Nitazoxanide inhibits human norovirus replication and synergizes with ribavirin by activation of cellular antiviral response

Wen Dang,[a] Lei Xu,[a#] Buyun Ma,[a#] Sunrui Chen,[a] Yuebang Yin,[a] Kyeong-Ok Chang,[b] Maikel P. Peppelenbosch, [a] Qiuwei Pan[a]*

[a]Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands

[b]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, USA

Supplementary Methods

Reverse transcription PCR (RT-PCR)

Two primer sets were designed to detect HuNV replicons in HG23 cells. The primer pair designated Neo-F and Neo-R is located in the neomycin resistant gene (1) and is predicted to produce a product of 804 bases. Primers NVp36/35 are located in a highly conserved RNA polymerase region of HuNV GI genome and are predicted to give a product of 470 bases (2). For RT, cDNA was reverse transcribed from extracted cellular RNA by using TakaRa PrimeScript™ RT reagent Kit according to the manufacturer's instructions. Briefly, 8 µL of total cellular RNA (500 ng) was mixed with 2 µL of 5× PrimeScript Buffer on ice. The mixture was incubated at 37℃ for 15 mins; this was followed by heat inactivation at 85℃ for 5 s. The mixture was 20 times diluted and stored at -20℃ for further use. PCR was performed using Q5® High-Fidelity DNA polymerase kit (New England Biolabs Inc.) in a 25 µL reaction mixture containing 5 µL of 5× Q5 Reaction Buffer, 0.5 µL of a 10 mM dNTPs, 1.25 µL of 10 µM Forward Primer, 1.25 µL of 10 µM Reverse Primer, 5 µL of template cDNA and 0.25 µL of Q5 High-Fidelity DNA Polymerase. Amplification was carried out with initial denaturation at 98℃ [30 s]; 30 cycles of 98℃ [10 s], 50-70℃ [30 s] and 72 ℃ [30 s]; and an additional extension step of 72℃ for 2 min. The GAPDH primer set was used as a reference gene and was amplified in 25 cycles (3). The products were loaded onto 1-2% agarose gel containing SERVA DNA Stain G. DNA electrophoresis at 100 V for 45 min was performed. 100 bp DNA ladder (Promega) was also electrophoresed on each gel. Bands were visualized and recorded with the Gel.Doc 2000 system (Bio-Rad). All primer combinations were listed in Table S2.

MTT assay

Cytotoxicity of the compounds on host cells were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, cells were seeded into 96-well plates containing 0.05% DMSO (control) or increasing concentrations of drugs. After the time indicated, 10 mM MTT (Sigma, Zwijndrecht, Netherlands) was added. With another 3 hours of incubation, the medium was removed and 100 μL DMSO was added to each well. The plate was incubated at 37℃ for 50 min. The absorbance at 490 nm was recorded on the microplate absorbance reader (Bio-Rad, CA, USA).

Table S1 Overview of culture medium components for human intestinal organoids.

Components	Storage (℃)	Stocks	Final	Company
Wnt 3a	4	Conditioned medium	50%	Self-produced
R-Spondin 1	-20	Conditioned medium	20%	Self-produced
Noggin	-20	Conditioned medium	10%	Self-produced
B27	-20	50×	1	Gibco
N2	-20	100×	1	Gibco
N-Acetylcysteine (mol/L)	-20	0.5	1×10^{-3}	Invitrogen
Nicotinamide (mol/L)	-20	1	1×10^{-2}	Sigma-Aldrich
A83-01 (TGF-beta inhibitor) (mol/L)	-20	5×10^{-4}	5×10^{-7}	Tocris
SB202190 (p38 inhibitor) (mol/L)	-20	3×10^{-2}	3×10^{-6}	Sigma-Aldrich
Human EGF (g/L)	-20	5×10^{-2}	5×10^{-5}	Sigma-Aldrich
Prostaglandin E2 (mol/L)	-20	1×10^{-4}	1×10^{-8}	PeproTech
Gastrin (mol/L)	-20	1×10^{-4}	1×10^{-8}	PeproTech
Y-27632 (Rho kinase inhibitor) (mol/L)	-20	1×10^{-2}	1×10^{-5}	Bio-Connect BV

Table S2 Primers used by qRT-PCR.

