Supplementary information to

Inhibition of calcineurin or IMPDH exerts moderate to potent antiviral activity against norovirus replication

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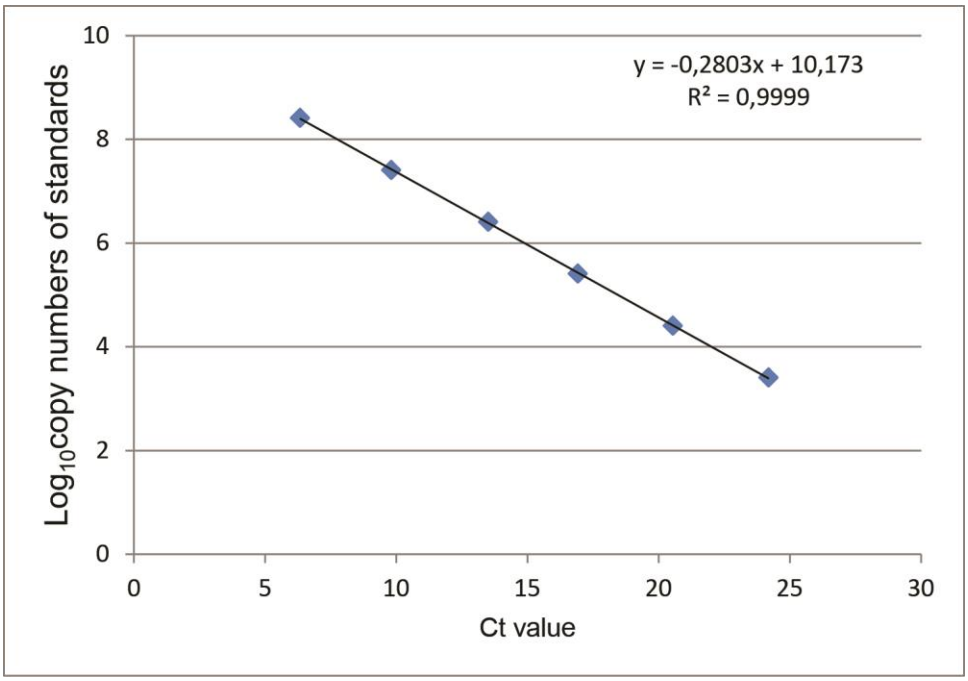
Abbreviations: BQR, brequinar; CNIs, calcineurin inhibitors; CsA, cyclosporin A; CPE, cytopathic effect; Ct, cycle threshold; CVID, common variable immunodeficiency; DEX, dexamethasone; CyPA/B, cyclophilin a/b; FK506, tacrolimus; HuNV, human norovirus; IMPDH, inosine monophosphate dehydrogenase; LEF, leflunomide; MNV-1, murine norovirus 1; MOI, multiplicity of infection; MPA, mycophenolic acid; PRED, prednisolone; RAP, rapamycin; shRNA, short-hair RNA; $TCID_{50}$, 50% tissue culture infective dose.

Supplementary Table S1

PCR primer sequences were listed.

Name	Sequences
Neomycin phosphotransferase	F: 5'-CCGGCTACCTGCCCATTC-3' R: 5'-CCAGATCATCCTGATCGACAA G-3'
MNV-1[a]	F: 5'-CACGCCACCGATCTGTTCTG-3' R: 5'-GCGCTGCGCCATCACTC-3'
Human GAPDH	F: 5'-GTCTCCTCT GACTTCAACAGCG-3' R: 5'-ACCACCCTGTTGCTGTAGTAGCCAA-3'
Murine GAPDH	F: 5'-TTCCAGTATGACTCCACTCACGG-3' R: 5'-TGAAGACACCAGTAGACTCCACGAC-3'
CyPA	F: 5'-TAAAGCATACGGGTCCTGG-3' R: 5'-TCGAGTTGTCCACAGTCAG-3'
CyPB	F: 5'-ATGTAGGCCGGGTGATCTTT-3' R: 5'-TTTATCCCGGCTGTCTGTCT-3'
FKBP12	F: 5'- TGCTAGGCAAGCAGGAGGTGAT-3' R: 5'- GTGGCACCATAGGCATAATCTGG-3'
FKBP8	F: 5'- ACTCCTACGACCTCGCCATCAA-3' R: 5'- GGTAGTGGTCGAGCTTCAGCTG-3'
PPP3CA	F: 5'-GCCCTGATGAACCAACAGTTCC-3' R: 5'-GCAGGTGGTTCTTTGAATCGGTC-3'
IMPDH1	F: 5'-GCACACTGTGGGCGAT-3' R: 5'-GAGCCACCACCAGTTCA-3'
IMPDH2	F: 5'-TCTTCAACTGCGGAGAC-3' R: 5'-CTGTAAGCGCCATTGCT-3'

