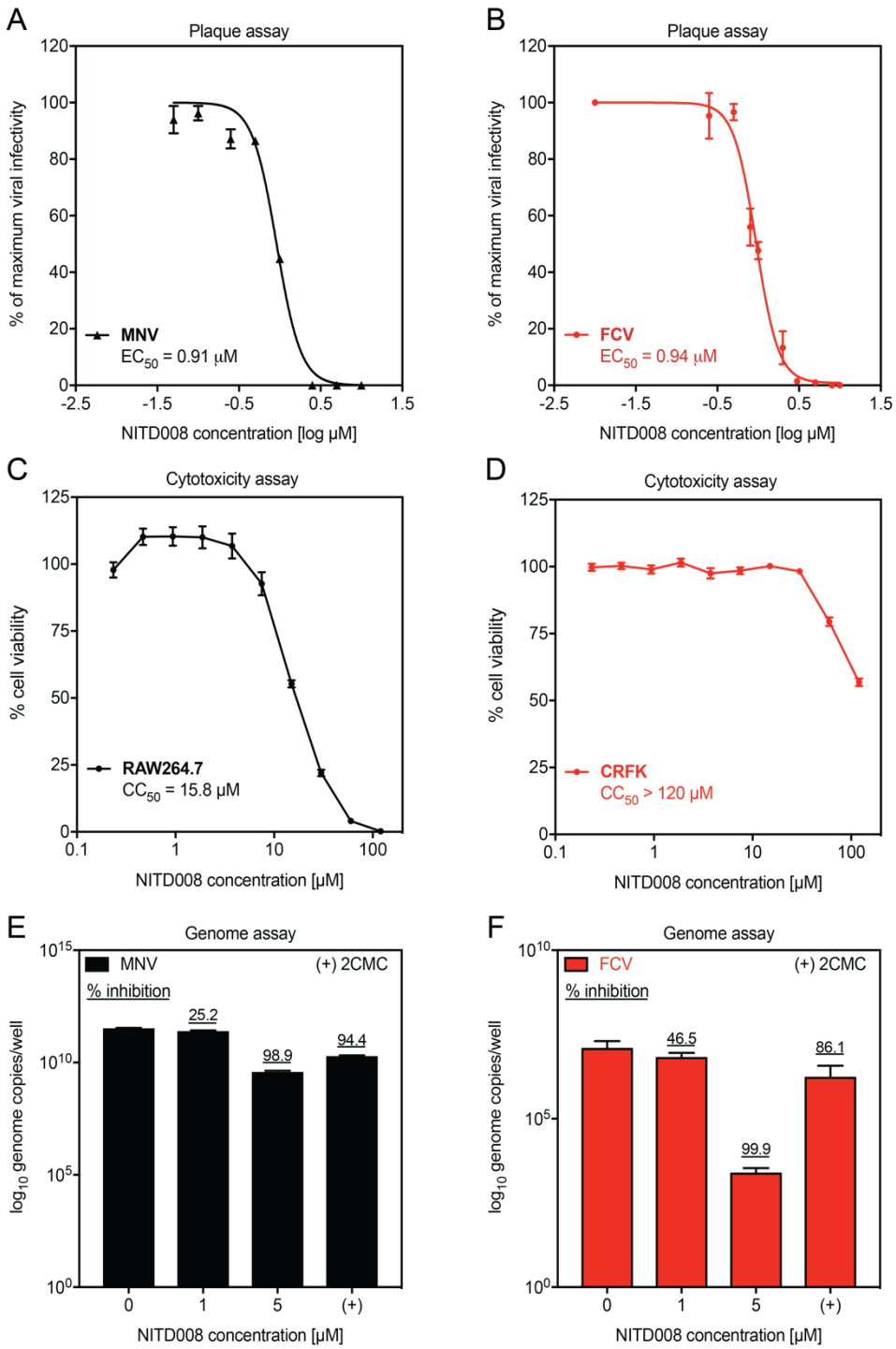


Figure 5.1. NITD008 is a potent inhibitor of FCV and MNV infection

The antiviral potential of NITD008 was explored against two animal caliciviruses closely related to human norovirus. Panels with black data points/bars are related to MNV and involve 48 h incubations whereas panels with red data points/bars are related to FCV and involve 24 h incubations. (A-B) Infectious virus levels following NITD008 treatment (0.01-10 µM) were quantified by plaque reduction assay. The percentage of maximal viral infectivity is relative to DMSO controls and is plotted as a dose-response curve. The cytotoxic effects of NITD008 (0.2-120 µM) against (C) RAW264.7 and (D) CRFK cell lines, permissive for MNV and FCV respectively, were quantified after

treatment using the fluorescent CellTiter-Blue assay. The percentage cell viability is relative to DMSO controls. (E-F) Viral genome levels following NITD008 treatment (1 µM and 5 µM) were quantified by RT-qPCR. The nucleoside analogue 2CMC (10 µM) and DMSO were used as positive and negative controls respectively. Triplicate data from at least two independent experiments are presented for each panel. Error bars represent the mean ± standard deviations.