Name	Sequences[a]	Location
HuNoV GI-specific primer	COG1F (+): 5'- CGYTGGATGCGNTTYCATGA-3'[b] COG1R (-): 5'- CTTAGACGCATCATCATTYAC-3'[b]	5291[b] 5375[b]
FeCV	F: 5'- GAACTACCCGCCAATCAACAT -3' R: 5'- CGGCTCTGATGGCTTGAAACTG-3'	2420-2440 2507-2528
DDX60	F: 5'-GGTGTTTTCACCAGGGAGTATCG-3' R: 5'-CCAGTTTTGGCGATGAGGAGCA-3'	
Human GAPDH	F: 5'-TGTCCCCACCCCCAATGTATC-3' R: 5'-CTCCGATGCCTGCTTCACTACCTT-3'	
Feline GAPDH	GAPDH.57f: 5'- GCCGTGGAATTTGCCGT-3' GAPDH.138r: 5'- GCCATCAATGACCCCTTCAT-3'	
IFIT1	F: 5'-GCCTTGCTGAAGTGTGGAGGAA-3' R: 5'-ATCCAGGCGATAGGCAGAGATC-3'	
IRF9	F: 5'- CCACCGAAGTTCCAGGTAACAC-3' R: 5'- AGTCTGCTCCAGCAAGTATCGG-3'	
IFI27	F: 5'- CGTCCTCCATAGCAGCCAAGAT-3' R: 5'- ACCCAATGGAGCCCAGGATGAA-3'	
IFITM3	F: 5'-CTGGGCTTCATAGCATTCGCCT-3' R: 5'-AGATGTTCAGGCACTTGGCGGT-3'	
ISG15	F: 5'-CTCTGAGCATCCTGGTGAGGAA-3' R: 5'-AAGGTCAGCCAGAACAGGTCGT-3'	
IRF-1	F: 5'-GAGGAGGTGAAAGACCAGAGCA-3' R: 5'-TAGCATCTCGGCTGGACTTCGA-3'	
IFI6	F: 5'-TGATGAGCTGGTCTGCGATCCT-3' R: 5'-GTAGCCCATCAGGGCACCAATA-3'	

MDA5 (IFIH1)	F: 5’-GCTGAAGTAGGAGTCAAAGCCC-3’ R: 5’-CCACTGTGGTAGCGATAAGCAG-3’
Mx1	F: 5’- GGCTGTTTACCAGACTCCGACA-3’ R: 5’- CACAAAGCCTGGCAGCTCTCTA-3’
OAS3	F: 5’-CCTGATTCTGCTGGTGAAGCAC-3’ R: 5’-TCCCAGGCAAAGATGGTGAGGA-3’
OASL	F: 5’-GTGCCTGAAACAGGACTGTTGC-3’ R: 5’-CCTCTGCTCCACTGTCAAGTGG-3’
PKR	F: 5’-GAAGTGGACCTCTACGCTTTGG-3’ R: 5’-TGATGCCATCCCGTAGGTCTGT-3’
RIG-I	F: 5’-CACCTCAGTTGCTGATGAAGGC-3’ R: 5’-GTCAGAAGGAAGCACTTGCTACC-3’
RSDA2	F: 5’-CCAGTGCAACTACAAATGCGGC-3’ R: 5’-CGGTCTTGAAGAAATGGCTCTCC-3’

[a]Y = C + T; R = A + G; W = A+T; N = any

[b]Corresponding nucleotide position of Norwalk/68 virus (accession no. M87661) of the 5’end.

