An Emerging Application of Cholesterol-lowering Therapy in Inhibition of Rotavirus Infection

Shihao Ding (tingtingv25@gmail.com)
Erasmus Medical Centre: Erasmus MC
https://orcid.org/0000-0002-0490-3608

Bingting Yu
Erasmus Medical Centre: Erasmus MC

Anneke van Vuuren
Erasmus Medical Centre: Erasmus MC

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Abstract

Despite the huge impact of rotavirus infection on global public health, there is no normally available drug against the virus worldwide. We have revealed the interaction of cholesterol metabolism and rotavirus replication, as well as identified statin as a promising drug to repress rotavirus infection, but the medical resources are greatly different across countries, so more drugs are needed for anti-rotavirus treatment in clinical activity. Two cell lines and a human small intestinal organoids were used as the models, which were infected by rotavirus SA11 strain. A clinically derived rotavirus virion, 026K strain, was measured intracellular virus RNA copies in Caco2 cells. We investigated the effects of different cholesterol-lowering drugs, including bisphosphonates (zoledronic acid, ZA), fibrate class (fenofibric acid, FA), vitamin B3 (nicotinic acid, NA), and ezetimibe on rotavirus replication in the pre-clinical models. All these cholesterol-lowering drugs resulted in significant decreases of rotavirus replication. The combinations of FA / ezetimibe with the statins had not the obvious synergies in the inhibition of rotavirus replication than any of them alone. Compared to the other drugs, ezetimibe showed the additional preventive and interference effects towards rotavirus infection. We describe an emerging application of clinical cholesterol-lowering therapy for anti-rotavirus treatment. These results could be directly considered when physicians treat with rotavirus-caused diseases worldwide.

Introduction

Rotavirus is the major cause of severe gastrointestinal diarrhea among infants and young children, even causes brain immaturity and cardiac muscle damage [1],[2]. Although rotavirus infection is self-limiting, given the treatment of combating the virus-induced diseases is conservative, it is always a heavy burden to the public healthcare worldwide. Annually, the estimated 2.3 million hospitalization cases and more than 200,000 deaths are caused by the virus infection globally [3],[4]. Besides children, adults can still be infected by the unusual rotavirus strains or high doses common rotavirus strains to cause severe symptoms [5]. The vaccination is a preventive strategy against rotavirus infection, but the vaccines may induce an increase in intussusception [6], which is a lethally intestinal emergency to infants. Moreover, because most cases of rotavirus-induced diseases occur in developing countries where basic medical facilities are imperfect and the vaccination is not popular, the improvement of the therapeutic strategy is urgent. Currently, the vaccine hesitancy is getting more all over the world that also raises the urgency of medical treatment.

The replications of multiple viruses have been proved to be associated with intracellular cholesterol [7],[8]. Before, our study has exposed that rotavirus replication is related to cholesterol metabolism, and identified statin might be a potential medical option for treating rotavirus infection. Specifically, inhibition of cholesterol biosynthesis significantly repressed rotavirus replication; in contrast, enhancement of cholesterol production provoked the viral infection. The combinations of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) inhibition, which is the rate-limiting enzyme of cholesterol synthesis, with cholesterol treatment markedly stimulated rotavirus replication. However, the major goal of the anti-rotavirus drug development is not only to discover a single but also to provide variously clinical drugs that should be
available in different countries or regions against unforeseen outbreaks of rotavirus. Herein, following the concept that blocking cholesterol synthesis suppresses rotavirus infection, we explore whether other clinical cholesterol-lowering drugs except statin also exert the anti-rotavirus effects. In this study, four first-line cholesterol-lowering drugs: zoledronic acid (ZA), nicotinic acid (NA), fenofibrate/fenofibric acid (FA) and ezetimibe were used for verifying their anti-rotavirus functions, because these drugs not only were available and cheap in most countries but also had excellent safeties. Given the anti-rotavirus effects of the medicines could be more directly explained in vitro and processing clinical trial were not available due to the deficiency of the basic investigation, we tested the potential effects of the drugs towards rotavirus in two rotavirus infected cell models and a primary human small intestinal (HSI) organoid model. All these drugs indicated the anti-rotavirus functions on the pre-clinical models. Unexpectedly, unlike the other drugs, ezetimibe showed the preventive and interference effects on rotavirus infection. The combinations of FA / ezetimibe with the statins did not show the synergy on the anti-rotavirus effect compared with their single-use. By the pharmacological mechanism of FA / fenofibrate and ezetimibe, it implied that the cellular cholesterol pool, but not cholesterol biosynthesis, plays a role in the anti-rotavirus infection function. Therefore, we concluded that the clinical application spectrum of the existing cholesterol-lowering drugs could be expanded to treat rotavirus infected, and the results would provide an important reference for the therapy of rotavirus infection by clinicians.