5.4.2 NITD008 combined with 2CMC has an antagonistic antiviral effect

NITD008 has been reported to inhibit the replication of numerous flaviviruses and functions as a chain terminator through interaction with the polymerase active site[294-299]. To determine whether NITD008 shares the same binding site in caliciviruses we performed a drug combination analysis with NITD008 and 2CMC (a known calicivirus NA). We tested five concentrations of NITD008 (0 - 1 µM) and 2CMC (0 - 3 µM) in combination to assess for synergistic or antagonist effects using a MNV plaque reduction assay. The Lowe-Additivity model demonstrated a strong antagonism between the two compounds (average delta synergy score -39.58 across the matrix), which was more prominent with increasing drug concentrations (delta synergy score – 76.79 at the combined maximum drug concentration (Figure 5.2).

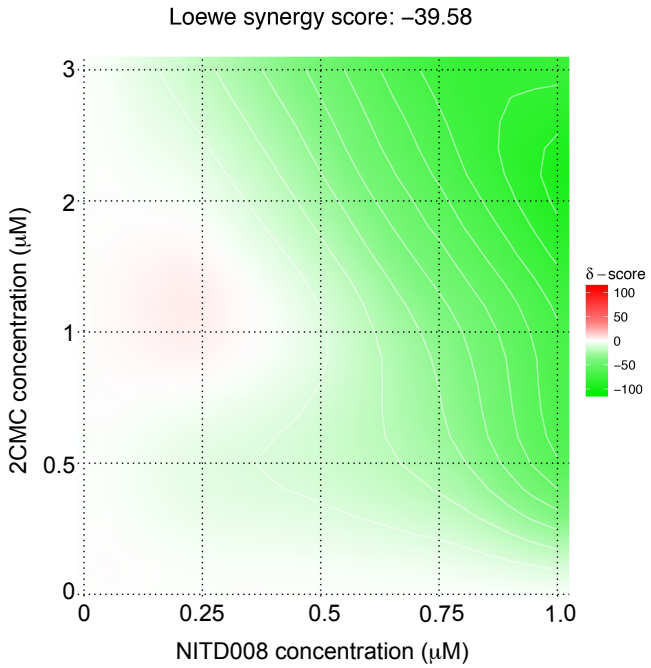


Figure 5.2. The interaction between the NITD008 and 2CMC is strongly antagonistic

The combinational effects of 2CMC (0-3 μM) and NITD008 (0-1 μM) against MNV infection were tested over a range of concentrations using a plaque reduction assay. The percentage of inhibition observed across all drug combinations were tabled into a dose-response matrix and analysed for synergy and antagonism using the Lowe-Additivity model on SynergyFinder. The synergy scores obtained for each drug combination across the entire matrix are presented as a heatmap. Synergistic (positive δ-score) and antagonistic (negative δ-score) interactions are represented in red and green respectively, and no observable effect (δ=0) is represented as white.

5.4.3 NITD008 is a potent inhibitor of human norovirus replication

To determine if NITD008 has antiviral activity against human norovirus, we examined its inhibitory effects against the Norwalk virus replicon using RT-qPCR to measure RNA levels (Figure 5.3). NITD008 effectively reduced Norwalk replicon levels in a dose-dependent manner (EC_{50} of 0.21 μM, 95% CI: 0.17-0.26) (Figure 5.3A) and displayed no toxicity against the HG23 cell line when tested up to 120 μM (Figure 5.3B). The TI for NITD008 in this replicon system was calculated at ≥ 571.4.

