

Assessment of the anti-norovirus activity in cell culture using the mouse norovirus: Early mechanistic studies

Antiviral Chemistry and Chemotherapy
2021, Vol. 29: 1–6
© The Author(s) 2021
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/20402066211025175
journals.sagepub.com/home/avc



Jana Van Dycke , Jasper Rymenants , Johan Neyts and Joana Rocha-Pereira

Abstract

Human norovirus is the main cause of viral gastroenteritis, resulting annually in ~700 million infections and 200,000 deaths, of whom most are children <5 years. Mouse norovirus-infected macrophages are the most widely used *in vitro* system to screen and characterize the antiviral effect of norovirus-targeting small molecules. We have previously established antiviral assays using this system, identified novel inhibitors and performed additional studies in order to have a first insight into their mechanism of action. After the identification of novel small molecules with anti-norovirus activity (part I of this protocol), we here describe the logical next step which entails the generation of early information of their mode of action. This information together with a continuous improvement of the potency of compounds will contribute to the optimization of a compound class towards *in vivo* efficacy and a successful preclinical development.

Keywords

Compounds, drug resistance, mutations, virus

Date received: 19 February 2021; accepted: 26 May 2021

Introduction

We here describe the first steps towards the characterization of the molecular mechanism of action of a norovirus antiviral, as a follow-up to the identification of active molecules (in part I of this protocol).¹ After running a set of assays that identify novel norovirus inhibitors, we propose an early characterization of these inhibitors by means of time of drug addition studies and tentative selection of drug-resistant variants.

To estimate the time-point when the compounds act on the viral replication cycle of MNV a time-of-drug-addition experiment can be performed as shown in Figure 1. This is most efficient to distinguish molecules acting before or at moment a virus enters the host cell from those molecules targeting post-entry steps. The use of known compounds with an effect on a specific step of the virus life cycle, in parallel with a new molecule, might allow additional conclusions about the targeted step by this new molecule.

More information will arise by a repeated cultivation of the virus in the presence of the novel inhibitor. On one hand, a notion of barrier to resistance will be

acquired as rapid occurrence of drug-resistant virus variants has been observed in the clinic with some approved antivirals (e.g. anti-HIV or anti-influenza drugs). Importantly, by exposing the virus to multiple rounds of compound treatment in the laboratory, the acquisition of mutations that hamper the antiviral effect of compound is stimulated. Knowing where these mutations are located in the virus genome (i.e. after sequencing) will lead to a more precise target identification. From here on, further studies can be tailored to more precisely elucidate the interaction of the antiviral molecule with its target.

KU Leuven – Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology and Chemotherapy, Leuven, Belgium

Corresponding author:

Joana Rocha-Pereira, KU Leuven – Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology and Chemotherapy, Leuven, Belgium.
Email: joana.rochapereira@kuleuven.be

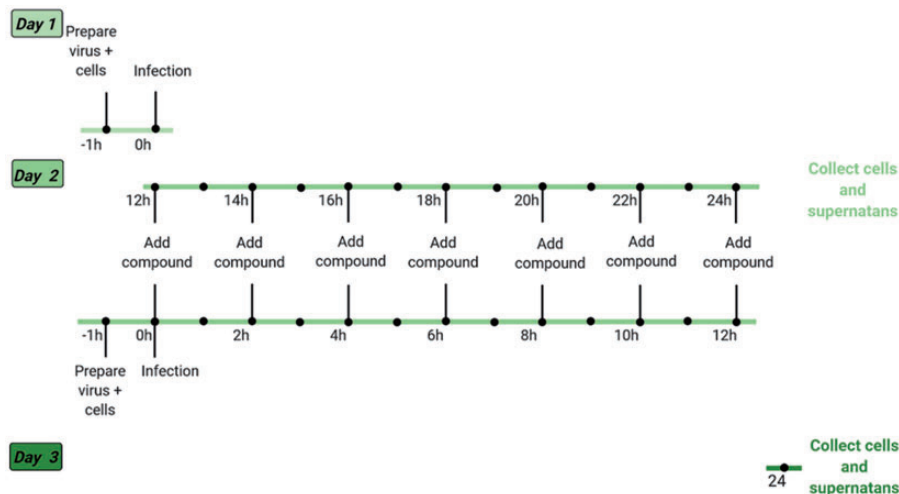


Figure 1. Layout of the timeline of a time-of-drug-addition experiment. Created with BioRender.com.

