



Advances in the Development of Antiviral Compounds for Rotavirus Infections

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ABSTRACT Group A rotaviruses (RVAs) are the major cause of severe acute gastroenteritis (AGE) in children under 5 years of age, annually resulting in nearly 130,000 deaths worldwide. Social conditions in developing countries that contribute to decreased oral rehydration and vaccine efficacy and the lack of approved antiviral drugs position RVA as a global health concern. In this minireview, we present an update in the field of antiviral compounds, mainly in relation to the latest findings in RVA virion structure and the viral replication cycle. In turn, we attempt to provide a perspective on the possible treatments for RVA-associated AGE, with special focus on novel approaches, such as those representing broad-spectrum therapeutic options. In this context, the modulation of host factors, lipid droplets, and the viral polymerase, which is highly conserved among AGE-causing viruses, are analyzed as possible drug targets.

KEYWORDS antiviral, lipid droplets, rotavirus

Approximately 1.3 million deaths are caused by diarrheal diseases among all ages, with the main impact being on children under 5 years of age (1). Fatal gastroenteritis occurs mainly in developing countries, primarily due to poor hygiene conditions and malnutrition, causing about 300,000 deaths/year in sub-Saharan Africa. However, fatality should not be neglected in high-income countries, where approximately 700 deaths occurred in 2015 (2). Group A rotavirus (RVA) is the most frequent diarrhea-causative agent and continues to be the leading cause of diarrhea-associated mortality among children younger than 5 years, responsible for nearly 130,000 deaths worldwide in 2016 (3). In the small intestine, sodium transport and glucose transport are coupled, which means that the latter also promotes the absorption of solutes and water; therefore, the clinical management of RV-associated diarrhea and dehydration in children is based primarily on the administration of oral rehydration solutions (ORS) (4). ORS are balanced mixtures of glucose and electrolytes that stimulate water absorption to prevent dehydration. Although ORS are effective, their formulation still needs optimization (5, 6).

To decrease the prevalence of diarrhea-associated mortality, the World Health Organization (WHO) recommended the inclusion of RVA vaccines in all national immunization programs of the European region and the Americas in 2006, and this recommendation was extended to all regions worldwide in 2009 (7). The positive impact of RVA vaccination in public health has been demonstrated. However, the incidence and mortality rate of RV infection in high- and low-income locations continue to be quite different from each other. This scenario strongly supports the necessity of antiviral compounds to decrease the number of child deaths as a consequence of acute gastroenteritis (AGE) dehydration.

RV is a segmented double-stranded RNA (dsRNA) virus of the *Reoviridae* family (8). Under an electron microscope, RV is observed as a nonenveloped icosahedral triple-layered particle

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(TLP) of ~100 nm in diameter that resembles a wheel (Latin *rota*) (9). RV has an ~18,500-bp segmented genome with 11 dsRNA molecules that encode six structural proteins (viroplasm 1 [VP1] to -4, VP6, and VP7) and five or six nonstructural proteins depending on the strain (NSP1 to -5/6) (10, 11). Each of the 11 dsRNA segments is associated with one copy of the RNA-dependent RNA polymerase (RdRp) VP1 and the RNA-capping enzyme VP3 (12, 13). Each of the 11 complexes is located beneath each of the 12 5-fold symmetry axes of the innermost layer (14, 15). The innermost icosahedral shell is formed by 60 dimers of the VP2 protein organized in a T=1 lattice, which is an architectural hallmark among nearly all dsRNA viruses and which, once assembled, will never be disassembled during the viral cycle; it constitutes the core or single-layered particle (SLP) of RV (8). The SLP is surrounded by an ~15-nm-thick icosahedral T=13 capsid made up of 260 pear-shaped VP6 trimers to form the double-layered particle (DLP) (15, 16). In addition to being highly abundant and conserved, VP6 proteins have been shown to be both antigenic and immunogenic (17–21). The DLP is the RV transcriptional machinery that produces capped, nonpolyadenylated, positive-sense, single-stranded RNA (+ssRNA) molecules, capable of initiating an infectious cycle when released into the host cell cytoplasm (22, 23). The third layer is formed by the outer capsid proteins VP4 and VP7. With the same icosahedral triangulation (T=13) as the virion second layer, the glycoprotein VP7 forms 260 calcium-stabilized trimers that sit atop of each VP6 trimer (24). The VP7 layer constitutes the shell from which VP4 spikes protrude. Three copies of VP4 from each viral spike are anchored in the depressions formed in the center of VP7 hexamers (25). This third, outermost layer of the TLP contains the main viral antigens mediating viral entry into the host cell (26).