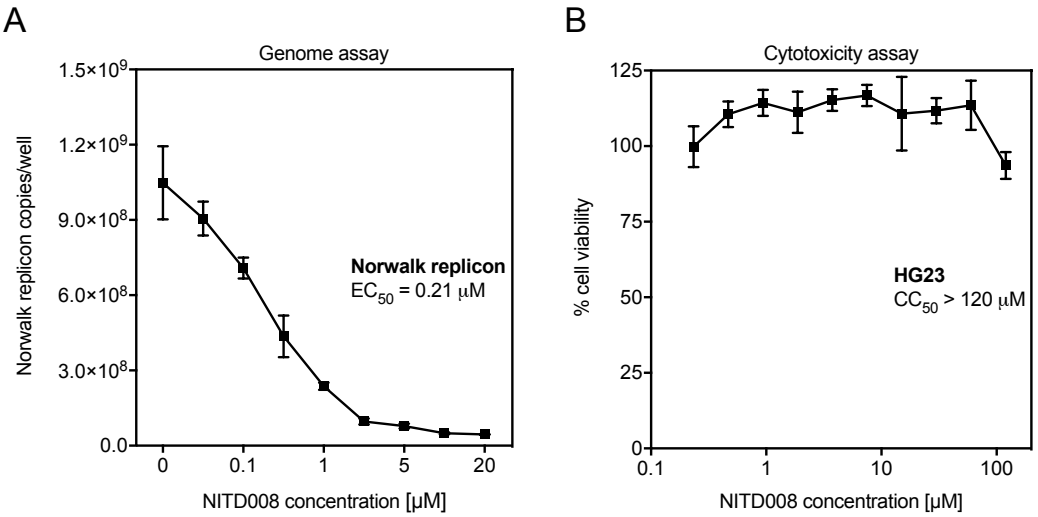


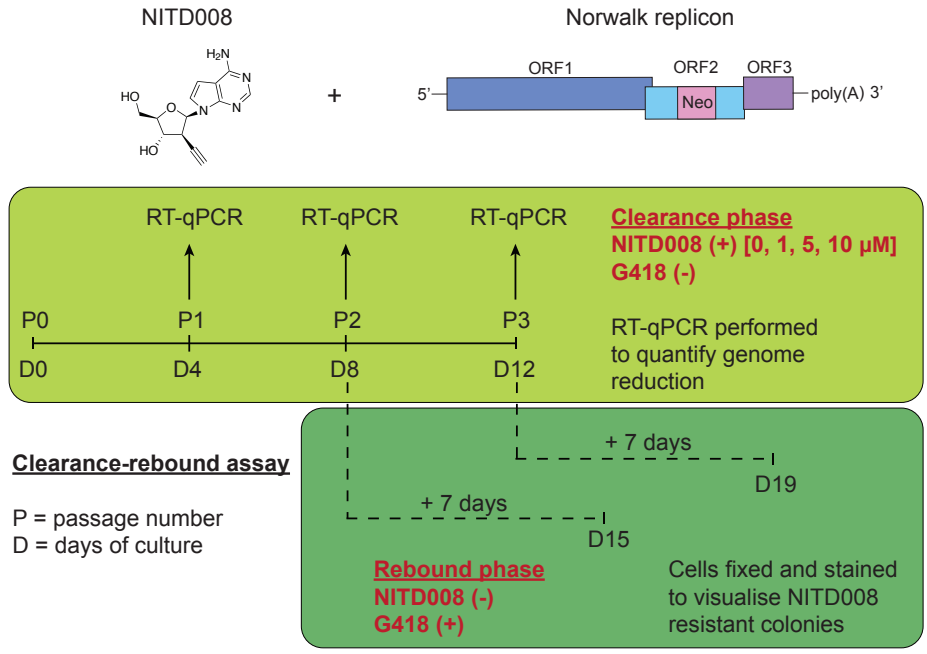
Figure 5.3. NITD008 is a potent inhibitor of the human norovirus replication

(A) Norwalk replicon levels were measured using RT-qPCR following NITD008 treatment (0-20 μM) for 48 h. The norovirus RdRp sequence was amplified for the replicon and *β-actin* was amplified as the house-keeping gene. The ΔΔCt method was used to normalise cell number between experimental conditions prior to quantitation of replicon levels. (B) The cytotoxic effects of NITD008 (0.2-120 μM) against the Norwalk replicon-bearing cell line (Huh7) was assessed after 48 h using the fluorescent CellTitre-Blue assay. The percentage cell viability is relative to DMSO controls. Triplicate data from two independent experiments are presented for each panel. Error bars represent the mean ± standard deviations.