Expertise needed to implement the protocol

The scientist needs to be trained to work with cell culture and pathogens at BSL-2 level.

Materials and reagents

- Appropriate personal protection to work in a bio-safety level 2 (BSL-2) laboratory (gloves, lab coat, shoe covers, safety glasses)
- Disinfectant: bleach (5,000 ppm) or Virkon S.
- Cryotubes (Thermo Fisher Scientific, Thermo Scientific, catalog number: 377224)
- Pipet tips (10 μ L, 100 μ L, 1,000 μ L)
- Disposable serological pipets (5 mL, 10 mL, 25 mL)
- Disposable aspirator pipets (Falcon, catalog number: 357558)
- Murine macrophage cells (RAW 264.7, ATCC, catalog number: TIB-71)
- Dulbecco's phosphate buffered saline (PBS) (Thermo Fisher Scientific, catalog number: 14190094)
- Dulbecco's modified eagle's medium (DMEM) (Thermo Fisher Scientific, GibcoTM, catalog number: 41965039)
- Fetal calf serum (FCS) (Thermo Fisher Scientific, GibcoTM, catalog number: 10270106)
- Sodium bicarbonate (Thermo Fisher Scientific, GibcoTM, catalog number: 25080060)
- L-Glutamine (Thermo Fisher Scientific, GibcoTM, catalog number: 25030024)
- HEPES (Thermo Fisher Scientific, GibcoTM, catalog number: 15630056)
- Penicillin/streptomycin (P/S) (Thermo Fisher Scientific, GibcoTM, catalog number: 15140148)
- Sodium pyruvate (Thermo Fisher Scientific, GibcoTM, catalog number: 11360039)
- Colorless Minimal Essential Medium (MEM) (Thermo Fisher Scientific, GibcoTM, catalog number: 51200046)
- Eppendorf safe-lock tubes, 1.5 mL (Eppendorf, catalog number: 0030120086)
- 2'-C-methylcytidine (2CMC, Carbosynth, catalog number: NM07918)
- Falcon tubes 50 mL (Greiner Bio-one, catalog number: 227261)
- Falcon 5 mL polystyrene Round-Bottom Tube (Falcon, catalog number: 352054)
- CellTiter 96 AQueous MTS Reagent Powder (MTS, Promega, catalog number: G1111)
- 96-well plate (Falcon, catalog number: 353072)
- Phenazine methosulfate (PMS, Sigma, catalog number: P9625)
- Filter 0.22 μ m (Thermo Scientific Nalgene catalog number: 161-0020)
- Trypan Blue Stain, 0.4% (Logos Biosystems; Catalog number: T13001)
- MNV forward primer: 5'-CAC GCC ACC GAT CTG TTC TG -3' (Integrated DNA Technologies)²
- MNV reverse primer: 5'-GCG CTG CGC CAT CAC TC-3' (Integrated DNA Technologies)²
- MNV RT-qPCR standard: 5'- tagaagtgtgtctccc acgccaccgatctgttctgcg ctgggtgcgctttggaacaatgga tgctgagaccccgaggaacgctcagcagctttgtggaatgaggatg agtgtatggcgcagcgccaaaagccaatggct -3' (Integrated DNA Technologies)³
- MNV probe: 6-FAM-MGB 5'-CGC TTT GGA ACA ATG-3' (Thermo Fisher Scientific)³
- qPCR kit (iTaq Universal Probes One-Step Kit Catalog number: 1725141)

Equipment

1. Biosafety hood in a BSL-2 laboratory
2. Incubator (37°C, 5% CO₂, humidified)
3. Pipet set (P10, P100, P1000)
4. Pipetboy (Integra Biosciences, catalog number: 155016)
5. Multipette® M4 (Eppendorf, catalog number: 4982000012)
6. Multichannel pipette (Eppendorf, catalog number: 3122000035)
7. –80°C freezer
8. PCR Workstation
9. Vortex
10. Centrifuge with a rotor suitable for 1.5 mL tubes
11. Centrifuge with a rotor suitable for 50 mL tubes
12. Inverted light microscope
13. Cell counter (Logos Biosystems Luna-II automated Cell counter, catalog number L400002)
14. Aspirator (Integra BioSciences Vacusafe catalog number: 158320)
15. Plate reader (SPARK® Multimode Microplate Reader)

16. Stirring plate
17. pH meter

Procedures

Note: Culture cells under a biosafety hood.