Materials And Methods
Reagents, antibodies and plasmids
ZA, FA, NA and ezetimibe were purchased from Sigma-Aldrich (St Louis, MO, USA). All the reagents were dissolved in 100% dimethyl sulfoxide (DMSO). The mouse monoclonal antibody against rotavirus VP4 protein was a gift from Prof. Harry Greenberg (Medicine-Gastroenterology & Hepatology, Stanford University School of Medicine, USA). The mouse antibody against the rotavirus VP6 protein was purchased from Abcam (Cambridge, MA, USA). The mouse antibody against β-actin was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The secondary antibodies were obtained from Dako (for immunoblotting; Amstelveen, Netherlands) and Invitrogen (immunofluorescence; CA, USA).

Cells, human intestinal organoids and rotavirus strains
Caco2 cells (human colon cancer cell line) and MA104 cells (African green monkey fetal kidney cell line) were originally purchased from ATCC. Caco2 and MA104 cells were cultured in Dulbecco’s modified eagle medium (DMEM; Lonza; Verviers, Belgium) supplemented with 20% and 10% (v/v) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich; St. Louis, MO, USA) respectively and 100 U/ml Penicillin / Streptomycin (P/S; Gibco; Grand Island, NY, USA) solution. Both of the cells were incubated at 37°C with 5% CO2 and confirmed to be mycoplasma negative.

Intestinal tissues were surgically resected and transferred into a 15 ml falcon tube including 10 mL complete chelating solution (CCS; MilliQ H2O was supplemented with 1.0 g/L Na2HPO4·2H2O, 5.6 g/L
NaCl, 1.08 g/L KH$_2$PO$_4$, 15 g/L Sucrose, 0.12 g/L KCl, 10 g/L D-Sorbitol and 80 µg/L DL-dithiothreitol). The tissue was washed 3 times by phosphate-buffered saline (PBS; Lonza; Verviers, Belgium) and rocked with 8 mM EDTA for 15 min at 4°C. This study was approved by the ethics committee of Erasmus University Medical Center in Rotterdam. All patients gave written informed consent.

Simian rotavirus SA11 strain was a gift from Karen Knipping in Nutricia Research Utrecht, the Netherland. Rotavirus SA11 strain was used and prepared as described previously [9]. A patient-derived rotavirus isolate, 026K strain, was isolated from the stool sample from rotavirus infected patient during their diarrhea period, that was stored in Erasmus MC biobank, and examined for parechovirus, enterovirus, norovirus genogroups I and II, astrovirus, adenovirus and sapovirus by qRT-PCR.

**Rotavirus inoculation and drug treatment**

Caco2 or MA104 cells in T75 flask were washed and suspended, subsequently seeded into a 48-well plate (5 x 10$^4$ cells / well). When the confluence of the cells in each well was up to 80%, the culture medium was removed and the monolayer cells were washed 3 times by PBS. 100 µL of FCS-free DMEM medium supplemented with 5 µg/mL of trypsin (Gibco; Paisley, UK) and rotavirus SA11 or 026K strain was added and incubated at 37°C with 5% CO$_2$ for 90 min, followed by washing for 3 times with PBS. Subsequently, the cells were added with the FCS-free DMEM medium containing the corresponding concentrations of specific drugs.

The inoculation and drug treatment process of HSI organoids by rotavirus SA11 was the same as the process of the cells.

**RNA extraction, cDNA synthesis and qRT-PCR analysis**

RNA extraction, cDNA synthesis and qRT-PCR analysis of the intra- and extracellular rotavirus RNA were operated as described before [10]. All the used primers were purchased from Sigma-Aldrich and described in supplementary table 1.

**Virus RNA copies assay by long-term**

qRT-PCR was used for measuring the intracellular rotavirus RNA copies of Caco2 cells by long-term. After rotavirus SA11 infection, Caco2 cells were collected immediately, followed by RNA isolation and the measurement of the rotavirus RNA genome by qRT-PCR. During the next 96 hours, every 24 hours the intracellular rotavirus RNA copies were assayed as before and compared to the original expression of the control or the drug treatment respectively.

**Western blot**

Western blot process was described as described before [10]. Proteins were detected and quantified by LI-COR Odyssey infrared scanner (LI-COR Bioscience; Lincoln, NE, USA). The scanned data was analyzed using Odyssey version 3.0 software (LI-COR Bioscience; Lincoln, NE, USA).

**Immunofluorescence staining**
The immunofluorescence (IF) staining was performed as described before [11]. Imaging of Caco2 and MA104 cells was performed by an Olympus IX70 fluorescence microscope.

IF staining of HSI organoids was similar to the staining process for the cells. Imaging was performed by Leica TCS LSI confocal microscope.