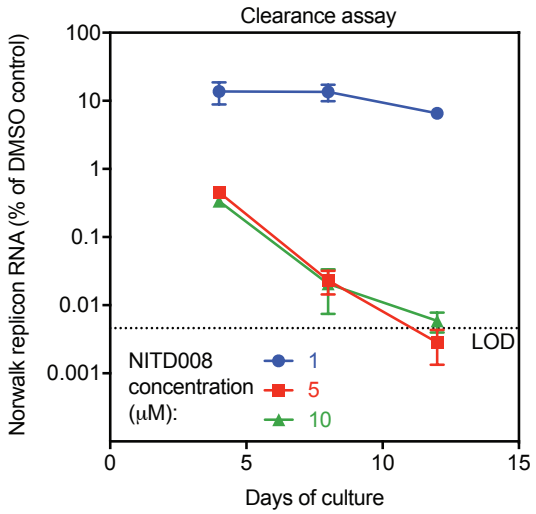
5.4.4 NITD008 effectively clears the human norovirus replicon from cells

To evaluate whether repeated NITD008 treatment could eliminate the Norwalk replicon from host cells, clearance-rebound assays were performed, as described in Figure 5.4A. During the clearance phase, G418 was absent from the culture media and HG23 replicon-bearing cells were passaged in the presence of either 1, 5 or 10 μM of NITD008 for 12 consecutive days. Norwalk replicon RNA levels were measured by RT-qPCR at each passage for all concentrations of NITD008 tested and compared to the mock control (Figure 5.4A). Following the first passage treatment with 1 μM, NITD008 resulted in an 86.3% ± 4.9% reduction in replicon RNA levels, whereas treatment with 5 μM and 10 μM resulted in a 99.5% ± 0.1% and 99.7% ± 0.1% reduction, respectively (Figure 5.4B). At the third passage, 1 μM reduced the replicon population by 93.4% ± 0.8% and treatment at 5 and 10 μM reduced the population by more than 99.9% (Figure 5.4B). To determine whether the genome reduction observed in the clearance phase was sufficient to prevent a resurgence of replicon replication, a colony formation assay was to check for rebound (Figure 5.4C). Cells from the second and third passage (P2 and P3) at each drug concentration of the clearance-phase were incubated in the presence of G418 to select for replicon-bearing cells (Figure 5.4A). We observed no rebound for NITD008 treatment at 5 μM and 10 μM at either P2 or P3, however, there was complete rebound with 1 μM treatment at P2 and partial rebound at P3 (Figure 5.4C). In summary, the effectiveness of both clearance and prevention of replicon rebound was enhanced by increasing both NITD008 dosage and treatment duration.

A


NITD008
Norwalk replicon
ORF1
ORF2
ORF3
5'
Neo
poly(A) 3'
RT-qPCR
RT-qPCR
RT-qPCR
Clearance phase
NITD008 (+) [0, 1, 5, 10 µM]
G418 (-)
P0
P1
P2
P3
D0
D4
D8
D12
RT-qPCR performed to quantify genome reduction
Clearance-rebound assay
P = passage number
D = days of culture
+ 7 days
D19
+ 7 days
D15
Rebound phase
NITD008 (-)
G418 (+)
Cells fixed and stained to visualise NITD008 resistant colonies

B


Clearance assay
Norwalk replicon RNA (% of DMSO control)
100
10
1
0.1
0.01
0.001
LOD
NITD008 concentration (µM):
1
5
10
0
5
10
15
Days of culture

C

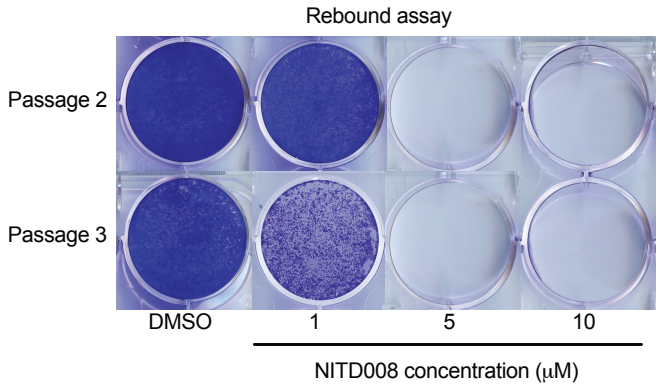

Rebound assay
Passage 2
Passage 3
DMSO
1
5
10
NITD008 concentration (µM)

