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Repurposing of Rutin for the Inhibition of Norovirus Replication

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Abstract

Drug repurposing is a strategy employed to circumvent some of the bottlenecks involved in drug development, such as the cost and time needed for developing new molecular entities. Noroviruses cause recurrent epidemics and sporadic outbreaks of gastroenteritis associated with significant mortality and economic costs but no treatment is approved to date. Herein, a library of molecules previously used in man was screened to find compounds with anti-noroviral activity. Antiviral testing for four selected compounds against murine norovirus infection revealed that rutin has anti-murine norovirus activity in cell-based assays.

Main text

The development cost of new drugs has exploded over the last decades and is now estimated to be at least \$2 billion per drug [27]. At the same time, the number of new molecular entities approved by FDA has saturated to about 25 per year [25]. The success rate of new molecular entities is estimated to be 10% from Phase II to market, and 50% from Phase III to market. These values increase to 25% and 64% for repositioned drugs [36]. Therefore, an increased effort is being made towards drug repurposing [32, 40, 48], with demonstrated success in part due to the fact that different diseases can have related biological origin [36, 37, 46]. Since toxicology and pharmacokinetic data of repurposed molecules are already available, the development process is accelerated and the risk of failures is lowered.

Noroviruses (NoV) cause almost 80% of non-bacterial gastroenteritis in humans and spread quickly in confined environments, leading to ~71,000 hospitalizations and ~800 deaths every year in the USA [4, 20, 34, 41]. The impact is even greater in resource poor nations, where an estimated 200,000 children under 5 years of age die every year from NoV infections [34]. However, no specific treatments to control or prevent norovirus illness are approved by the FDA to date. Norovirus vaccine efforts are in phase I clinical trials [45], however vaccines cannot be used in some at risk populations, such as immunocompromised patients [17]. Moreover, any effective vaccine design needs to overcome the large genetic diversity and rapid mutation rate of HuNoVs [26]. Thus, the development of low-molecular weight drugs with anti-norovirus activity has emerged as another strategy to treat or prevent norovirus infections. To date, three of the nine proteins encoded by the (+)-sense, single-stranded RNA genome have been researched extensively for their potential as antiviral targets [3]: the capsid protein VP1 [12, 35], the protease [24, 30, 44], and the RNA-dependent RNA-polymerase

(RdRp) [8, 38, 51]. In the absence of a robust HuNoV cell culture system [23], murine NoV (MNV) has proved to be an attractive and widely used surrogate virus for HuNoV, including for drug development efforts [39].

To identify molecules that potentially could be repurposed as a broad spectrum norovirus antiviral, we chose molecules that have already been used in humans and docked them *in silico* into the MNV RdRp protein (PDB ID: 3UPF [31]). The “ZINC In Man” library was downloaded in SMILES format (<http://zinc.docking.org/browse/subsets/special>) [21, 22]. The 11421 molecules were converted to 15990 3D structures with LigPrep [5] by preparing different stereoisomers or protonation states. This *in silico* library was first docked with Glide-SP on chain B of the MNV RdRp [2, 13, 19]. The 2500 best molecules (i.e. with the lowest scores) were kept and docked with the extra-precision mode (XP) of Glide [14] on the same grid. The 37 molecules, which can be represented as 90 states (stereoisomers or tautomers), was next docked on the three chains of the MNV RdRp with the QM-Polarized Ligand Docking approach and the scores were averaged [1, 7]. This set of 90 molecules was also docked into the human norovirus RdRp (PDB ID: 1SH2 [33]). The docking outcome was then analyzed, and we removed the molecules which appeared not promising for testing as a potential drug due to their ubiquity in cells (for example NADH or sugars). Sixteen molecules with the lowest score for MNV RdRp and the accompanying scores for HuNoV RdRp are presented in Table 1.