Note: Infect cells under a biosafety hood in a BSL-2 laboratory.

Time-of-drug-addition assay

When a compound results in a protective effect a time-of-drug-addition experiment can be performed to estimate the time-point when the compounds act on the viral replication cycle of MNV.

! The infection is performed at 4°C, this allows viral attachment but not entry. The latter will make sure that once the temperature is brought up to 37°C the entry step will occur at the same time in every cell, yielding a more accurate virus growth curve. Infection is also performed in suspension as to facilitate this synchronization of virus infection. In order to achieve infection of all cells at once, the MOI should be above 1.

Date	Hour	Steps
Day 1	19:45	<ul style="list-style-type: none"> • Prepare 6 mL virus dilution 1:50 in cold 2% medium. • Count the RAW 264.7 cells/mL and prepare 6 mL of cell suspension with 4,00,000 cells/mL in 2% culture media. • Infect cells in suspension by mixing the diluted virus (MOI>1) and cells in a falcon. Pipet up and down at least three times. • Incubate for 30 minutes at 4°C. • Vortex falcon with cells and virus gently.
	21:00	<ul style="list-style-type: none"> • Incubate for another 30 minutes at 4°C. • Centrifuge for 5 min at 1500 rpm and discard the supernatants. • Wash the cells with cold 2% medium. • Centrifuge for 5 min at 1500 rpm and discard the supernatants. • Add 12 mL of cold 2% medium and resuspend cells. • Add 1 mL of cell suspension to seven 5 mL falcon tubes. Label these as follows 12h, 14h, 16h, 18h, 20h, 22h and 24h. • Incubate at 37°C.
Day 2	07:45	<ul style="list-style-type: none"> • Perform the same steps as described in day 1 section 1.
	09:00	<ul style="list-style-type: none"> • Prepare compound. • Perform the same steps as described in day 1 section 2. But label the 5 mL falcon tubes as 0h, 2h, 4h, 6h, 8h, 10h, 12h.
	11:15	<ul style="list-style-type: none"> • Add compound to the cells in the falcon of the hours 0,12
	13:15	<ul style="list-style-type: none"> • Add compound to hours 2, 14
	15:15	<ul style="list-style-type: none"> • Add compound to hours 4, 16
	17:15	<ul style="list-style-type: none"> • Add compound to hours 6, 18
	19:15	<ul style="list-style-type: none"> • Add compound to hours 8, 20
21:15	<ul style="list-style-type: none"> • Add compound to hours 10, 22 • Add compound to hours 12, 24 • Vortex and transfer the full content of time points (12 to 24) (= infection has been ongoing for 24 h) to a clean Eppendorf tube. Centrifuge for 5 min at 1500 rpm. Transfer supernatant to an extra labelled Eppendorf (hence leaving the pelleted cells in the first Eppendorf). Store the series of tubes at –80°C 	
Day 3	09:15	<ul style="list-style-type: none"> • Proceed as described in previous point, but for time points (0–12)

To determine the virus growth curve of the same procedure can be used, however instead of adding the compounds at 2-hour intervals, harvest the supernatants and cells as described at 2-hour intervals and freeze at -80°C .

Resistance selection

When a compound results in a protective effect a resistance selection assay can be performed to investigate how fast drug-resistant virus variants occur. Moreover, sequencing the drug resistant variants and identifying the location of the acquired mutations in the virus genome will provide information on which viral proteins are targeted by the compounds.

! Always include a non-treated virus control, which is passaged in parallel to determine which mutations arise naturally in cell culture.

1. Start with an antiviral assay as described in PART 1,¹ but add the same compound to rows B to G. Use one plate per compound for which you aim to select for resistant variants. In each plate, 3 tentatively resistant variants will be passaged independently (rows B and C = resistant variant 1; rows D and E = resistant variant 2; rows F and G = resistant variant 3). See Figure 2.
2. After 72 h, score the CPE microscopically and collect, in each row, 100 μL of the supernatant (containing the virus) of the well where the highest concentration of added compound still allowed the viral CPE to completely destroy the cell monolayer. Pool supernatants from rows B and C together (as well as D+E and F+G) into a cryotube containing 800 μL of 2% media, as to achieve a 1:5 dilution and store at -80°C .
3. Prepare a new antiviral assay as described in PART 1,¹ with the same compounds. To create the next assay round, the virus harvested from the previous passage is used.