In this review, an update in the field of antiviral compounds is presented, mainly in relation to the latest findings in RVA virion structure and the infectious cycle. The possible treatments for RVA-caused AGE are also presented.

BIOCHEMICAL FEATURES OF THE RV INFECTIOUS CYCLE AND POTENTIAL TARGETS FOR THE DEVELOPMENT OF ANTIVIRAL COMPOUNDS AGAINST RV INFECTION

The RV infectious cycle is exquisitely synchronized with an ordered, stepwise disassembly and assembly of the viral particle layers. Updated data regarding RV life cycle, including the antiviral compounds described in this revision, have been incorporated into the model depicted in Fig. 1. The drug inhibitor class, the mechanism of action, and the stage of development for each compound are summarized in Table 1.

RV ENTRY INHIBITORS

The viral entry to the host cell is a multistep process occurring after viral attachment, endocytosis, and uncoating to allow the DLPs to reach the cytoplasm for +ssRNA transcription. RV entry is orchestrated by the continuous interaction of VP4 and VP7 with host cell molecules. *In vivo*, RV infects primarily mature enterocytes, but to become fully infectious, TLPs must be cleaved by trypsin-like proteases present in the intestinal lumen (27). This activation step cleaves VP4 to produce two main fragments, the N-terminal VP8* (at the tip of spikes) and the larger C-terminal VP5*.

There is a consensus that the initial virus attachment occurs through the interaction of VP8* with epithelial glycans (10, 28, 29). After that, and depending on the strain, RV interacts with integrins and the heat shock cognate (hsc) protein 70, which have been found to be associated with cell cholesterol-enriched membrane lipid microdomains (10, 28, 30–32). Also, it has been described that the presence of fatty acids enhances RV infectivity at a postattachment step for the SA11 RV strain (33). Using the bovine RV strain CH12, Cui et al. demonstrated that the pretreatment of cells with methyl- β -cyclodextrin (M β CD), a cholesterol-sequestering drug, decreased the levels of viral mRNA, VP4 accumulation, and RV progeny yield. These authors observed that the adsorption of viral particles to the cell membrane was not affected, and no effect was observed when the drug was added after the entry step. Therefore, the authors concluded that M β CD accomplished its antiviral effect by dispersing the cholesterol rafts, thus avoiding receptor-mediated endocytosis (34). Similar results were obtained by Dou et al. with