Figure 5.4. NITD008 effectively clears the Norwalk replicon from replicon-bearing cells

(A) Schematic overview of the clearance-rebound assays performed against the Norwalk replicon. The light green panel represents the clearance phase in which replicon-bearing cells were passaged every 4 days in the presence of DMSO or NITD008 at 1, 5, and 10 μM (~ 5, 25, 50 x the EC_{50} value). The dark green panel represents the rebound phase in which NITD008 is removed from the media and G418 is used to selected for replicon-bearing cells. (B) Clearance of the Norwalk replicon from cells was determined by RT-qPCR. Norwalk replicon levels following NITD008 treatment at every passage are plotted relative to the corresponding DMSO controls. The dotted line represents the qPCR limit of detection (LOD). (C) Rebound of the Norwalk replicon was assessed by performing colony formation assays on cells passaged in the presence of NITD008 for either 8 or 12 days. Colonies were fixed and stained with crystal violet before visualisation. Triplicate data from at least two independent experiments are presented for each panel. Error bars represent the mean ± standard deviations.

5.5 Discussion

In the absence of efficacious calicivirus vaccines, broad-spectrum antivirals represent a practical solution to limit calicivirus disease and transmission. NAs are a class of antivirals that generally display broad-spectrum antiviral activity which is attributed to the highly conserved polymerase active site that they interact with. So far, the only NAs that have been published against caliciviruses include 2CMC, T-705 and ribavirin (reviewed in [41]). Of these, 2CMC is the most effective and has low micromolar potency against caliciviruses, with reported EC_{50} values of 2.6 μM, 1.6 μM and 1.3 μM against FCV, MNV and the Norwalk replicon, respectively[75,286,292]. In this study, we report that NITD008 has EC_{50} values of 0.94 μM, 0.91 μM and 0.21 μM against FCV, MNV and the Norwalk replicon systems, respectively. Given the higher potency displayed by NITD008 across all three models, compared to previously described NAs, NITD008 is a good candidate for further refinement as an antiviral for calicivirus infections.

The potency displayed by NITD008 against caliciviruses is comparable to that against other viruses including ZIKV, DENV and EV71 with EC_{50} values of 0.24 μM, 0.64 μM and 0.67 μM respectively[294,295,299]. Despite this potency, NITD008 has not yet progressed to clinical trials for the treatment of virus infections. Pre-clinical animal testing of NITD008 for DENV infection revealed toxicity in dogs following two weeks of daily compound treatment[294]. However, *in vivo* toxicity was absent within the first week of this study and thus NITD008 may still be a viable treatment of virus infections with a short treatment schedule.

To confirm that NITD008 inhibited calicivirus replication thorough interaction with the active site, we tested the combined effects of NITD008 and 2CMC against MNV infection. We show that when combined, a strong antagonistic effect was observed. This antagonism is most likely a result of competition for the active site within the polymerase and demonstrates that NITD008 likely works by a typical NA mechanism. The pan-*Caliciviridae* activity displayed by NITD008 (cross-genera and

cross-genogroup) supports the idea that it could be used widely for the treatment of animal and human calicivirus infections including variants that emerge through rapid evolution.

Next, we wanted to assess whether NITD008 could effectively prevent viral rebound after completion of a short treatment regime. We exposed the Norwalk replicon to NITD008 for 12 days and quantified replicon RNA at multiple time points to determine whether treatment could reduce replication and subsequently prevent replicon rebound. A decrease in the replicon RNA levels was observed with each consecutive passage in the presence of NITD008 (1, 5 and 10 μM) and treatment with 5 μM for eight days was sufficient to completely prevent rebound of the Norwalk replicon. The ability of NITD008 to prevent viral rebound is likely to be a critical factor towards the therapeutic efficacy of this compound against caliciviruses. However, these experiments were performed *in vitro* and thus the concentrations of NITD008 used in this study may not reflect clinically effective dosages. Nonetheless, our preliminary findings warrant more extensive assessment of NITD008 using *in vivo* studies.