Based on the scores for murine and human NoV RdRp and the price of the molecules, four compounds were selected for experimental testing in cell culture: ZINC28639308 (AD132644), ZINC11726792 (Sinefungin), ZINC72206342 (Rutin), ZINC04216682 (Kasugamycin). The predicted dissociation constants of these molecules with the MNV RdRp are 1.4, 66, 78 and 588 nM, respectively. Rutin hydrate (Sigma, Cat. No. R5143) and kasugamycin hydrochloride from streptomyces kasugaensis (Sigma, Cat. No. K4013) were freshly prepared in DMSO as a 10 mM stock

solution or in water as a 1 mM stock solution, respectively. Sinefungin (Sigma, Cat. No. S8559) and AD132644 (AnalytiCon Discovery, Cat. No. NP-005514) were dissolved in water as a 26.22 mM stock solution, or in DMSO as a 10 mM stock solution, respectively, and stored at 4°C. Since no reproducible HuNoV cell culture model was available [11, 23], we tested the antiviral activity of these compounds against MNV in the murine macrophage cell line RAW 267.4 (ATCC; Manassas, VA). Cells were maintained as described previously [47], and the plaque-purified MNV-1 clone (GV/MNV1/2002/USA) MNV-1.CW3 (referred herein as MNV-1) was used at passage 6. Our first goal was to determine the cellular toxicity of the selected compounds in RAW 264.7 cells using the WST-1 assay (Roche, San Francisco, CA), which measures mitochondrial dehydrogenase activity [16]. Concentrations that maintained cell viability at or above 80% were considered non-toxic. Concentrations equal or above 100 µM for rutin (Figure 1a), 0.5 µM for kasugamycin (Figure 1b), 1 mM for sinefungin (Figure 1c), and 5 µM for AD132644 (Figure 1d) maintain cellular viability.

In order to identify whether the selected drugs exhibited antiviral efficacy against NoV, RAW 264.7 cells were infected with MNV-1 (MOI of 2.0) in the presence of rutin, kasugamycin, sinefungin, and AD132644 at previously determined non-toxic concentrations, or equal amounts of vehicle control (i.e., H₂O or DMSO) (Figure 2). RAW 264.7 cells were plated at 5×10^5 per well in 12-well plates the day before. Infection occurred for 1 h on ice, after which the viral inoculum was removed by washing cells with ice-cold PBS three times before adding media containing one of the four drugs at indicated concentrations. Eight hours later, viral titers were determined by plaque assay as described previously [15]. A statistically significant difference between rutin and vehicle-treated cells was observed at 1 µM and 100 µM of drug (Figure 2a). However, no antiviral effect was observed at any of the concentrations tested for kasugamycin (Figure 2b), sinefungin (Figure 2c), and AD132644 (Figure 2d). Taken together, these data suggest that rutin but not kasugamycin, sinefungin and AD132644

have antiviral efficacy against MNV-1 in cell culture.

Rutin is a flavonoid which belongs to the family of vitamin C2 and is found in many vegetables (olive, asparagus), fruits (raspberry, plum) or cereals (buckwheat) (<http://phenol-explorer.eu/contents/polyphenol/296>). It is a compound with known antioxidant [49], anti-inflammatory [18] and anti-cancer effects [9, 28] and some of its derivatives are commercialized under the name Venoruton. To get more insight on the mechanism of action of rutin, we performed RNA synthesis assays using recombinantly expressed and purified MNV-1 and GII.4 HuNoV RdRps [50]. Surprisingly, rutin exhibited no inhibitory activity against either RdRp at all concentrations tested up to 5 μ M (see Supporting Information). In the same assay, benzodiathiadine, a potent non-nucleoside inhibitor of the HCV RdRp [10], inhibited RNA synthesis by the HCV RdRp at low micromolar concentrations (data not shown). To determine whether rutin acts as a prodrug, its activity was tested in a mammalian cell-based assay where transiently expressed RdRp stimulates retinoic acid-inducible gene I (RIG-I)-dependent reporter luciferase production via the beta interferon promoter [42]. Again no inhibition was observed at all concentrations tested (data shown in Supporting Information).