Table S3 Primers used by RT-PCR.

Target	Primer		Sense	Sequence (5'-3')[a]	Location	Annealing
HuNV	HuNV GI-specific	NVp36	+	ATAAAAGTTGGCATGAACA	4487-4505[b]	57℃
		NVp35	_	CTTGTTGGTTTGAGGCCATAT	4936-4956[b]	
Neomycin phosphotransferase	Neo-F		+	ATGGGATCGGCCATTGAAC		59℃
	Neo-R		_	TCAGAAGAACTCGTCAAG		
GAPDH	GAPDH-F		+	TCGTGGAAGGACTCATGACC		68℃
	GAPDH-R		_	TCCACCACCCTGTTGCT		

[a]Y = C + T; M = A + C; D = A+T+G; W = A+T

[b]Nucleotide positions are the positions in Hu/NLV/Norwalk/68/US (accession no. M87661).

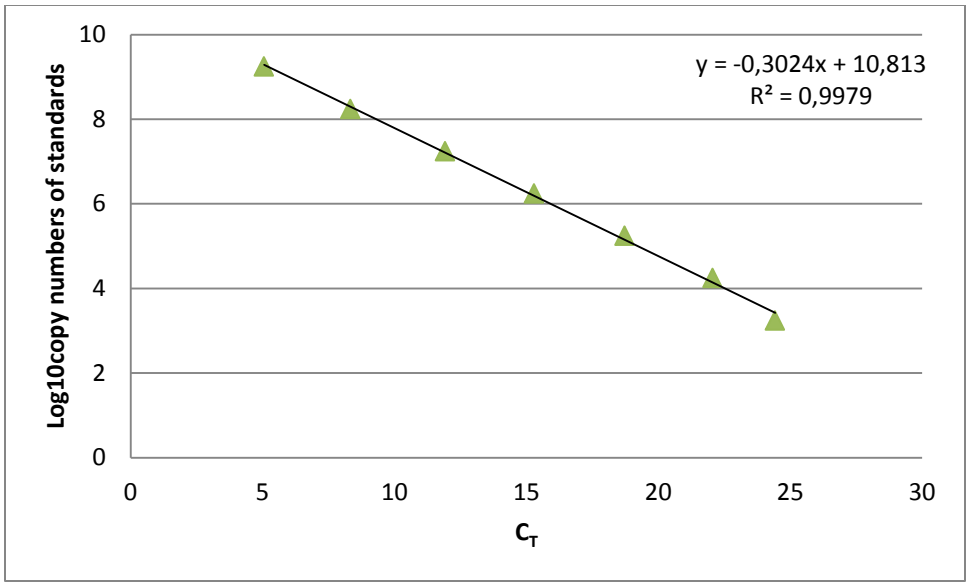

Fig. S1 Standard curve for quantifying FeCV genome copy numbers.

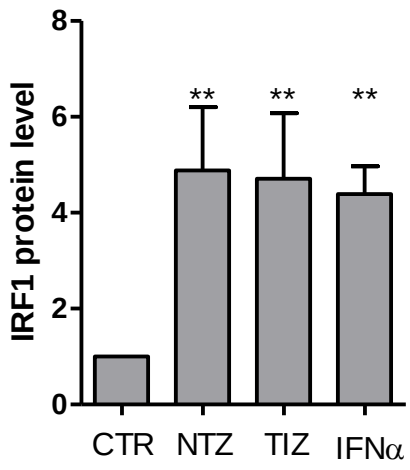

Fig. S2 Densitometric analysis of western blot of IRF-1 protein expression after treatment with NTZ (10 μg/mL), TIZ (10 μg/mL), IFNα (1000 IU/mL) or matched concentration of DMSO as vehicle control (CTR). The data were plotted as means ± SEM and statistical comparisons were analyzed by t test (n = 5). **P < 0.01.