The development of antiviral resistance is important clinically since rebound of resistant populations can have a dramatic impact on treatment success[229]. Several NITD008 resistance mutations have been documented in EV71[299] and HCV[296] following passage of these viruses with increasing concentrations of NITD008. For EV71, mutations that confer resistance are within the 3A and 3D domains which encode proteins that function to form a part of the replication complex and the polymerase respectively. For HCV, mutations occur within motif B of the polymerase as well the finger and thumb domains. In comparison, other studies have shown that long-term passage in the presence of NITD008 failed to induce mutations within DENV[294], WNV[294], ZIKV[295] and EV71[298]. The conflicting data observed for EV71 resistance to NITD008 is likely attributed to the different experimental techniques performed for resistance selection. However, it is clear from these studies that resistance only emerged with long-term exposure and overall NITD008 has a high barrier to

resistance. Although we show that the cell line was completely cured of the Norwalk replicon with NITD008 treatment, we did not perform analysis to detect escape mutants. Future evaluation of resistance to NITD008 within caliciviruses should be performed to provide further understanding of the applicability of this compound in the clinic.

In summary, we present the most potent NA described in the literature for caliciviruses to-date. The sub-micromolar potency, low toxicity, large therapeutic window and pan-*Caliciviridae* inhibition displayed by NITD008 *in vitro* suggests that this compound will be of considerable benefit. Moreover, since resistance profiles against NITD008 have been documented, combination therapy with other DAAs would be an appropriate strategy to limit antiviral resistance to NITD008. Our data provides strong evidence that NITD008 would likely be a suitable backbone for calicivirus therapies and further investigations into potential drug combinations are warranted.

6 General Discussion

Human norovirus is a leading cause of acute gastroenteritis worldwide and is estimated to be responsible for more 200,000 deaths each year[5]. The low infectious dose and relative ease of norovirus transmission results in both epidemic and pandemic outbreaks that cause a significant health and economic burden globally[11-13]. Although most norovirus infections are self-limiting, immunocompromised individuals are particularly susceptible to chronic infection and would benefit immensely from an effective therapy[8,9,21]. However, despite substantial research efforts, there is still no vaccine or norovirus-specific antiviral approved for clinical use[41].

Therefore, the main aims of this thesis were to (i) increase our understanding of norovirus pathogenesis, (ii) discover new host targets for norovirus antivirals, and (iii) assess whether antiviral compounds (DAA or host-targeting) in pre-clinical development, within clinical trials or clinically approved for other viruses could be repurposed for norovirus. Overall, this thesis aimed to increase the number of therapeutic options currently in the pipeline for the treatment of norovirus infections.

6.1 Findings and implications

6.1.1 MNV infection induces a transcriptional profile that indicates dampening of the innate immune response

Our understanding of human norovirus pathogenesis has been significantly hampered due to the lack of efficient tissue culture systems and animal models[64]. In recent years, new methods for human norovirus cultivation have been developed[56,57], however MNV is still widely used as a main surrogate model to investigate human norovirus replication and pathogenesis[132,303]. Indeed, knowledge of norovirus pathogenesis can illuminate features of the host that can be targeted for antiviral development.

RNA-sequencing is a technique that can provide unparalleled insight into host-pathogen interactions and help elucidate mechanisms involved in viral pathogenesis[304-307]. This technology can generate expression information of all the genes present within an organism[308] and studies have shown that a strong correlation exists between transcript and protein abundance[309,310]. Thus, this method can be used characterise the complex biological changes that occur within the host following viral infection.

In **chapter three**, RNA-sequencing was performed to characterise the transcriptional profile of the RAW264.7 cell line following MNV infection (time-course and end-point). Differential expression analysis revealed an upregulation of genes that encode proteins involved in antiviral defence including PRRs, innate signalling pathway proteins and cytokines. These findings were characteristic of type I IFN production. Moreover, we observed a downregulation in the expression of genes that encode Trim proteins (*Trim14*, *Trim2*, *Trim68*, *Trim47* and *Trim7*), pro-inflammatory cytokine receptors (*Cxcr3*, *Cxcr5*, *Il17rc* and *Il1rl1*), and TLR7 which is responsible for the detection viral nucleic acids (ssRNA)[311]. These changes suggested that MNV reduces viral recognition and cytokine stimulation of the host to avoid destruction by the immune response and can thereby continue to

replicate. Although evasion of the immune response is known to be a feature in the pathogenesis of multiple RNA and DNA viruses[312,313], further investigation is required to confirm whether this is also true for noroviruses. In addition to this dataset, a later study also performed RNA-sequencing of MNV infection in the RAW264.7 cell line in addition to primary murine macrophages (BMDMs)[314]. The authors reported a remarkably similar transcriptional profile for both upregulated and downregulated genes when compared to our analysis[314]. Furthermore, it would be beneficial to explore whether transcriptional changes in the genes mentioned above correlate at the protein level to provide further evidence into the role of such genes in MNV pathogenesis.