a accession# NC008311

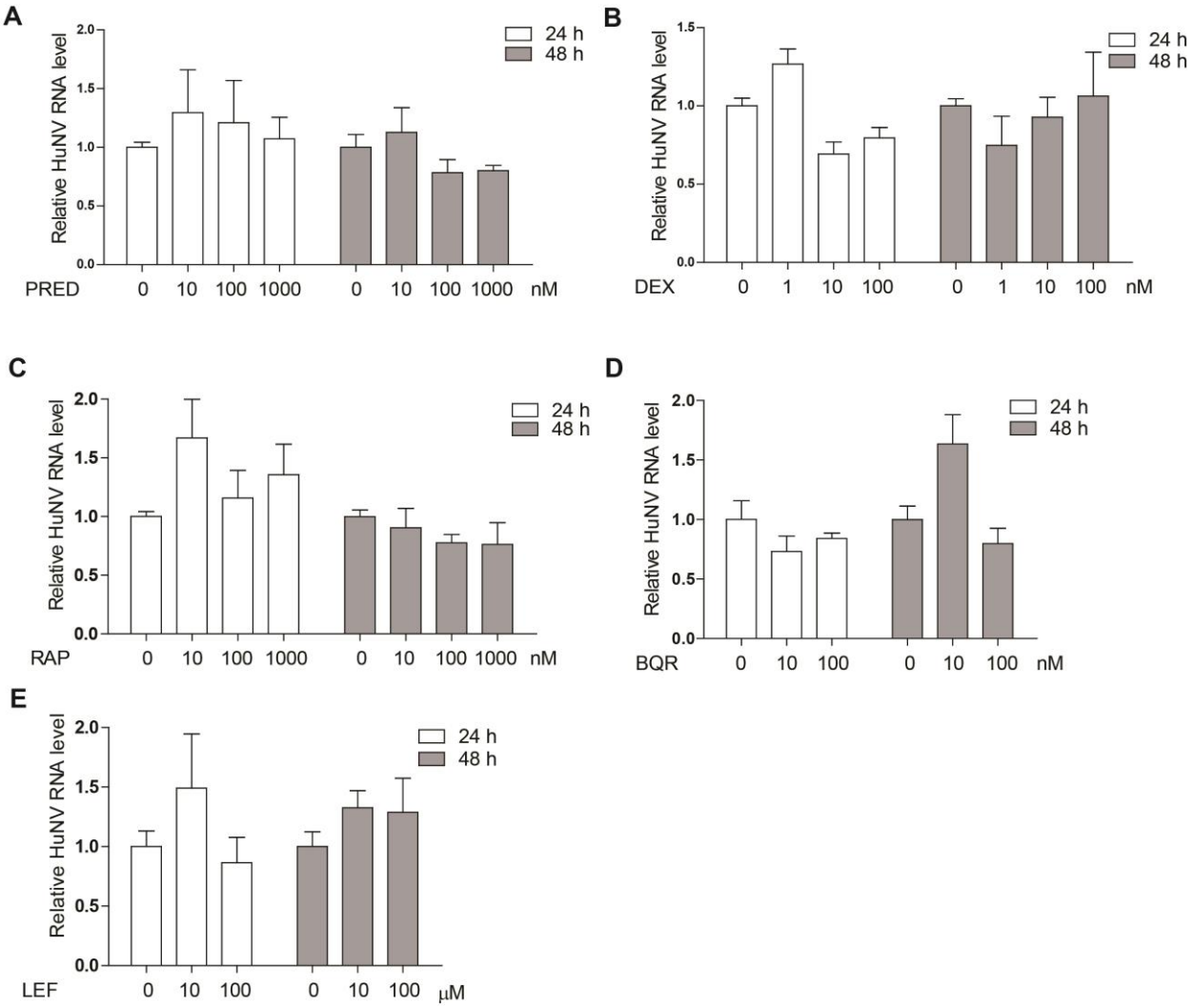


Supplementary Fig. S1. Standard curve for quantifying MNV-1 genome copy numbers.