**MTT assay and IC$_{50}$ assay**

50% inhibition concentration (IC$_{50}$) represents the concentration at which a compound exerts half of its maximal inhibitory effect. In this study, it was used to refer to the potential of a drug to cause a 50% decrease in certain cell survival. Briefly, the viabilities of Caco2 or MA104 cells were determined by 3-(4, 5 Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The cells were seeded 1 x 10$^4$ cells one well of a 96-well plate and the specific concentration of the drug was added to the cells. The viable cells were detected at 48 hours post the compound exposure through adding 10 µL of 5 mg/mL MTT each well, followed by incubation at 37°C for 3 hours and replaced the medium by 100 µL dimethyl sulfoxide (DMSO) and incubated for 30 min. MTT assay was performed by absorbance analysis (490 nm) on multi-well plate reader series 4000 reader (CYTOFLUOR).

Based on the results of an experimental set that had 3 independent MTT assay, the IC$_{50}$ value was calculated. A four parameters logistic regression model was used for the calculation: $Y = \text{Min} + \frac{(\text{Max} - \text{Min})}{1 + \left(\frac{X}{\text{C50}}\right)^{\text{Hill coefficient}}}$ by using online IC$_{50}$ calculator of AAT Bioquest at https://www.aatbio.com/tools/ic50-calculator.

**TCID$_{50}$ assay**

The original supernatant was serially diluted 11 times from 10$^{-1}$ to 10$^{-11}$ to measure 50% tissue culture infectious doses (TCID$_{50}$). Briefly, each dilution was inoculated into the monolayer confluent MA104 cells in each well of a 96-well plate with 8 repeated wells for 1 hour. After the incubation, the monolayer cells were washed by PBS, and cultured with DMEM for 72 hours. The Log$_{10}$ TCID$_{50}$ was calculated using the Reed-Muench method as described previously [12].

**Statistical analysis**

Statistical analysis was performed by the non-paired, non-parametric test (Mann-Whitney test; GraphPad Software; San Diego, CA, USA). Statistical significance was defined as *P < .05, **P < .005, ***P < .001.

**Results**

**Bisphosphonate repressed rotavirus replication**

ZA is a representative member of nitrogen-containing bisphosphonates (N-BPs) which are used widely to treat a variety of bone diseases, including osteoporosis and the high blood calcium which is secondary to bone tumors [13]. As an inhibitor of the mevalonate pathway / cholesterol synthesis pathway, ZA
specifically targets farnesyl diphosphate synthase (FDPS) to block the production of cholesterol [14]. By the rotavirus infected Caco2 model, we first tested the effect of ZA on the intra- and extracellular rotavirus RNA copies by qRT-PCR. ZA dose-dependently inhibited both rotavirus RNA replications (Fig. 1A). To exclude that the anti-rotavirus effect of ZA was due to the decline in the proliferation of the cells, the viabilities of Caco2 cells by ZA treatment was assayed using IC\textsubscript{50}. The result demonstrated the anti-rotavirus effect of ZA without triggering significant cytotoxicity (supplementary Fig. 1A). Further, compared to the non-treatment, 50 µM ZA treatment reduced the expressions of rotavirus VP4 and VP6 proteins by western blot analysis and immunofluorescence (IF) imaging respectively (Fig. 1B, C; supplementary Fig. 1C). To avoid potential issues relating to the specificity of the cell line, we proceeded to investigate the effect of ZA in MA104 cells which were previously reported to support favorably the replication of rotavirus SA11 [15]. Consistently, by ZA treatment, the notable and dose-dependent decreases of the intra- and extracellular rotavirus RNA copies were observed, and those dropped to 19% and 22% respectively at 50 µM ZA treatment (Fig. 1D). Also, the concentrations of ZA exhibited no significant cytotoxicity in MA104 cells (supplementary Fig. 1B). The inhibitory effect was also confirmed by western blot analysis and IF staining, indicating that ZA repressed VP4 and VP6 proteins of rotavirus (Fig. 1E, F; supplementary Fig. 1D). To better characterize the anti-rotavirus effect of ZA under physiological condition, we performed the confirmatory experiments in the HSI organoids which included many characteristics of the intestinal epithelium. The inhibitory effect on the inner rotavirus RNA copies which fell to 31% by 50 µM ZA treatment was observed (Fig. 1G). That was further verified on VP4 and VP6 proteins (Fig. 1H, I). To track dynamically the intracellular rotavirus RNA level by ZA treatment, we undertook 96 hours time course experiment in Caco2 cells. As Fig. 1J shown, ZA markedly suppressed the intracellular virus RNA level since the first 24 hours, and its anti-rotavirus effect was time-dependent. At the 96 hour, 50 µM ZA eventually minimized the rotavirus RNA level to 16%. Next, TCID\textsubscript{50} assay was performed to measure the titer of rotavirus infectious particles in the supernatant by 50 µM ZA treatment, and an obvious drop of the titer was observed (Fig. 1K). Collectively, our results demonstrated that ZA potently inhibited rotavirus infection.