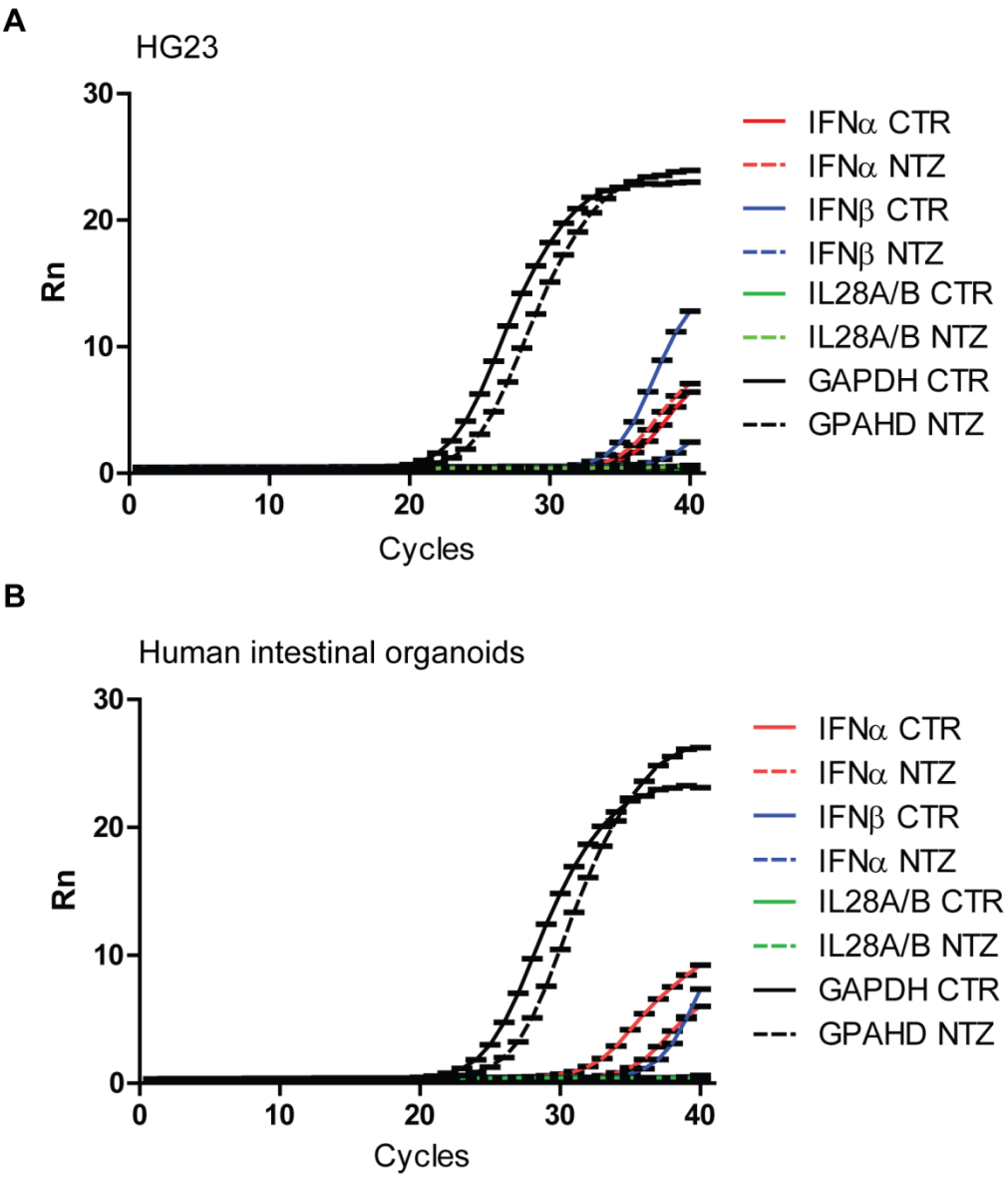

FIG S3 NTZ had no major effects on the induction of IFN genes. qRT-PCR analysis of IFN genes including IFNα, IFNβ and IL28A/B after NTZ treatment (10 µg/mL for 2 days) in HG23 (A) and human intestinal organoids (B). Data were representative of 3 independent experiments.