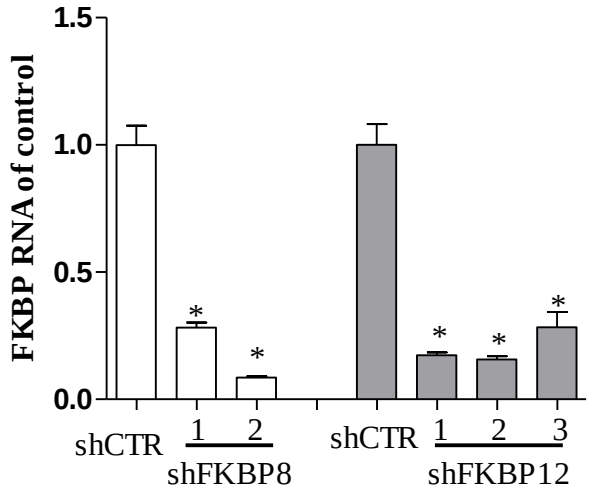
Supplementary Table S2

Sequences of shRNA-mediated vectors were listed.

Name	sequences
Scrambled vector	5’-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3’
CyPA	5’-CCGGTGGTGACTTCACACGCCATAACTCGAGTTATGGCGTGTGAAGTCACCATTTTTG-3’
CyPB	5’-CCGGGCCTTAGCTACAGGAGAGAAACTCGAGTTTCTCTCCTGTAGCTAAGGCTTTTTG-3’
shFKBP8-1	5’-CCGGACGTCGCTGGAGAATGGCACACTCGAGTGTGCCATTCTCCAGCGACGTTTTTT-3’
shFKBP8-2	5’-CCGGAGTGGACATGACGTTCGAGGACTCGAGTCCTCGAACGTCATGTCCACTTTTTT-3’
shFKBP12-1	5’- CCGGAGAGAGCCAAACTGACTATATCTCGAGATATAGTCAGTTTGGCTCTCTTTTTT-3’
shFKBP12-2	5’-CCGGGCCAAACTGACTATATCTCCACTCGAGTGGAGATATAGTCAGTTTGGCTTTTT-3’
shFKBP12-3	5’-CCGGGAGAGCCAAACTGACTATATCCTCGAGGATATAGTCAGTTTGGCTCTCTTTTT-3’
shPPP3CA-1	5’-CCGGCACCACAACATAAGATCACTACTCGAGTAGTGATCTTATGTTGTGGTGTTTTT-3’
shPPP3CA-2	5’-CCGGGAATAATAACAGAGGGTGCATCTCGAGATGCACCCTCTGTTATTATTCTTTTT-3’
shIMPDH1-1	5’-CCGGCCAGGATTCATAGACTTCATACTCGAGTATGAAGTCTATGAATCCTGGTTTTT-3’
shIMPDH1-2	5’-CCGGCGGAAGGTCAAGAAGTTTGAACTCGAGTTCAAACTTCTTGACCTTCCGTTTTT-3’
shIMPDH1-3	5’-CCGGGTGACGTTGAAAGAGGCAAATCTCGAGATTTGCCTCTTTCAACGTCACTTTTT-3’
shIMPDH1-4	5’-CCGGCCTGAAGAAGAACCGAGACTACTCGAGTAGTCTCGGTTCTTCTTCAGGTTTTT-3’
shIMPDH2-1	5’-CCGGCACCTACAATGACTTTCTCATCTCGAGATGAGAAAGTCATTGTAGGTGTTTTT-3’
shIMPDH2-2	5’-CCGGGACTGTTTCTTGGAAGAGATACTCGAGTATCTCTTCCAAGAAACAGTCTTTTT-3’



Supplementary Fig. S2 Not all immunosuppressants directly affect norovirus replication. Glucocorticoids, including PRED (A) and DEX (B) showed no effect on HuNV replication. Likewise, rapamycin (C), an mTOR inhibitor, produced no significant changes in HuNV replication. Similarly, BQR (D) and LFM (E), which are in clinical development for use in transplantation medicine, showed no significant effects on HuNV replication. Six replicates were performed (three independent experiments, each with two replicates) and data are presented as means ± SEM.