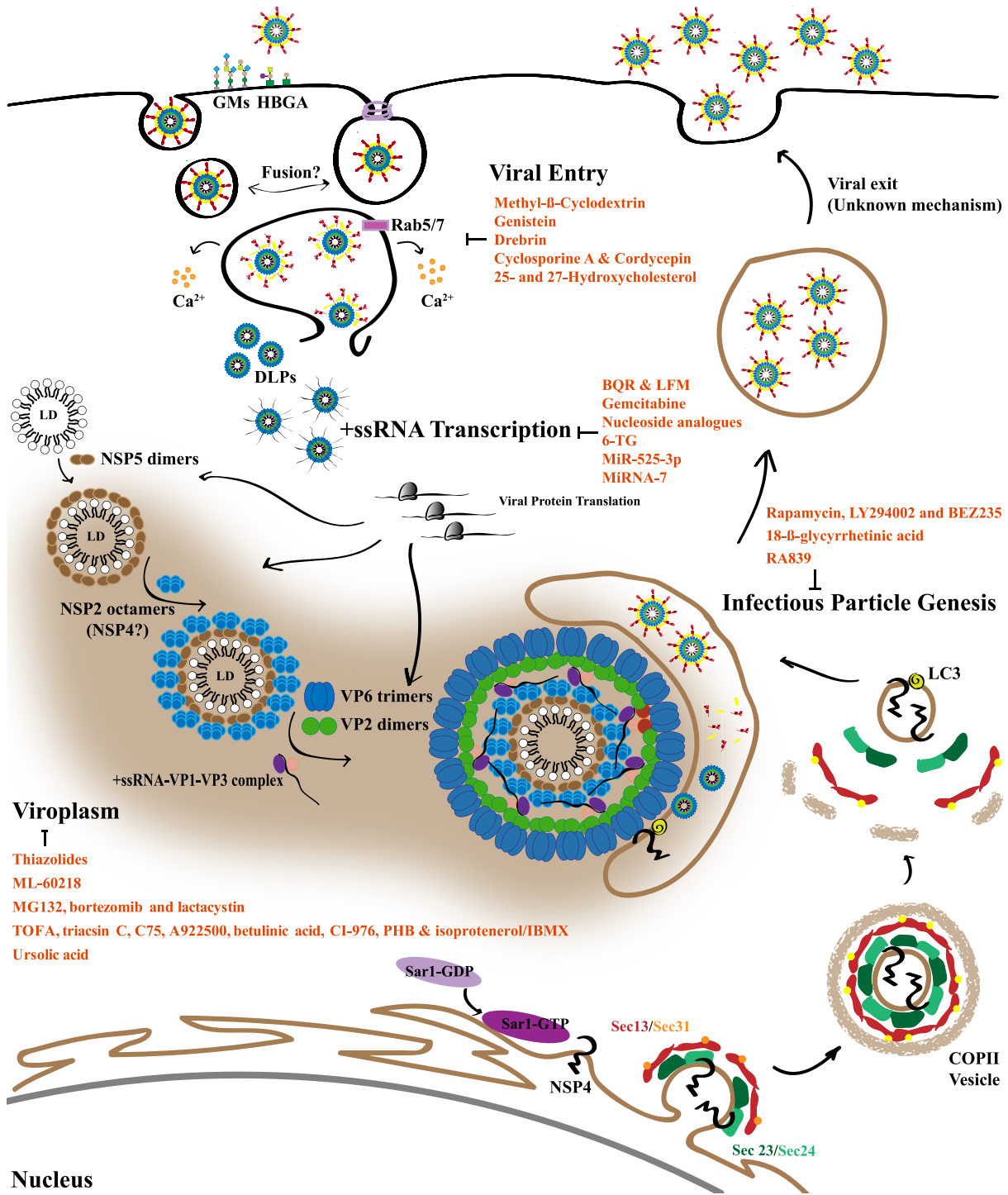


FIG 1 RV life cycle. The first step of the replication cycle consists of the interaction of the infectious particle with glycan attachment molecules. Then, the two models for RV entry are depicted. (Left) Engulfment of the virus in a tight-fitting membrane directed by viral components (38). (Right) Clathrin-dependent (or clathrin-independent) endocytosis, depending on strain. Once in the cytosol, homotypic fusion may occur for RV to finally reach a compartment where calcium ions are extruded from the vesicle to the cytoplasm, producing a progressive decrease of the calcium concentration and leading to the disassembly of the VP7/VP4 layer. Once devoid of the VP7 layer, the VP4 spike is able to perform the fold-back transition, leading to the vesicle bilayer burst and the DLP release into the cytoplasm to initiate viral +ssRNA transcription. After the translation of viral proteins, NSP5 and NSP2 form viroplasm (VPs), where morphogenesis occurs. VPs are large, membrane-less, electron-dense cytoplasmic structures. NSP2, in turn, associates with precursors (consisting of VP1, VP3, and segmental +ssRNA), VP2, and VP6 to give rise to immature DLP particles, which interact with NSP4 inserted into the ER. NSP4 is synthesized as an ER transmembrane protein and interacts with Sec24 to be incorporated into COPII vesicles that are released into the cytoplasm. GDP/GTP exchange on Sar1 induces the insertion of Sar1 into the ER and the subsequent recruitment of Sec23/Sec24. Sar1 interacts with Sec23, which allows Sec24 to bind the cargo protein, NSP4, and concentrates these proteins into the nascent COPII vesicle. LC3 interacts with NSP4 and inserts into the NSP4-containing, COPII-derived membranes. The LC3/NSP4-containing membranes traffic to VPs. DLPs produced in the VP interact with NSP4, which triggers particle budding through the LC3/NSP4-containing membranes and infectious particle assembly. The exit of TLPs is by cell lysis or budding (polarized cells).