To further investigate whether ZA had a preventive effect on rotavirus, the effect of ZA pre-treatment was tested in Caco2 cells. After ZA treatment for 2 hours before rotavirus SA11 infection, we found that no obvious effect on the intracellular virus RNA copies and VP4 protein was observed (supplementary Fig. 1E, F). During the rotavirus infection process, treating with ZA also had no influence (supplementary Fig. 1G, H). The data suggested that ZA had not the preventive and interference effects on rotavirus infection.

**NA represses rotavirus replication**

NA, also known as Niacin, is a form of vitamin B3. NA is effective in lowering low-density lipoprotein (LDL) cholesterol and raising high-density lipoprotein (HDL) cholesterol, which makes this agent of unique value in dyslipidemia therapy [16]. As shown in Fig. 2A, compared to the extracellular result, the intracellular result by NA treatment demonstrated a clearer inhibitory effect on rotavirus RNA replication in Caco2 cells. To be specific, 100 µM NA reduced the intracellular rotavirus RNA level to 72% and that of
the extracellular to 55% respectively. Western blot assay and IF staining also showed the inhibitory function of NA on the viral protein level (Fig. 2B, C; supplementary Fig. 2C). As expected, in MA104 cells, the anti-rotavirus effects of NA were also confirmed (Fig. 2D, E, F; supplementary Fig. 2D). The measurements on NA cytotoxicity showed the used concentrations did not induce significant cell death in both Caco2 and MA104 cells (supplementary Fig. 2A, B). On the HSI organoid model, compared with the significant inhibition on the inside rotavirus RNA level and VP6 protein (Fig. 2G, I), rotavirus VP4 protein seemed insensitive to NA treatment (Fig. 2H). We further undertook the time course experiment, and NA gained a persistently significant reduction on the inner virus RNA level (Fig. 2J). Following TCID\textsubscript{50} measurement, a comparably low titer of rotavirus in the supernatant was observed with 100 µM NA treatment (Fig. 2K). Consistent with the results of ZA, NA also had the anti-rotavirus effect.

NA did not show the prevention and interference to rotavirus infection (supplementary Fig. 2E, F; supplementary Fig. 2G, H).

**Fibrates suppresses rotavirus replication**

Fibrates (Fibric acid derivatives) is a class of medication that can lower the level of triglyceride and increase the level of HDL cholesterol. As a characteristic member of fibrates, fenofibrate is recommended as the first-line drug therapy to patients with dyslipidemia. In vitro, to achieve the effect of first-pass hepatic metabolism, we chose FA to represent fenofibrate in our study [17]. As shown in Fig. 3A, 50 µM FA lowered the intra- and extracellular rotavirus RNA copies to 19% and 22% respectively. The anti-rotavirus effect of FA was further confirmed by the lessened expressions of rotavirus VP4 and VP6 proteins (Fig. 3B, C; supplementary Fig. 3C). Similar results were obtained in MA104 cells (Fig. 3D, E, F; supplementary Fig. 3D). Specifically, 50 µM FA dropped the intra- and extracellular rotavirus RNA levels to 41% and 55% respectively. The declining degree of rotavirus RNA replications by FA treatment was more significant in Caco2 cells than that in MA104 cells. In parallel, the viabilities of Caco2 and MA104 cells were not markedly affected by the concentrations of FA treatment (supplementary Fig. 3A and B). On HSI organoid model, the anti-rotavirus effect of FA was similar to the results of the cellular models (Fig. 3G, H, I). Besides, the anti-rotavirus effect of FA was proved to be long-term (Fig. 3J). On average $5.1 \pm 0.2$ and $4.3 \pm 0.2 \log_{10}$ progeny infectious particles were produced from non-treated and FA-treated samples respectively, which corresponded to a 15.7% reduction of rotavirus titer by FA treatment (Fig. 3K), revealing that FA had antivirus effect on rotavirus progeny infectious particles. All the data indicated that the anti-rotavirus effect of FA was obvious.

Analogously, FA did not show the preventive (supplementary Fig. 3E, F) and the interference effects (supplementary Fig. 3G, H) on rotavirus infection.