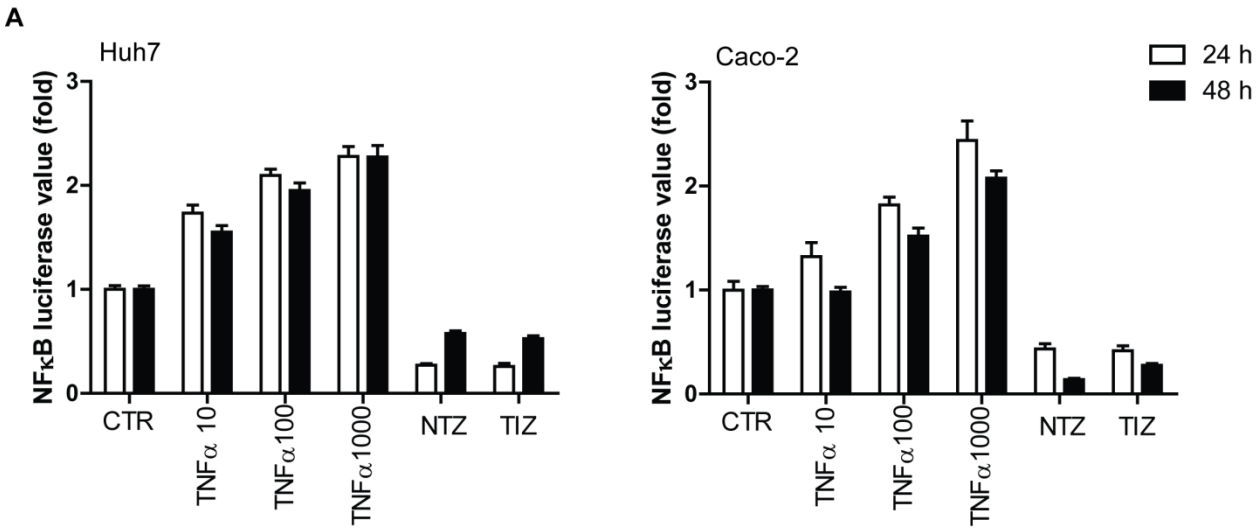

Figure S4 Stimulation of ISGs by TZD was independent of NFκB pathway. Huh7 and Caco-2-based NFκB luciferase reporter cells were mock-treated (DMSO) or stimulated for 24 and 48 hours with TNFα (10, 100 and 1000 ng/mL), NTZ (1 µg/mL) or TIZ (1 µg/mL). Luciferase activities were determined and normalized to the DMSO control (n = 3 independent experiments with duplicates each). TZD treatment failed to induce NFκb-dependent reporter gene activity.