TABLE 1 Antiviral compounds for rotavirus infections included in this minireview^a

Antiviral compound(s)	Class(es) of inhibitor(s)	Mechanism(s) of action (reference[s])	Stage of development
Methyl- β -cyclodextrin	Pharmacological cholesterol-sequestering agent	Dispersion of cholesterol rafts and inhibition of RV receptor-mediated endocytosis (34, 35)	Investigational <i>in vitro</i> studies
Genistein	Flavonoid, tyrosine kinase inhibitor	Inhibition of integrin phosphorylation, reducing RV binding affinity to integrins and entry (37)	Investigational <i>in vitro</i> studies
Drebrin	Cytoskeleton-associated protein	Interaction with VP4 protein and its C-terminal VP5* fragment, impairing RV cell entry (41)	Investigational <i>in vitro</i> studies; also tested in human primary enteroids and in an <i>in vivo</i> model (C57BL/6 mice)
Cyclosporine (CsA) and cordycepin	Cyclic peptide and adenosine analog, respectively	Increase in the expression of type I interferon (47, 48), promoting antiviral activity	Investigational <i>in vitro</i> studies; also tested in a mouse model (BALB/c infant mice); CsA is a well-known and widely used immunosuppressive agent
25- and 27-hydroxycholesterol	Oxysterols	Sequestering of cholesterol in late endosomes, impeding the DLPs from reaching the cytosol (54)	Investigational <i>in vitro</i> studies
Brequinar (BQR) and leflunomide (LEFM)	DHODH inhibitors	Reducing the host cell nucleoside pool and interfering with viral transcription and replication (63)	Investigational <i>in vitro</i> studies, tested in human primary enteroids; both compounds are well-known immunosuppressive agents
Gemcitabine	Cytidine analog	Inhibition of pyrimidine biosynthesis and reduction in the host cell nucleoside pool, interfering with viral transcription and replication (66)	Investigational <i>in vitro</i> studies, tested in human primary enteroids; gemcitabine can be found in the "safe-in-man broad-spectrum antiviral agents' library" and is widely used for cancer treatment
2'-C-methyl nucleoside analogs	Nucleoside analogs	Inhibition of viral RdRps (69)	Investigational <i>in vitro</i> studies
6-Thioguanine (6-TG)	Thio- analog of guanine	GTP-Rac1 inhibitor (71)	Investigational <i>in vitro</i> studies, tested in human enteroids; 6-TG has been clinically used since the 1950s as an anticancer and immune-suppressive agent
MIR-525-3p	Cellular microRNA	Binding the 3' UTR of RV NSP1 and increasing the concn of interferon and cytokines (74)	Investigational <i>in vitro</i> studies, tested in a mouse model (BALB/c infant mice)
miRNA-7	Cellular microRNA	Targeting the 11th RV gene that encodes NSP5, affecting VP formation (75)	Clinical trial for RV treatment; clinically approved anti-infective treatment
Nitazoxanide and tizoxanide	Thiazolides	Hampering NSP5-NSP2 interactions (88–90)	Investigational <i>in vitro</i> studies
ML-60218	Indazole sulfonamide	Disrupting assembled VPs and hampering the formation of new ones; structural damage on VP6 (97)	Investigational <i>in vitro</i> studies
MG132, bortezomib, and lactacystin	Tripeptide aldehyde, dipeptidylboronate, and antibiotic, respectively	Proteasome inhibitors; hampering the formation of VPs (100); altering VP1 localization (101)	Investigational <i>in vitro</i> studies
TOFA, triacsin C, C75, A922500, betulinic acid, CI-976, PHB, and isoproterenol/IBMX	Pharmacological enzyme inhibitors of the lipid metabolism pathway	Modulation of LD biogenesis and degradation (reviewed in reference 102)	Investigational <i>in vitro</i> studies
Ursolic acid	Triterpenoid	Modulation of LD metabolism (?) (106)	Investigational <i>in vitro</i> studies
Rapamycin, LY294002, and BEZ235	Macrolide, morpholine-containing chemical compound, and imidazoquinoline derivative, respectively	mTOR and PI3K inhibitors, respectively; activation of the autophagy cascade (125)	Investigational <i>in vitro</i> studies, tested in human enteroids; rapamycin is an FDA-approved drug for transplant patients
18- β -Glycyrrhetic acid	Aglycone	Modulation of the PI3K/Akt pathway; antiviral activity (127, 128)	Investigational <i>in vitro</i> studies, tested in a mouse model (C57BL/6 male mice)
RA839	Small molecule	Activation of the Nrf2/ARE pathway, modulating the redox stress response (132)	Investigational <i>in vitro</i> studies

^aAbbreviations: RV, rotavirus; LE, late endosomes; DLPs, double-layer particles; DHODH, dihydroorotate dehydrogenase; RdRp, RNA-dependent RNA polymerase; VPs, viroplasm; LD, lipid droplet; Nrf2, nuclear factor 2; ARE, antioxidant response elements; mTOR, mechanistic target of rapamycin; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B.