- a. Dilute the collected virus in a 1:25 ratio in 2% media. Prepare enough to infect the treated cells, 1 mL for 2 rows. Store the remaining virus of the previous passage at -80°C .
 - b. Add 50 μL of the diluted virus into the according wells, meaning that virus collected from rows B and C are again used to infected cells treated with the same compound in rows B and C. Do not add virus to the cell control column
4. Prepare a RAW 264.7 cell suspension of 10,000 cells/50 μL in 2% culture media, as described in PART 1.¹
 5. Add 50 μL of the cell suspension to the inner 60 wells of the 96-well plate.
 6. Incubate the 96-well plate for 72 to 96 h (i.e. until CPE stops progressing) in a CO_2 incubator at 5% CO_2 and 37°C .
 7. Repeat steps 3–6 until a resistant virus variant is selected or after sufficient passages (~ 30 passages would be suggested). A virus variant is considered resistant if the EC_{50} value is 10-times higher than at the start. Meaning the resistant virus is 10 times less sensitive to the compound.
 8. Confirm resistance by re-growing the virus (in presence of the highest concentration of compound the mutant was still giving full CPE) in a 6-well plate (160,000 cells/well). If successful, semi-purify by titrating the virus in a 96-well plate in the presence and absence of compound. In a successful assay, the virus should be able to grow to an equal extent in both conditions. In rows B, C and D add the dilution series of the compound and in rows E, F and G do not add the compound to determine the replication efficacy of the mutant virus. After 3 days, select the highest dilution of virus that still gives full CPE. Use this virus to grow a stock using the same concentration of compound selected above in a T-25 flask as described in PART 1.¹

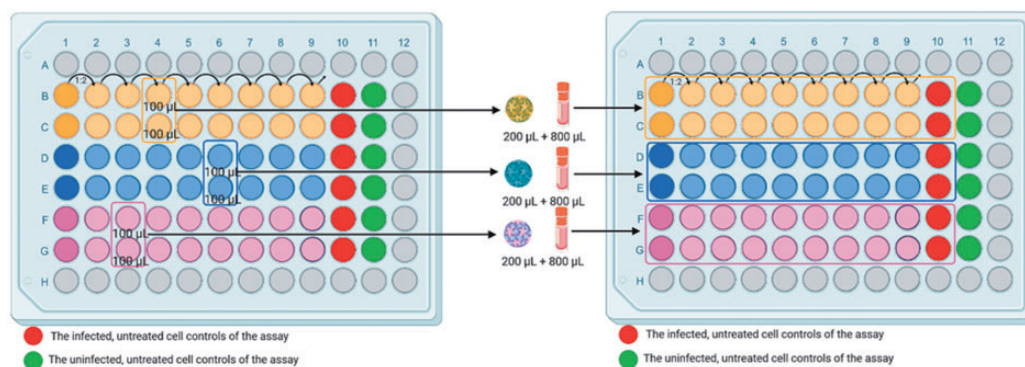


Figure 2. Layout of the resistance selection assay as a dilution series of selected compounds on RAW 264.7 cells (1×10^4 cells/well). Created with BioRender.com.

9. Confirm resistance by performing an antiviral assay as described in PART 1,¹ using in one plate the resistant MNV variant and a second plate the wild type virus at the same MOI. Add a serial dilution of the compound against the resistance has been acquired and of a reference compound in both plates.
10. If the resistance is confirmed with a clear shift in EC₅₀, sequence the resistant virus to determine acquired mutations by sanger sequencing by using primers selected for the genome region of interest (if molecular target is known) or perform a full-length virus genome sequencing.