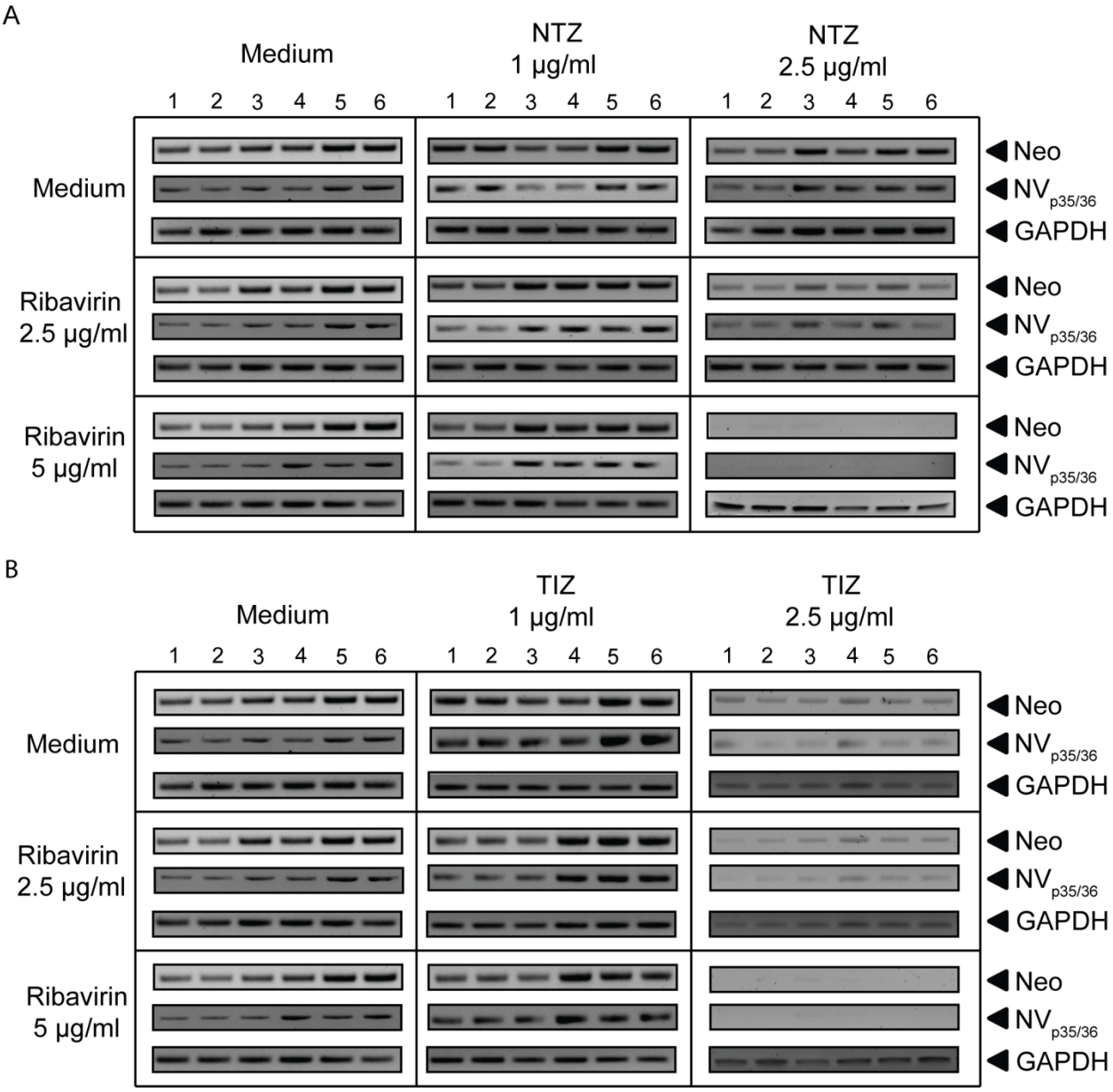

FIG S5 Combination of TZD with ribavirin completely depleted HuNV replicons from HG23 cells after prolonged treatment. After 10 days of treatment total cellular RNA was isolated from HG23 cells and gel-based reverse transcription polymerase chain reaction (RT-PCR) was performed to detect HuNV replicons with two primer pairs: (1) Neo-F/R were designed to amplify the full length neomycin phosphotransferase (804 bp); (2) $NV_{p35/36}$ specifically detected genogroup I (GI) norovirus and yielded a 470 bp DNA

amplicons. Human GAPDH primer set was used as a reference gene. A total of 6 samples were detected and were indicated by numbers.

Supplementary Reference

1. **Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY.** 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353:**463-473.
2. **Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, et al.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J Virol **68:**5982-5990.
3. **Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z, Hsieh JT.** 1999. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. Cancer Res **59:**325-330.