the porcine RV strain DN30209 (35). Integrins are also a target for small-molecule inhibitors. Taking into account that the activity of integrins is controlled by phosphorylation events (36), López et al. treated MA104 cells with the tyrosine-kinase (TK) inhibitor genistein to find a dose- and strain-dependent decrease of RV infectivity. They showed that the anti-RV activity may be attributed to an impairment of RV attachment. Simian SA11 and bovine RF strains were the most sensitive to the drug; the bovine UK, rhesus RRV, neuraminidase-resistant nar3, porcine YM, and human Wa strains were slightly affected, while the porcine TRF-41 strain was insensitive to genistein. Since the less affected strains were those that do not use integrins to invade the host cells, the authors hypothesized that the inhibition of the integrin phosphorylation mediated by TK might be responsible for the reduction of the RV binding affinity to integrins. Even though genistein was found to exert a dramatic reduction of RV infectivity, the drug was highly cytotoxic; therefore, it could not be used as an anti-RV treatment (37).

After the RV attachment to the cell surface, TLPs are internalized into the cytoplasm by receptor-mediated endocytosis (10, 32, 38). Several endocytic routes have been reported to be involved in RV entry, depending on the viral strain (recently reviewed in reference 39). Drebrin, a cytoskeletal protein involved in the stabilization of actin filaments, inhibits endocytosis (40). Li et al. observed that this protein interacts with VP4 and its C-terminal VP5* fragment within the TLP, impairing RV entry at a postattachment stage. Taking into account these biological effects and the fact that actin plays a role in the viral cycle, the authors proposed that the development of drebrin agonists may be a potential broad-spectrum antiviral strategy (41).

Independently of the entry route, all RV strains seem to be incorporated into early endosomes (EEs), since their infectivity depends on the activities of the well-known EE functional regulators Rab5 and EEA1 and the ESCRT (endosomal sorting complex required for transport) machinery (42, 43). On the other hand, the infectivity of several RV strains tested so far depends on the expression of Rab7, suggesting that these viruses travel farther inside the cell to reach late endosomes and behave as late-penetration (LP) viruses (44). The decrease of intraluminal calcium levels promotes the disassembly of the VP7 layer, and soon (typically within 10 min), the outer layer proteins are lost and transcriptionally active DLPs enter the cytosol (23). Infected cells then activate innate immunity mechanisms to reach an antiviral status through the synthesis and secretion of high levels of interferon (IFN) in response to the presence of viral RNA (45). RV has been demonstrated to evade this immune attack by inhibiting the production of IFN (46). Therefore, maintaining high IFN levels in infected cells may be an effective anti-RV strategy. Cyclosporine (CsA), a well-known immunosuppressant agent, has been evaluated as an anti-RV agent. CsA is known to increase the expression of type I IFN both *in vitro* and *in vivo*, causing a significant decrease in RV titers (47). Similar results were found with cordycepin, an adenosine analogue that induces a type I IFN response within the cells (48). Oxysterols are a family of cholesterol oxidation derivatives that exert a wide variety of biochemical effects, one of them being the inhibition of viral replication (49). The bulk of the studies on the antiviral activity of oxysterols has been focused on 25-hydroxycholesterol (25HC), which emerged as a wide-spectrum antiviral molecule against a large number of viruses *in vitro* (49). IFN is known to induce a marked increase in the levels of 25HC in macrophages and dendritic cells. It is an essential cytokine involved in the establishment of the cellular antiviral state through the upregulation of hundreds of interferon-stimulated genes (ISGs). Thus, 25HC can be considered an IFN effector (50). *Ch25* (IFN-inducible cholesterol 25-hydroxylase) is an antiviral ISG whose product converts cholesterol to 25HC, directly linking the innate antiviral response elicited by IFN to the sterol metabolic pathways. Importantly, the *Ch25* antiviral role against the emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has recently been described (51, 52). Civra et al. first tested 25HC and 27HC against the human Wa RV strain and other nonenveloped viruses, observing marked antiviral activities for these compounds (53). Later studies of Civra et al. aimed at ascertaining the anti-RV mechanism of 25HC and 27HC included the WI61, HRV 408, HRV 248, and DS-1 RV strains and

demonstrated marked antiviral activities for these compounds (53). All these strains are LP viruses, which require Rab7 to enter the cytosol. The authors showed that the anti-RV effect was mediated by the sequestering of cholesterol in late endosomes (LEs), preventing DLP exit from the LEs to reach the cytosol (54).