**Ezetimibe inhibits rotavirus replication**

Ezetimibe inhibits the cholesterol absorption which depends on Niemann-Pick C1-like 1 (NPC1L1) at the brush border of the intestine [18]. The dose-dependently inhibitory effects of ezetimibe were obtained on both intra- and extracellular rotavirus RNA copies (Fig. 4A). The rotavirus RNA levels decreased to 68%
intracellularly and 32% extracellularly respectively at 50 µM ezetimibe treatment. Rotavirus VP4 and VP6 proteins were indicated to be visibly suppressed (Fig. 4B, C; supplementary Fig. 4C). In MA104 cells, the inhibitory effect of ezetimibe was similar to the results of Caco2 cells (Fig. 4D, E, F; supplementary Fig. 4D). In both cell lines, the dose-dependent cytotoxic effects were observed with an IC$_{50}$ value of 219 µM in Caco2 cells and 238 µM in MA104 cells respectively, demonstrating that the tested concentrations of ezetimibe had no apparent cytotoxicity to these cells (supplementary Fig. 4A, B). Next, in the HSI organoids, the inner rotavirus RNA level declined significantly to 24% compared to the non-treated control (Fig. 4G). The similarly decreases of rotavirus VP4 and VP6 were also observed (Fig. 4H, I). As Fig. 4J shown, ezetimibe durably suppressed the virus RNA replication during the whole course. Eventually, 50 µM ezetimibe minimized the intracellular virus RNA level to 57% at the end of the 96-hour course. Moreover, in the presence of ezetimibe, 4.7 ± 0.1 Log$_{10}$ infectious particles were produced compared to the non-treatment control 5.3 ± 0.2 Log$_{10}$, illustrating that ezetimibe had an evident adverse effect on the infectious rotavirus particles (Fig. 4K). Altogether, ezetimibe exerted a significant antiviral effect on rotavirus in all the pre-clinical models.

Unlike the other drugs, we observed that ezetimibe pre-treatment indicated a significant prevention effect to rotavirus infection (supplementary Fig. 4E, F). By ezetimibe co-treatment, an obvious interference effect was also observed (supplementary Fig. 4G, H). It prompted that ezetimibe might be a potential clinical option for preventing rotavirus infection.

**Combinations of FA / ezetimibe with statin do not exhibit significant synergistic anti-rotavirus effects**

Statin, fenofibrate and ezetimibe are all cholesterol-lowering drugs with distinct pharmacological mechanisms. In clinical practice, the combinations of statin with fenofibrate or ezetimibe respectively could benefit patients who have not achieved expected goals on hyperlipidemia-lowering management by monotherapy [19],[20]. By the mechanism of intracellular cholesterol and rotavirus interaction, we further investigated the anti-rotavirus effects of combining two statins, lovastatin and simvastatin, with FA or ezetimibe respectively. As shown in Fig. 5A, B; supplementary Fig. 5C, when 50 µM lovastatin or simvastatin combined with 50 µM FA respectively in Caco2 cells, the combinations did not demonstrate the general beneficial effects. In parallel, no obvious increased cytotoxicity was observed (supplementary Fig. 5A). The similar results of FA and the statins combinations were also obtained on HSI model (Fig. 5E, F).

We next examined the potential effect of the two statins with ezetimibe combinations towards rotavirus. The results revealed no significant synergistic anti-rotavirus effects by these combinations in Caco2 and HSI models (Fig. 5C, D; supplementary Fig. 5D; Fig. 5G, H). MTT analysis showed no significant difference between the combinations of the drugs and their monotherapy in Caco2 cells (supplementary Fig. 5B).
All these drugs repress the replication of a clinical rotavirus isolate

To avoid these results were rotavirus strain-dependent specificity and close to clinical practice, we decided to test the anti-rotavirus effects of all the drugs by a clinical rotavirus isolate, rotavirus 026K strain. Because the secreted rotavirus 026K strain RNA copies in the supernatant were too low to be tested by qRT-PCR, only the intracellular virus RNA copies were measured. Compared to the non-treated control, there was a notable decrease to 21% by ZA treatment on the intracellular virus RNA level (Fig. 6A). As the data from Fig. 6B, C and D, the treatments of NA, FA and ezetimibe also showed the declines on the intracellular virus RNA level respectively, but the reductions were not significant. However, by the statins and FA / ezetimibe combinations, there was no significant difference between the combinations and the monotherapy of the drugs on the rotavirus 026K RNA copies (Fig. 6E, F). Generally, these results indicated that the anti-rotavirus profiles of the drugs were not specific on rotavirus strain, but their inhibitory effects might be different among distinct rotavirus strains.

Discussion

Currently, oral or intravenous rehydration constitutes the main treatment for rotavirus-induced diarrhea [21], but the therapy is conservative and passive. Even though several drugs have been reported to treat rotavirus infection in recent years, there are the uncertain issues on their use. Some meta-analysis suggested that probiotics might reduce the duration of rotavirus-caused diarrhea [22], however, another study did not demonstrate a certain benefit from its use [23]. Nitazoxanide as a broad-spectrum antiviral drug has been investigated its anti-rotavirus function in the pre-clinical stage [24], and reported to significantly reduce the median time of rotavirus-associated intestinal symptoms [25]. However, the conclusion was from a small-scale clinical trial which was a limited reference. Therefore, the new and effective therapeutic approach is expected for rotavirus infection. The previous study conducted by our team has reported that block of intracellular cholesterol biosynthesis could inhibit rotavirus replication. Even if statin has been identified as a promising drug to combat rotavirus infection in our study, given the availability of medical resources has a huge difference across countries, more drugs should be identified to provide diverse choices against rotavirus in clinical activity. Following the disclosed interaction of cholesterol metabolism and rotavirus replication, we tested the potential effects of a variety of clinical cholesterol-lowering drugs towards rotavirus, and we demonstrated that ZA (cholesterol biosynthesis inhibitor), FA (fibrate class drug), NA (vitamin B3) and ezetimibe (cholesterol absorption inhibitor) significantly inhibited rotavirus infection in vitro. However, the combinations of FA / ezetimibe and the statins did not show synergy. The tested concentrations of all these drugs covered their clinical dosages, thus we proposed that the results should be a valuable reference regarding the choice of the antiviral therapy for rotavirus infected patients.