Therefore, we next hypothesized that rutin may be targeting another viral protein. Recent studies have reported that rutin is efficient for the inhibition of enterovirus 3C protease [29], which is a picornavirus, the most closely related virus family to caliviruses. Thus, we docked rutin in the norovirus protease (PDB ID: 3UR6 [24]): the predicted K_d was found to be of 588 nM (docking score of -8.5). Therefore, we performed FRET-based rutin inhibition assays with the MNV-1 and GII.4 HuNoV proteases [6] at concentrations of up to 100 μ M. Unfortunately, no inhibition of protease activity by rutin was observed (data shown in Supporting Information).

Taken together, the *in vitro* assays demonstrated that the antiviral activity of rutin is not mediated by the viral polymerase or protease, suggesting the antiviral activity is mediated by one or

more of the remaining viral proteins and/or unknown cellular proteins. MNV encodes nine proteins, which all could be potential targets [43]. However, it is unlikely that rutin targets the major capsid protein VP1, as no similarities were observed between rutin and other known small molecules targeting the VP1 capsid protein (such as suramin, heparin, citrate or fucose analogs) [3]. Another potential explanation is that rutin is a prodrug of the active compound, for example via its sugar part (rutinose), which is synthesized in the cell. Moreover, amongst the non-nucleoside analog RdRp inhibitors are styrylchromones [3], which share with rutin a chromone moiety. Styrylchromones [38] are capable of inhibiting MNV replication in cell culture through an unknown mechanism, but which is hypothesized to be via targeting the RdRp. However, since the cell-based RdRp assay tests demonstrated no inhibition, it is unlikely that rutin acts as a prodrug on the MNV RdRp. At this point, we cannot rule out that rutin interacts with one of the remaining six viral proteins NS1-5, VF1 or VP2. Time of addition experiments were performed and results are presented in Figure 3. We observed that no differences are found when 100 μ M of rutin are added one hour before infection, at the time of infection, one hour or four hours later. However, when 1 μ M of rutin is added, the % of virus titer drops from 76 to 39 depending if addition is made before infection or four hours after. These data lead us to hypothesize that rutin may act at the level of replication or later in the life cycle, but not at the stage of adhesion or internalization. Alternatively, the antiviral activity of rutin may be mediated through interaction with one or more cellular proteins needed during infection. This appears to be the most likely explanation as rutin inhibited MNV infection at a concentration of 1 μ M (Figure 2a), a concentration far below the cytotoxic concentration of rutin, 100 μ M (Figure 1a). However, identification of targets will require additional studies in the future.

In summary, we report that rutin, a flavonoid derivative, has antiviral efficacy against MNV-1 in cell culture. No inhibition was observed in *in vitro* assays with the viral polymerase or protease. The

exact mechanism of action of rutin that leads to antiviral effects remains to be identified; nonetheless, we hypothesize that rutin may act at the level of replication or later in the life cycle.

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Figure Legends:

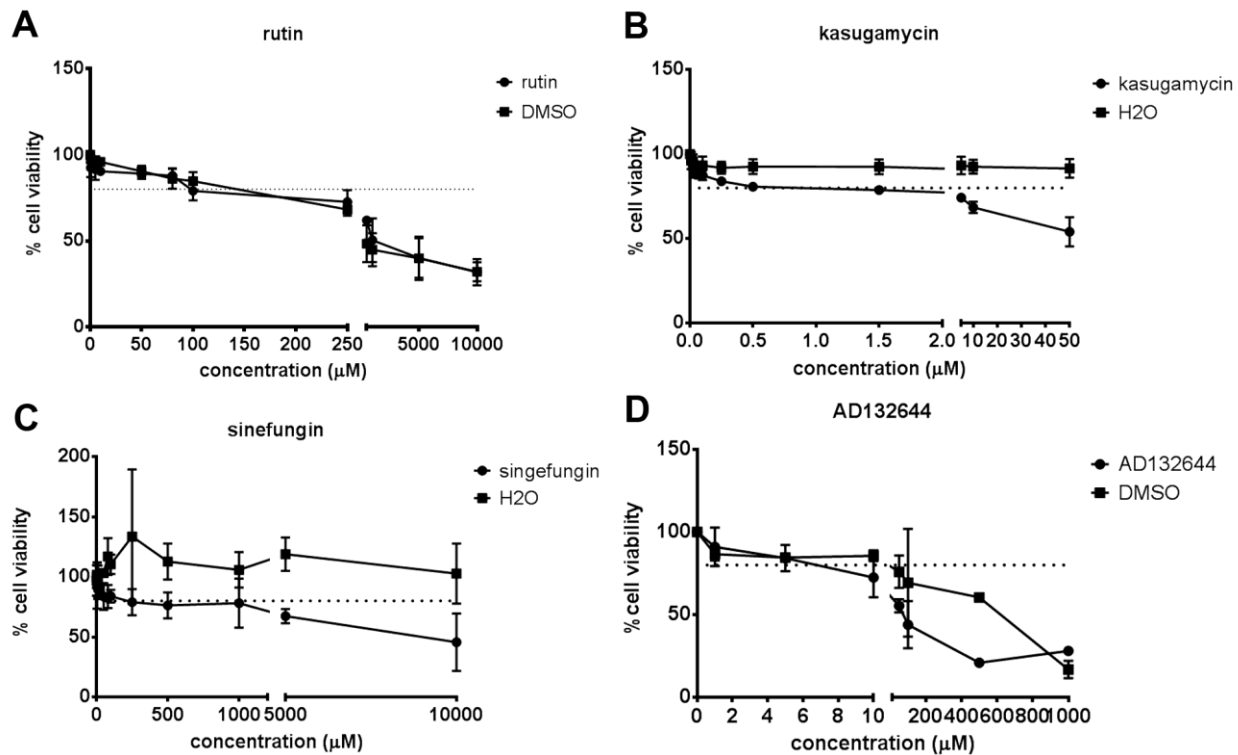


Figure 1. Cell viability following drug treatment. Cells were treated for 8 hours with indicated concentrations of (a) rutin; (b) kasugamycin; (c) sinefungin; (d) AD132644. Cells treated with vehicle were set to 100%. Drug concentrations resulting in cell viability above 80% were considered safe to use and are indicated by the dashed line. Data are presented as means ± SE of three independent experiments.