Many viruses have evolved counter-measures to interfere with one or more steps in antigen presentation to evade immune recognition, including; viral peptide generation via the proteasome or other proteases (host or viral), packaging of MHC proteins with peptides, intracellular trafficking of MHC-peptide complexes or cell surface expression of MHC[315,316]. Poliovirus[317], HSV[318], HPV[319] and HIV[320] are examples of viruses that manipulate MHC class I protein expression to limit antigen presentation and enhance resistance against cytotoxic lymphocytes[321,322]. In this study, MNV infection resulted in significant downregulation of genes involved in multiple steps of MHC class I maturation. This included members of the *Psmb* family that encode proteins which make up the catalytic core of the 26S proteasome, *Psme2* which encodes an activator of the i-proteasome, and *Ap1s1* which is reportedly involved in trans-Golgi trafficking[203,204]. From these findings, we hypothesised that norovirus decreases MHC class I protein expression to limit antigen presentation. Importantly, this hypothesis was subsequently confirmed by another group that showed MNV infection reduced the surface expression of MHC class I proteins on both macrophages and dendritic cells which prevented $CD8^+$ T cell activation[323].

Chapter three highlights the power of RNA-sequencing as a tool to probe host-pathogen interactions. Broadly, we identified that a dampening of the innate immune response is likely to be a major driver in norovirus pathogenesis. Moreover, the use of RNA-sequencing to better understand norovirus biology prompted the discovery of a new norovirus antiviral target which is discussed further in section 6.1.2. Overall this study has generated an incredibly rich source of data that researchers can investigate further to help understand the complex interplay between noroviruses and the host.

6.1.2 TLR7 represents a new target for norovirus antivirals and agonists of this receptor could be repurposed for developing anti-norovirus therapies

Antivirals that target the host generally have a higher barrier to resistance compared to DAAs[119] and can be used in combination with DAAs. Despite these benefits, DAAs represent the vast majority of antiviral compounds that have been screened against noroviruses, whereas host-targeting compounds are far less studied. Given the small number of host-targeting antivirals with efficacy demonstrated against norovirus[41], new therapeutic options of this class should be explored as potential therapeutics.

TLR agonists are a class of host-targeting compounds which have been widely explored as antivirals for HPV, HSV, HIV and HBV[142,143,146,225-227]. Several TLR agonists are progressing through clinical trials [228]and the TLR7 agonist, R-837, is currently approved for the treatment of HPV-associated genital warts[146,225]. In **chapter three**, we observed that *TLR7* expression was downregulated with MNV infection and postulated whether this receptor could be targeted to inhibit norovirus replication, and thus represent a new antiviral target.

In **chapter four**, the TLR7 agonists Gardiquimod, R-837, R-848 and GS-9620 were screened for antiviral activity against MNV using *in vitro* plaque reduction assays. Potent inhibition was displayed by all compounds with EC_{50} values in the range of 23.5 nM – 1.5 μM. RNA-sequencing analysis of TLR7 stimulated cells confirmed robust induction of the type I IFN response and additional analyses revealed that these cells also secreted soluble molecules capable of inhibiting MNV replication in a dose dependent manner. However, the most potent agonist (R-848) was ineffective against the Norwalk replicon, only reducing replicon copies by 54% at 100 μM. We hypothesised that the lack of potency displayed against the replicon by R-848 was likely a result of poor TLR expression within the replicon-bearing cell line[266-268].

Following publication of **chapter four**, another research group identified that the TLR4 agonist, poly-γ-glutamic acid, inhibits MNV in cell culture and in mice[144]. Similar to our TLR7 antiviral study, treatment with the TLR4 agonist failed to effectively inhibit the Norwalk replicon[144]. The authors of this study also concluded that absent or low TLR expression within the replicon cell line (hepatocyte origin) is likely the cause of poor efficacy of TLR4 agonist the Norwalk replicon[144]. Both studies illustrate that although TLR agonists are potent, their activity is largely dependent on the presence of the target receptor within infected cells. However, since noroviruses have been shown to replicate in macrophages[132], dendritic cells[132], B-cells[56] and within intestinal enterocytes[46,324], TLR agonists are suitable antiviral candidates since these cell types express TLRs[266,325-327]. Nonetheless, future studies should explore whether the same antiviral effect is observed against human norovirus in the B-cell and enteroid culture systems which have unique TLR expression profiles.