Supplementary Fig. S3. qRT-PCR analysis of FKBP8 and FKBP12 RNA levels after shRNA-based knockdown.

Supplementary Table S3

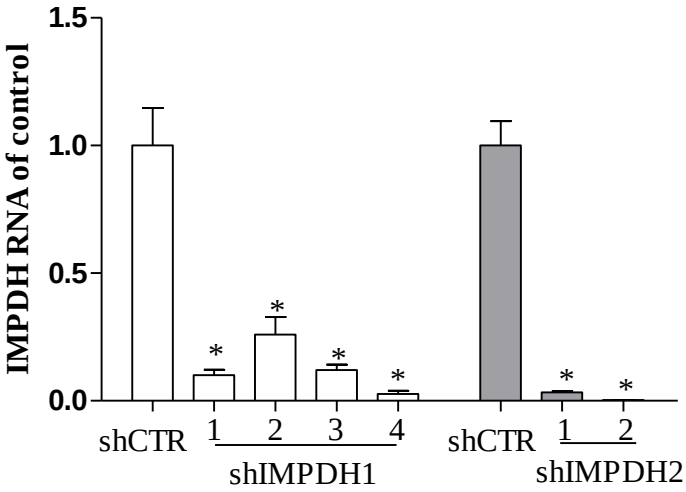
Table S3. In vitro anti-norovirus and cytotoxic activity of compounds

	Compounds	Inhibition of viral RNA replication EC_{50}, μg/ml	Inhibition of cell proliferation CC_{50}, μg/ml	Selectivity index
Human norovirus model	Cyclosporin A (CsA)	3.90	14.34	3.67
	Voclosporin (VCS)	0.94	4.74	5.04
	Tacrolimus (FK506)	2.92	3.72	1.27
	Mycophenolic acid (MPA)	0.13	4.11	31.61
	Ribavirin	1.25	14.94	11.95
Mouse norovirus model	Mycophenolic acid (MPA)	0.05	0.80	40
	Ribavirin	0.74	30.87	41

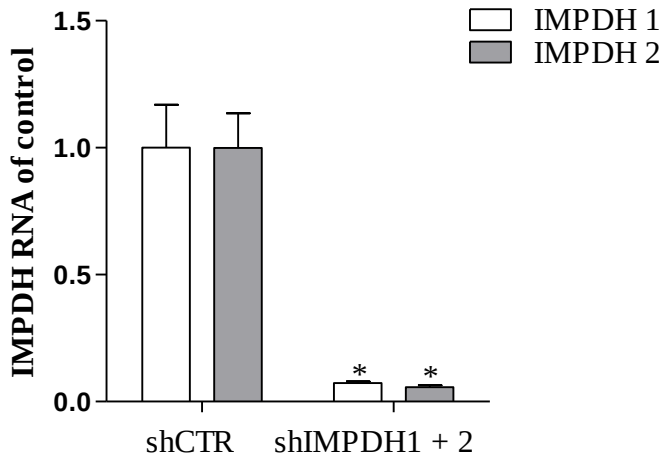
Supplementary Table S4

The Ki values of 4 IMPHD inhibitors towards IMPDH1 and IMPDH2. Because of confidentiality, the structures are not shown.