Besides the traditional efficacy against bone disorders, growing reports indicate the antivirus effect of ZA on multiple viruses. ZA was showed to have anti-viral activity against H5N1 and H1N1 viruses [26]. ZA
also could repress West Nile virus (WNV) replication by activating Vδ2 T-cells to release soluble factors [27]. In the present research, we observed the inhibitory effect of ZA towards rotavirus, and the effect was dose- and time-dependent. The anti-rotavirus effect of ZA was consistent with its pharmacological characteristics on managing cholesterol, suggesting that ZA might inhibit rotavirus infection through blocking cholesterol synthesis as statin.

NA is used as a lipid-lowering drug at higher concentrations, and it can inhibit diacylglycerol acyltransferase-2 (DGAT2) to reduces triglyceride synthesis and the level of low-density lipoprotein (LDL) cholesterol, but to increase the level of high-density lipoprotein (HDL) cholesterol by decreasing its hepatic catabolism [28]. Besides, NA also markedly decreases total cholesterol in mice [29]. The common side effects of NA include temporary flushing, pruritus and skin rashes [30]. In terms of antiviral effect, NA has been reported to inhibit HIV infection at a post-integration step of the viral life cycle [31]. When testing an analogue of NA, 6-amino-nicotinamide (6-AN), significant antiviral effect towards hepatitis B virus (HBV) through inhibition of cccDNA transcription to achieve the anti-HBV effect were seen [32]. These are consistent with our finding that NA also potently suppressed rotavirus replication. Considering the excellent safety of NA, we propose that NA might be a valuable choice against rotavirus infection in primary health care.

Fenofibrate acts by stimulating peroxisome proliferator activated receptor-α (PPARα) that is a member of nuclear receptors PPARs family which can regulate the expressions of fatty acid and cholesterol metabolism genes [33]. Although fenofibrate is used chiefly to lower triglyceride level in clinical practice [34], it also inhibits the absorption of cholesterol in the intestine through PPARα-dependent modulation of NPC1L1 expression [35],[36], that is similar to ezetimibe. Fenofibrate not only plays a role in lowering triglyceride and cholesterol but also shows the antivirus ability. For instance, fenofibrate pretreatment significantly decreased the titer of Japanese encephalitis virus (JEV) in BV-2 cells [37]. Besides, fenofibrate also repressed severe fever with thrombocytopenia syndrome virus (SFTSV) infection [38]. Pharmacologically, fenofibrate is a pro-drug which needs to be hydrolysed by the esterases in tissue and plasma to the active metabolite, FA, for performing its hypolipidemic function [17]. In our study, we used directly FA representing fenofibrate and observed a significant decrease in rotavirus replication with FA treatment. It is still uncertain whether FA / fenofibrate exerted the anti-rotavirus effect by reducing cholesterol and / or triglyceride, or the activations of some genes. Here, we assume that the reduction of rotavirus infection is achieved by lessened cholesterol absorption with FA / fenofibrate treatment, and it may correct the original concept to that the intracellular cholesterol pool, but not cholesterol biosynthesis, is involved in rotavirus infection. The results from ezetimibe support the updated concept. Ezetimibe is an FDA-approved drug used to lower serum cholesterol by blocking the cholesterol absorption of intestine [39], but it also has shown the antiviral effects to the infections of multiple viruses, such as Zika virus and HCV [40],[41]. In our study, as expected, ezetimibe showed a marked inhibitory effect on rotavirus like the other cholesterol-lowering drugs; interestingly, it even solely displayed the preventive and interference effects towards the virus infection. Combining the pharmacological mechanisms of FA / fenofibrate and ezetimibe on cholesterol reduction, we are inclined to consider that the intracellular cholesterol pool may
play a key role in rotavirus infection. Further investigations are needed to validate or disprove the hypothesis.

Fenofibrate / ezetimibe may be used in combination therapy with statin for the therapy of hyperlipidemia in clinical practice [19],[20], which inspired us to test the effects of these combinations on rotavirus. Statin has been indicated to be effective towards rotavirus by our previous experiment in vitro. Herein, we documented that the combinations of lovastatin or simvastatin with FA / ezetimibe respectively did not exert synergy on the anti-rotavirus effect in the pre-clinical models. The mechanism of the substantial limitation of the cholesterol-lowering drugs combinations is not yet clear, and it warrants more investigations.