Chapter four highlights that TLR7 agonists have potent antiviral activity against MNV *in vitro* and thus represent a new category of host-targeted norovirus antivirals. Moreover, this study has illustrated that repurposing antivirals (clinically approved or in trials) may offer an accelerated pathway to develop a cure for norovirus infections.

6.1.3 NITD008 is a broad-spectrum antiviral that is effective against noroviruses

Inhibitors of the viral polymerase (NA or NNI) and protease are the most intensely studied class of norovirus antivirals[41]. PIs are mostly virus-specific due to subtle differences in the catalytic mechanisms of viral proteases[328], whereas NAs generally display broad-spectrum antiviral activity[329]. The latter is attributed to the conserved binding site (active site) of NAs within the viral RdRp. Several broad-spectrum NAs have previously been tested against noroviruses including RBV[116,117], T-705[109,113,114], 2CMC[106,107,238,291] and CMX512[118]. As mentioned in section 1.6.2, RBV and T-705 have limited potency against noroviruses whereas 2CMC, although potent, induces side effects[105]. Thus, new NAs with anti-norovirus activity are highly desired to expand the current library of NAs (i.e. CMX512) that could progress to clinical trials.

NITD008 is a NA that has been shown to be effective against several members of *Flaviviridae* and *Picornaviridae* amongst others[295,296,298,299]. Given the broad-spectrum nature of NAs, we screened the antiviral activity of NITD008 against several caliciviruses to assess its potential as a norovirus therapeutic. In **chapter five** of this thesis, NITD008 was shown to inhibit both FCV, MNV and the human norovirus replicon with EC_{50} values of 0.94 μM, 0.91 μM and 0.21 μM respectively. NITD008 displayed minimal toxicity across all three systems and therapeutic indexes were in the range of 17.2 - ≥571.4. These values indicate that NITD008 is the most potent NA described to date for the caliciviruses.

A successful antiviral should efficiently clear norovirus and prevent rebound after drug removal. Clearance-rebound experiments with the Norwalk replicon revealed that exposure to NITD008 at a concentration 25 times the EC_{50} (i.e. 5 μM) for eight days was sufficient to clear the replicon from cells and prevent rebound. In comparison, the clinically approved NA, Sofosbuvir, prevents rebound of the HCV replicon with 14 days exposure at a concentration 10 times the EC_{50}[330]. This comparison provides insight into the therapeutic applicability of NITD008. Sofosbuvir clearly displays greater

efficacy in preventing viral rebound, however the ability of NITD008 to clear the Norwalk replicon at a clinically relevant concentration over a short duration (8 days vs 14 days for Sofosbuvir) illustrates that NITD008 (or derivatives of NITD008) should be explored further as a norovirus therapy.

As NITD008 has yet to progress to clinical trials for any virus, additional research efforts are needed before clinical development of this compound for caliciviruses. First, since the experiments in this chapter were *in vitro*, further antiviral testing against MNV *in vivo* and resistance mutation screening would be beneficial. Moreover, NITD008-induced toxicity has been observed in rats and dogs when dosed daily for two-weeks[294]. Although this may not be significant for short-term treatment of acute infections, it could play a crucial role in long-term treatment of chronically infected patients. Thus, structural derivatives of NITD008 could be screened for improved safety and pharmacokinetic profiles. Lastly, antivirals that display synergy with NITD008 should be identified so combination therapy can facilitate a dose reduction and thereby reduce the risk of toxicity *in vivo*.

Chapter five has identified a new NA, NITD008, as a potent inhibitor of calicivirus replication and a potential therapeutic option for human norovirus infection. The preliminary findings in this study show that NITD008 could be used as a scaffold for the generation of derivatives with superior drug qualities or alternatively used in combination with DAAs or host-targeted therapies in an anti-norovirus drug cocktail.

6.1.4 Final remarks

This thesis has identified new features of norovirus pathogenesis (**chapter three**) and harnessed such knowledge to show that TLRs represent a new target for norovirus antivirals (**chapter four**). By repurposing therapeutic compounds, this study identified several new drugs with novel anti-norovirus activity including TLR7 agonists (**chapter four**) and the NA NITD008 (**chapter five**). Overall this thesis has used a multi-faceted approach to antiviral discovery and thereby expanded the repertoire of compounds in pre-clinical development for the treatment of norovirus infections.

7 References

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