RV RNA TRANSCRIPTION AND GENOME REPLICATION INHIBITORS

The loss of the outermost layer and the arrival of DLPs to the cytosol promote the activation of VP1 and VP3 inside the DLP, allowing viral RNA transcription and the production of +ssRNA (10, 55, 56). The X-ray crystallographic structure of VP1 revealed that the RV RNA-dependent RNA polymerase (RdRp) domain lies within a cage-like sequence with two access channels to accommodate the template RNA and the nucleoside triphosphate substrates and two channels for the exit of ssRNAs in the transcription function and of dsRNA in the replication function. This unique fingers, palm, and thumb organization of RdRp VP1 is shared only with the mammalian orthoreovirus RdRp, λ 3 (57–59). Two recent *in situ* studies on RdRp structures have shed light on the transcription and replication mechanisms mediated by VP1 (60, 61). For this step, VP1 requires the biosynthesis of nucleosides by the host cell (62). Chen et al. tested the anti-RV effect of brequinar (BQR) and leflunomide (LFM), two inhibitors of dihydroorotate dehydrogenase, which is an enzyme involved in the biosynthesis of pyrimidines. The authors described the anti-RV effect both *in vitro* and in human enteroids, observing a reduction in viral protein synthesis and a decrease in RV yields for the simian SA11 RV strain and the human clinical isolate 2011K. Based on these observations, the authors claimed that the antiviral effect of BQR and LFM could not be attributed to the strain but to a reduction in the host cell nucleoside pool. The concentrations of both compounds needed to exert the antiviral activity were much lower than the doses used for the treatment of other infections, thus achieving high antiviral efficacy with few side effects (63). On the other hand, gemcitabine is a cytidine analog that inhibits the pyrimidine biosynthesis that is used as an anticancer agent (64). Previous studies showed that gemcitabine is an antienterovirus agent (65). Thus, Chen et al. evaluated the effect of gemcitabine on the rhesus RRV strain and five clinical isolates *in vitro* using Caco2 and MA104 cells and enteroids. Gemcitabine exerted a significant inhibition of RV replication, as evidenced by a decrease in the viral yield, a decrease in the accumulation of viral proteins, and a higher degree of conservation of organoid morphology. Thus, the authors proposed gemcitabine as a feasible anti-RV treatment, mainly in infections of patients with cancer (66). Importantly, the antiviral effect of gemcitabine has already been described for other RNA viruses (67, 68); therefore, the possible use of gemcitabine as a broad-spectrum antiviral cannot be ruled out.

Despite the unique organization of the RV VP1 RdRp domains, its functioning closely resembles that of other viral RdRps (59). Thus, the inhibition of the viral polymerase is a promising antiviral target, not only against RV but also against several related RNA viruses. In this context, Van Dycke et al. studied the effect of four analogs of 2'-C-methyl nucleoside (2'-C-methylguanosine, 2'-C-methyladenosine, 2'-C-methylcytidine, and 7-deaza-2'-C-methyladenosine) against the main RNA AGE-causing viruses, i.e., RV, norovirus, and sapovirus, to find a significant antiviral effect against the three of them. All nucleoside analogues studied proved to be effective anti-RV agents at noncytotoxic concentrations, as evidenced by a decreased accumulation of viral RNA and VP formation, a reduced cytopathic effect, and lower viral yields (69). The nucleoside analogs have been proposed as broad-spectrum antivirals against AGE-causing viruses (70). The clinical symptoms of all viral AGE are very similar; therefore, the availability of a broad-spectrum treatment might allow early treatment initiation, i.e., before virus identification. Finally, 6-thioguanine (6-TG), a thio-analog of guanine widely used as immunosuppressive drug in transplant patients, was tested by Yin et al. (71) in RV infections. The authors demonstrated that 6-TG promoted a decrease in RV +ssRNA levels and viral protein synthesis. The potent anti-RV effect of 6-TG was due to the inhibition of Rac1. Rac1 is a member of the Rho GTPases that is involved in multiple

biological processes (72). The role of GTP-Rac1 in RV infection is not clear, but it seems to be necessary for RV replication. This hypothesis was further confirmed when NSC23766, another GTP-Rac1 inhibitor, was demonstrated to have anti-RV properties similar to those of 6-TG (71). It is noteworthy that the anti-RV effect of 6-TG was tested in culture cells and in human organoids using the simian SA11 RV strain and clinical strains to obtain the same results in all cases. Although further research is needed, these authors proposed 6-TG as a promising anti-RV treatment.