Expected results

To have an estimate of the moment in which the compound interferes with the replication cycle of norovirus a time-of-drug-addition assay can be performed. In the MNV replication cycle, the levels of extracellular viral RNA of untreated infected cultures is detectable (above background) as of 18 h pi, the onset of intracellular RNA synthesis can be detected at 6 h pi.^{3,4} For 2CMC and T-705 the compounds blocked the formation of both extracellular and intracellular viral RNA synthesis when added during the first 6 h pi.^{3,4} When 2CMC and T-705 was first added at a time-point later than 6 h pi, a gradual increase of both extra- and intracellular viral RNA was observed, reflecting the loss of the antiviral effect.^{3,4} A direct comparison to these molecules in a TOA experiment may provide an extra clue on whether the targeted step by novel inhibitors could be the same.

The selection of resistant variants can be fast (occurring after a handful of passages), slow (requiring multiple months of continuous passaging) or ultimately unsuccessful. Assessment of a significant shift in EC₅₀ can be done with a Mann-Whitney U test. Picornavirus capsid binders are the quintessential example of rapid selection of resistance thus compounds targeting entry-related steps are often regarded as having a low barrier to resistance. In contrast, compounds targeting the viral polymerase are expected to have a high barrier to resistance, even more so if these target the active site of the enzyme, thus selecting for resistant variants for this target is expected to be challenging.⁵ Ultimately, it will depend from whether a single or multiple mutations are needed to confer resistance to the drug and whether these mutations will impact the viral fitness. If these result in a non-viable virus, the process may fail. An important follow-up step to validate the importance of these mutations to the viral fitness is to use reverse genetics systems, as they are one of the most important and powerful tools to study the molecular

biology of viruses.^{6,7} Of note is that compounds targeting host cell proteins are also likely quite challenging to select for resistance. Eventually, other approaches are likely required to characterize the effect of antivirals targeting host cell factors.

Conclusion

In parallel with the assessment of the antiviral effect of novel compounds against other noroviruses, it is highly relevant to understand how the molecules exerts its antiviral effect. We here proposed two assays that can be broadly used to gather a first round of information about their mode of action. After these first steps, a more specific approach will be required to further characterize this. For those compounds targeting a protein endowed by enzymatic activity, a biochemical assay can be performed. These are available for norovirus proteases and polymerases of various genogroups.^{8,9} For other viral proteins, alternative approaches will be necessary.



Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iDs

Jana Van Dycke  <https://orcid.org/0000-0003-1632-3499>
Jasper Rymenants  <https://orcid.org/0000-0003-3838-310X>

References

1. Dycke et al. Assessment of the antinorovirus activity in cell culture using the mouse norovirus: identification of active compounds. *Antivir Chem Chemother* (In press). DOI: 10.1177/20402066211026852.
2. Baert L, Wobus CE, Van Coillie E, et al. Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Appl Environ Microbiol* 2008; 74: 543–546.
3. Rocha-Pereira J, Jochmans D, Dallmeier K, et al. Favipiravir (T-705) inhibits in vitro norovirus replication. *Biochem Biophys Res Commun* 2012; 424: 777–780.
4. Rocha-Pereira J, Jochmans D, Dallmeier K, et al. Inhibition of norovirus replication by the nucleoside analogue 2'-C-methylcytidine. *Biochem Biophys Res Commun* 2012; 427: 796–800.
5. Deval J, Jin Z, Chuang YC, et al. Structure(s), function(s), and inhibition of the RNA-dependent RNA polymerase of noroviruses. *Virus Res* 2017; 234: 21–33.

6. Sandoval-Jaime C, Green KY and Sosnovtsev SV. Recovery of murine norovirus and feline calicivirus from plasmids encoding EMCV IRES in stable cell lines expressing T7 polymerase. *J Virol Methods* 2015; 217: 1–7.
7. Kitano M, Hosmillo M, Emmott E, et al. Selection and characterization of Rupintrivir-resistant Norwalk virus replicon cells in vitro. *Antimicrob Agents Chemother* 2018; 62: 3–14.
8. Emmott E, Sweeney TR and Goodfellow I. A cell-based fluorescence resonance energy transfer (FRET) sensor reveals inter- and intragenogroup variations in norovirus protease activity and polyprotein cleavage. *J Biol Chem* 2015; 290: 27841–27853.
9. Croci R, Pezzullo M, Tarantino D, et al. Structural bases of norovirus RNA dependent RNA polymerase inhibition by novel suramin-related compounds. *PloS One* 2014; 9: e91765.