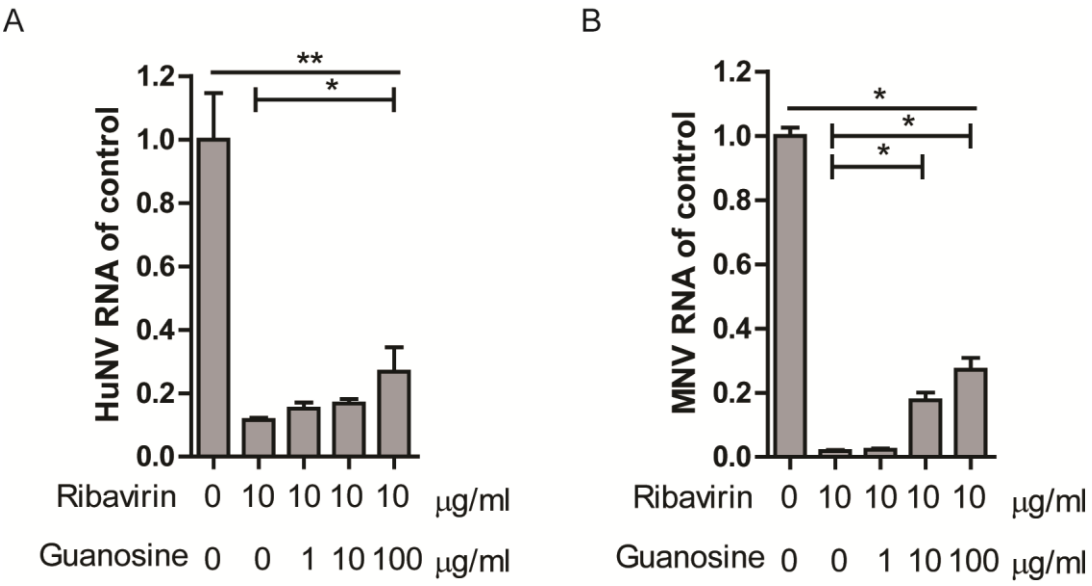
#	IMPDH I Ki in nM	IMPDH in nM	MW
1315	0.60	13.90	744.53
1346	859.1	243.9	546.54
1351	618.40	185.90	549.59
1356	70.12	65.69	588.67



Supplementary Fig. S4 qRT-PCR analysis of IMPDH RNA levels in IMPDH1 or IMPDH2 knockdown cells.



Supplementary Fig. S5 qRT-PCR analysis of IMPDH RNA levels after simultaneous knockdown of IMPDH1 and IMPDH2.



Supplementary Fig. S6 The effect of exogenous guanosine on the antiviral effect of ribavirin towards HuNV (A) and MNV (B) model.

Supplementary Discussion

Glucocorticosteroids were widely used during the early years of organ transplantation. Nowadays clinicians generally attempt to avoid prescribing steroids in case of active infection because of their potential of facilitating the infectious process and concern about long-term complications associated with such treatment regimens (1). For instance, steroids boluses used to treat acute rejection in hepatitis C virus (HCV) infection-related liver transplantation are often associated with an increase in viral load and the severity of HCV recurrence (1, 2). In cell culture, steroids are reported to have no effect on HCV replication (3), but can specifically facilitate viral entry by enhancing the expression of the HCV co-receptors (4). In the present study, however, we observed no direct effect of steroids on norovirus replication in cell culture, although their impact on norovirus infection in patients remains to be addressed. Rapamycin when complexed to FKBP12 to form the FKBP-rapamycin complex that directly binds to mTOR complex 1 and thus inhibits the mTOR pathway, appears likewise safe but is associated with anti-norovirus activity *per se.* This contrasts the situation with HCV infection. Rapamycin potently inhibits HCV RNA replication, but does not influence the early replication cycle steps associated with its biology such as cell entry and viral RNA translation (5). Diametrically opposing the situation with HCV, another study showed that rapamycin facilitated hepatitis E virus (HEV) replication through inhibition of PI3K-PKB-mTOR pathway. The latter pathway limits HEV infection and acts as a gate-keeper with respect to HEV in human HEV target cells. In our study, rapamycin treatment resulted in no change on norovirus replication in cell culture models and thus effects of mTOR inhibitors on viral life cycle appear highly virus specific, hampering design of

rational immunosuppressive therapies using this medication. Brequinar and leflunomide, two best-known inhibitors of dihydroorotate dehydrogenase (DHOD), interfere with cell proliferation by inhibiting pyrimidine nucleotide biosynthesis (6). Leflunomide has been extensively studied and is approved for the treatment of psoriatic arthritis and rheumatoid arthritis (7). Leflunomide has been shown to inhibit HIV-1 replication mainly through pyrimidine nucleotide pool depletion (8). In our study, both leflunomide and brequinar showed no effect on HuNV replication and appear thus not specifically useful for the management of patients at risk for norovirus infection.

Supplementary References

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