MicroRNAs (miRNAs) are cellular short RNA molecules that regulate gene expression by controlling mRNA transcription and stability. The antiviral immunity mediated by IFN also involves the overexpression of multiple miRNAs (73). Among them, MiR-525-3p and miRNA-7 have been studied for their anti-RV properties. MiR-525-3p binds the 3' untranslated region of RV NSP1, decreasing its expression, and the accumulation of MiR-525-3p is sufficient to inhibit the RV cycle by increasing the concentration of IFN and other cytokines produced by infected cells. Importantly, its anti-RV effect seems not to be strain dependent, since human Wa, simian RRV, and simian SA11 RV strains were effectively inhibited (74). miRNA-7, whose expression is regulated by the host cell, has been described to exert antiviral effects by targeting viral genes or host pathways hijacked by RV. Zhou et al. showed that miRNA-7 is significantly upregulated in RV-infected cells in a multiplicity of infection (MOI)-dependent, strain-independent manner. The wild-type ZTR-68 and ZTR-18, simian SA11, human S2, and porcine Gottfried RV strains were tested. The authors determined that miRNA-7 targets RV gene 11, which encodes NSP5, thereby affecting VP formation and ultimately inhibiting the production of a new RV progeny. The anti-RV effect exerted by miRNA-7 was observed both *in vitro* and *in vivo* (75).

VP BIOGENESIS AND STABILIZATION INHIBITORS

Replication and packaging of the viral genome into the progeny DLPs take place in specialized, large, membrane-less, electron-dense cytoplasmic inclusions called viroplasm (VPs). NSP5, the main VP protein, together with NSP2 (76), VP2 (77), and lipid droplets (LDs) (78, 79), is crucial for VP formation. The coexpression of NSP5 with either NSP2 or VP2, in the absence of other viral structures, leads to the formation of cytosolic inclusions, named VP-like structures (VLS), which are morphologically similar to VPs but are unable to produce viral progeny (76, 77, 80–82). By reverse-genetics approaches, a phosphorylation-dependent mechanism for VP formation in which the phosphorylation of both NSP2 and NSP5 was required for them to interact and give rise to VPs was demonstrated (83–85). Thiazolides are a big family of widely used anti-infective compounds. The use of thiazolides, particularly nitazoxanide, has been licensed in the United States for the treatment of several AGE-causing agents (86, 87). Rossignol and El-Gohary (88) evaluated its anti-RV effect in a randomized, placebo-controlled, double-blind clinical trial conducted in the Cairo University Children's Hospital to evaluate pediatric patients. A similar trial was conducted simultaneously in the Benha University Hospital and at the Alexandria University Hospital to evaluate adolescent to adult patients. In all cases, the administration of nitazoxanide significantly reduced the duration of RV AGE, compared to that in control groups (88, 89). Then, La Frazia et al. studied the mechanism of nitazoxanide and its active metabolite, tizoxanide, in an *in vitro* RV model. The authors observed that the treatment of cells with the compounds at 0 to 3 h after RV infection caused the highest reduction in VP size without affecting either viral attachment or viral entry or protein synthesis. Finally, they demonstrated that thiazolides hamper the NSP5-NSP2 interaction, concluding that thiazolides disturb VP formation and stabilization, leading to reduced RV yields (90). The use of nitazoxanide as anti-RV therapy may be a feasible option since it is an approved drug and clinical trials in humans have been conducted to test its effectiveness (91, 92).

Several other RV proteins are involved in VP formation. Buttafuoco et al. recently studied the role of NSP5-VP2 interaction in VLS formation. The authors identified the interacting domains on both proteins and showed that a mutant version of VP2, which is unable

to interact with and promote NSP5 phosphorylation, also impedes the VLS formation induced by NSP5-VP2 or NSP5-NSP2 coexpression (93). Aside from these nonstructural proteins, VP1, VP3, VP6, and NSP4 constitute the VPs (94–96). VPs play a key role in the maintenance of the RV infection; therefore, any compound affecting either their synthesis or their structure can be considered a good candidate for the treatment of AGE. One of these compounds is the small molecule called ML-60218 (ML). Eichwald et al. demonstrated that ML affects the formation of VPs and disrupts the assembled ones. Besides, ML-60218-treated DLPs exhibited structural damage that could be attributed to an impairment in the interaction among VP6 trimers that affected their transcriptional activity. However, the authors could not postulate this compound as a possible anti-RV treatment because of its high cytotoxicity and low solubility (97).