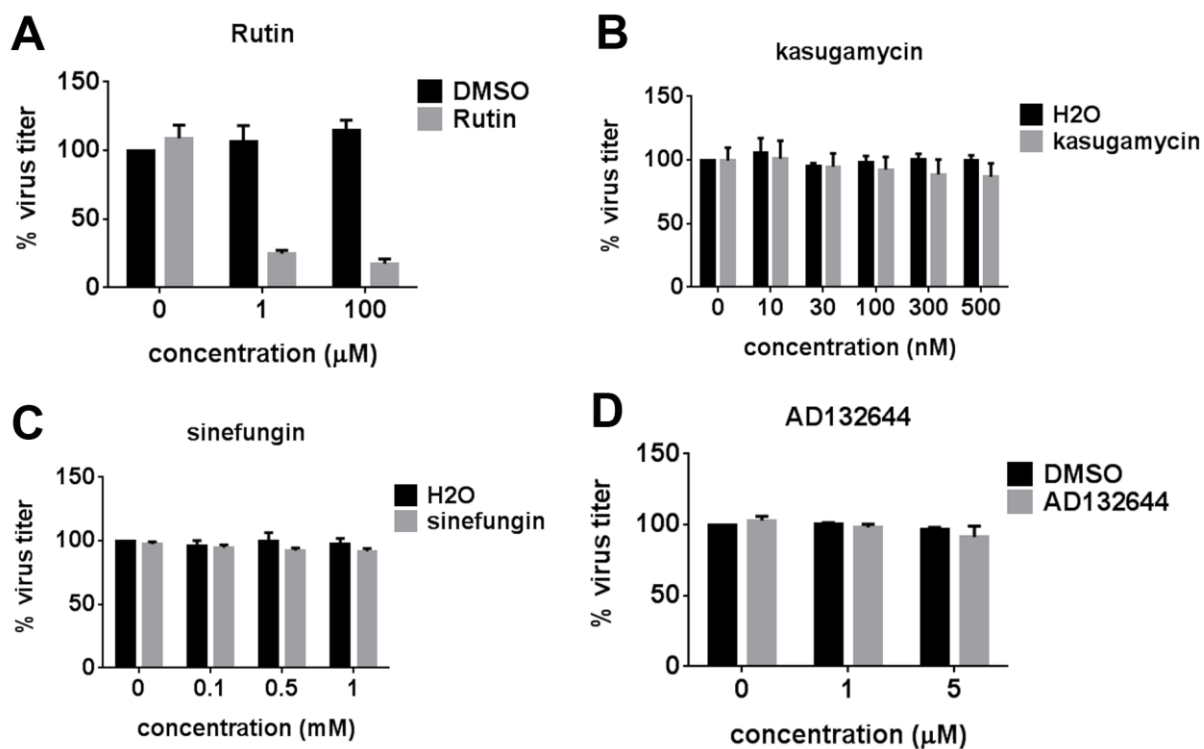


Figure 2. Antiviral efficacy of predicted RdRp inhibitors. Raw 264.7 cells were infected with MNV-1 (MOI of 2.0) for 8 hours in the presence of indicated concentrations of (a) rutin; (b) kasugamycin; (c) sinefungin; (d) AD132644. Viral titers were determined by plaque assay. Infected cells in the presence of the vehicle control were set to 100%. There is a statistically significant difference between the rutin and vehicle control group ($P < 0.05$, t test), but no significant difference between kasugamycin, sinefungin and AD132644 treatment and control groups. Data are presented as means \pm SE of three independent experiments.

Table 1. **Select candidates with highest docking scores^a after computational screening.**

ZINC ID	MNV Score^b	HNV Score^c	Price (\$)/$\mu$mol	Popular name
ZINC28639308	-12.1	-10.5	38	AD132644
ZINC11726792	-9.8	-9.6	17	Sinefungin
ZINC72206342	-9.7	-9.2	0.001	Rutin
ZINC08143614	-9.7	-7.2	1.32	Ouabain
ZINC04216676	-9.1	-8.4	not avail.	Kaempferitrin
ZINC26143524	-9.1	-6.8	163	Tezosentan
ZINC33359852	-8.9	-8.1	27	Lymecycline
ZINC04097715	-8.8	-9.9	4	Antirrhinin
ZINC04216682	-8.5	-10.4	0.01	Kasugamycin
ZINC03869383	-8.4	-8.9	1	(none)
ZINC01902827	-8.1	-9.1	15	Fidexaban
ZINC01910616	-8.1	-8.6	5	Dabigatran
ZINC03993831	-7.9	-8.7	81	Tomopenem
ZINC08214483	-7.9	-10.9	0.03	Amikacin
ZINC53684226	-7.4	-8.9	2	APAD
ZINC04557129	-7.3	-8.2	7	(none)

^a Docking scores are estimations of the binding free energy in kcal/mol.

^b Mean docking scores for murine norovirus (MNV) RdRp between the three chains of PDB 3UPF.

^c Docking scores for human norovirus (HNV) Norwalk virus RdRp from PDB 1SH2.

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