In summary, our investigation indicates that the cholesterol-lowering drugs can inhibit rotavirus infection in vitro. Repurposing a group of used drugs from one application field to another could save much resources and time needed for new medication development, and the application experience of the old drugs could be beneficial to formulate the mature treatment plans soon. Although the experimental research in vitro alone will not be able to clarify the complicated but important clinical issues, our results are certainly a valuable reference to clinical activity and promote the initiations of follow-up clinical trials to address these potential issues. Nevertheless, further studies regarding their efficacy and pharmacokinetics in vivo are essential to evaluate their potentials in clinical application.

Declarations

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Authors’ contributions

Shihao Ding: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Visualization; Writing - original draft; Bingting Yu: Investigation; Validation; Anneke J. van Vuuren: Funding acquisition; Supervision; Writing - review & editing.

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Availability of data and materials
All data generated or analyzed during this current study are available from the corresponding author on reasonable request.

**Declaration of competing interest**

The authors declare no conflict of interest.

**Ethics approval and consent to participate**

The use of all biological samples in this study was approved by the ethics committee of Erasmus University Medical Center in Rotterdam.

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Figures
Figure 1

Zoledronic acid (ZA) impairs rotavirus SA11 replication. In Caco2 cells, A the cells were infected by rotavirus SA11 strain (MOI 0.7) and subsequently treated with a series of concentrations of ZA for 48 hours, then the intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 6); B the content of rotavirus VP4 protein was measured by western blot with 50 µM ZA treatment for 48 hours (n = 3); C the expression of rotavirus VP6 protein was shown by immunofluorescence (IF) staining. In MA104
cells, the rotavirus SA11 infection and ZA treatment were operated as the description in the Caco2 cells. D The intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 5); E The content of rotavirus VP4 protein was measured by western blot (n = 3); F The expression of rotavirus VP6 protein was showed by IF staining. In HSI organoids, by 50 µM ZA treatment for 48 hours post rotavirus SA11 infection, G the inner rotavirus RNA level was measured by qRT-PCR (n = 5); H the content of rotavirus VP4 protein was measured by western blot (n = 3); and I the expression of rotavirus VP6 protein was showed by IF staining. J The 96 hours time-course experiment of ZA treatment on the intracellular rotavirus RNA level. K The titer of infectious rotavirus particles in supernatant was measured by TCID50 assay upon 50 µM ZA treatment in MA104 cells (n = 6). The mean value ± standard error was indicated. (*P < 0.05; **P < 0.01; ***P < 0.001). For all the IF staining, VP6 protein was stained as green, and Nuclei were visualized by DAPI (blue).
Figure 2

Nicotinic Acid (NA) impairs rotavirus SA11 replication. In Caco2 cells, A the cells were infected with rotavirus SA11 strain (MOI 0.7) and subsequently treated with a series of concentrations of NA for 48 hours, then the intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 6). B the content of rotavirus VP4 protein was measured by western blot (n = 3). C the expression of rotavirus VP6 protein was shown by immunofluorescence (IF) staining. In MA104 cells, the rotavirus SA11 infection and
NA treatment were operated as the description in the Caco2 cells. D The intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 6). E The content of rotavirus VP4 protein was measured by western blot (n = 3). F The expression of rotavirus VP6 protein was showed by IF staining. In HSI organoids, by 100 µM NA treatment for 48 hours post rotavirus SA11 infection, G the inner rotavirus RNA level was measured by qRT-PCR (n = 5); H The content of rotavirus VP4 protein was measured by western blot (n = 3); I The expression of rotavirus VP6 protein was showed by IF staining. J The 96 hours time course experiments of 100 µM NA treatment on the intracellular rotavirus RNA level. K The titer of infectious rotavirus particles in supernatant was measured by TCID50 assay upon 100 µM NA treatment in MA104 cells (n = 6). The mean value ± standard error was indicated. The mean value ± standard error was indicated. (*P < 0.05; **P < 0.01; ***P < 0.001). For all the IF staining, VP6 protein was stained as green, and Nuclei were visualized by DAPI (blue).
Figure 3

Fenofibric acid (FA) impairs rotavirus SA11 replication. In Caco2 cells, A the cells were infected with rotavirus SA11 strain (MOI 0.7) and subsequently treated with different concentrations of FA for 48 hours, then the intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 6). B The content of rotavirus VP4 protein was measured by western blot (n = 3). C The expression of rotavirus VP6 protein was showed by IF staining. In MA104 cells, the rotavirus SA11 infection and FA treatment were operated...
as the description in the Caco2 cells. D the intra- and extracellular rotavirus RNA levels were measured by qRT-PCR (n = 6); E the content of rotavirus VP4 protein was measured by western blot (n = 3); F the expression of rotavirus VP6 protein was showed by IF staining. In HSI organoids, by 50 µM FA treatment for 48 hours post rotavirus SA11 infection, G the inner rotavirus RNA copies were measured by qRT-PCR (n = 5). H the content of rotavirus VP4 protein was measured by western blot (n = 3). I the expression of rotavirus VP6 protein was showed by IF staining. J The 96 hours time course experiment of 50 µM FA treatment on the intracellular rotavirus RNA level. K The titer of infectious rotavirus particles in supernatant was measured by TCID50 assay upon 50 µM FA treatment in MA104 cells (n = 6). The mean value ± standard error was indicated. (*P < 0.05; **P < 0.01; ***P < 0.001). For all the IF staining, VP6 protein was stained as green, and Nuclei were visualized by DAPI (blue).
Ezetimibe impairs rotavirus SA11 replication. In Caco2 cells, A the cells were infected with rotavirus SA11 strain (MOI 0.7) and subsequently treated with different concentrations of ezetimibe for 48 hours, then the intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 6). B The content of rotavirus VP4 protein was measured by western blot (n = 3). C The expression of rotavirus VP6 protein was showed by IF staining. In MA104 cells, the rotavirus SA11 infection and ezetimibe treatment were
operated as the description in the Caco2 cells. D The intra- and extracellular rotavirus RNA copies were measured by qRT-PCR (n = 5). E The content of rotavirus VP4 protein was measured by western blot (n = 3). F The expression of rotavirus VP6 protein was showed by IF staining. In HSI organoids, by 50 µM ezetimibe treatment for 48 hours post rotavirus SA11 infection, G the inner rotavirus RNA copies were measured by qRT-PCR (n = 5); H the content of rotavirus VP4 protein was measured by western blot (n = 3); I the expression of rotavirus VP6 protein was showed by IF staining. J The 96 hours time course experiments of 50 µM ezetimibe treatment on the intracellular rotavirus RNA level. K The titer of infectious rotavirus particles in supernatant was measured by TCID50 assay upon 50 µM ezetimibe treatment in MA104 cells (n = 6). The mean value ± standard error was indicated. (*P < 0.05; **P < 0.01; ***P < 0.001). For all the IF staining, VP6 protein was stained as green, and Nuclei were visualized by DAPI (blue).
Figure 5

The combinations of lovastatin/simvastatin with FA / ezetimibe did not show significant synergy. In Caco2 cells were infected with rotavirus SA11 strain (MOI 0.7) and subsequently treated with 50 µM FA, 50 µM lovastatin, 50 µM simvastatin, the combination of 50 µM FA and 50 µM lovastatin, and the combination of 50 µM FA and 50 µM simvastatin respectively for 48 hours: A the intracellular rotavirus RNA copies were measured by qRT-PCR (n = 6) (left), and the extracellular rotavirus RNA copies were
measured by qRT-PCR (n = 6) (right). B The content of rotavirus VP4 protein was measured by western blot (n = 3). In Caco2 cells were infected with rotavirus SA11 strain (MOI 0.7) and subsequently treated with 50 µM ezetimibe, 50 µM lovastatin, 50 µM simvastatin, the combination of 50 µM ezetimibe and 50 µM lovastatin, and the combination of 50 µM ezetimibe and 50 µM simvastatin respectively for 48 hours: C the intracellular rotavirus RNA copies were measured by qRT-PCR (n = 6) (left), and the extracellular rotavirus RNA copies were measured by qRT-PCR (n = 6) (right). D The content of rotavirus VP4 protein was measured by western blot (n = 3). In HSI organoids, by 50 µM FA, 50 µM lovastatin, 50 µM simvastatin, the combination of 50 µM FA and 50 µM lovastatin, and the combination of 50 µM FA and 50 µM simvastatin respectively post rotavirus SA11 infection for 48 hours: E the inner rotavirus RNA copies were measured by qRT-PCR (n = 5); F the content of rotavirus VP4 protein was measured by western blot (n = 3). In HSI organoids, by 50 µM ezetimibe, 50 µM lovastatin, 50 µM simvastatin, the combination of 50 µM ezetimibe and 50 µM lovastatin, and the combination of 50 µM ezetimibe and 50 µM simvastatin respectively post rotavirus SA11 infection for 48 hours: G the inner rotavirus RNA copies were measured by qRT-PCR (n = 5); H the content of rotavirus VP4 protein was measured by western blot (n = 3). The mean value ± standard error was indicated. (*P < 0.05; **P < 0.01; ***P < 0.001).
All these clinical drugs exert the anti-rotavirus effects towards a clinical rotavirus isolate 026K in Caco2 cells. Caco2 cells were infected with rotavirus clinical 026K strain subsequently treated with A 50 µM ZA (n = 6), B 100 µM NA (n = 5), C 50 µM FA (n = 6), D 50 µM ezetimibe (n = 6), E the combination of FA with lovastatin / simvastatin (n = 6), and F the combination of ezetimibe with lovastatin / simvastatin respectively (n = 6). The mean value ± standard error was indicated. (*P < 0.05; **P < 0.01; ***P < 0.001).
Supplementary Files

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