The ubiquitin-proteasome system has been described to be modulated by some viruses during infection (98). The proteasome is a complex system that degrades ubiquitinated, altered proteins (99). Contin et al. tested the proteasome inhibitors MG132, bortezomib, and lactacystin in RV infections. These compounds induced an arrest of VP formation, leading to a significant reduction of RV yields of the simian SA11, porcine OSU, and bovine RF strains *in vitro*. The authors observed that the main effect was achieved at early time points of infection but after RV attachment and entry, while no effect was observed on assembled VPs. Thus, they hypothesized that an active proteasome may be necessary to degrade a host protein or proteins that hamper the formation of VPs at early time points of RV infection (100). On the other hand, López et al. also tested the effects of the proteasome inhibitors MG132, lactacystin, and proteasome inhibitor I on the RRV strain. The authors evidenced a significant reduction in RV yields, with MG132 having the strongest effect. These results suggested that the anti-RV effect of MG132 might be partially due to a reduction in the free-amino acid pool, which is necessary for the cellular and viral biosynthetic machinery, and by the accumulation of ubiquitinated proteins. They also observed a reduction in RV replication that might be explained by the presence of smaller VPs and an altered distribution of VP1 (101).

Among the cellular factors, lipid droplets (LDs) are ER-derived intracellular organelles for neutral lipid storage that are required for VP formation. The formation of complexes between VPs and LDs is crucial for RV replication, since compounds disrupting LDs significantly decrease the numbers and sizes of VPs and the RV yield (78, 102). In a recent study, Suárez et al. proposed a model of the nanometric-scale organization of VPs (103). The authors showed that the viral components in the VPs form porous circumferences that are arranged as quite discrete concentric layers. Interestingly, this model proposes the existence of a hollow center inside the first layer formed by NSP5, which might be complemented with a previous observation demonstrating the role of LDs (102). In this context, several inhibitors of lipogenic enzymes have proved to interfere with RV infection. Thus, triacsin C was demonstrated to reduce VP size and number with the corresponding reduction in the viral yield (78, 104). Likewise, tall oil fatty acid (TOFA), alone or in combination with C75, reduces both the infectivity of progeny virus and viral dsRNA production in a time- and dose-dependent manner (105). Reduced viral yields were also observed in RV-infected cells treated with A922500, betulinic acid, CI-976, or polyhydroxybutyrate (PHB) (104). On the other hand, targeting the dispersion of LDs with isoproterenol and isobutylmethylxanthine (IBMX) in RV-infected cells resulted in a reduced number and size of VPs, a decreased production of viral dsRNA, and a 20- to 50-fold-lower yield of infectious progeny (78). In our laboratory, we showed that ursolic acid (UA), a natural pentacyclic triterpenoid, is a potent anti-RV compound *in vitro*. UA significantly decreased RV yields at noncytotoxic concentrations, and its anti-RV effect was maintained when the RV MOI was increased. UA affected the early stages of the RV replication cycle, as evidenced by a significant reduction in the number and size of VPs, the level of viral proteins, and RV yields (106). Since UA is a hypolipidemic agent, we can hypothesize that the anti-RV activity exerted by UA *in vitro* is mediated by the decrease in the availability of LDs for the virus to build the VPs. Due to its multiple biological properties, UA has been extensively

studied over the last years (107, 108). Its low bioavailability is the main disadvantage; however, several chemical modifications have successfully been introduced to overcome this obstacle, while none of them has yet been proved to treat RV infection. UA is an FDA-approved drug, representing an attractive candidate for the treatment of viral infections in humans (109, 110).

Within the VPs, NSP2 sorts the RNA complex containing VP1, VP3, and viral +ssRNA. This complex engages VP2 for SLP assembly, while VP1 within newly formed SLP directs the dsRNA synthesis using +ssRNAs as the templates (60, 78, 111, 112). Within the RVA 3' untranslated region (3' UTR), Ren et al. (113) identified ATP5B, a core subunit of the mitochondrial ATP synthase, which binds to the consensus region of the 3' UTR. During RV infection, ATP5B colocalizes with the viral RNA and the VP. The small interfering RNA (siRNA)-mediated genetic depletion of ATP5B reduced the production of infectious viral progeny, with significant alteration of intracellular viral RNA levels and RNA translation. Moreover, the treatment of conventional cell cultures and human enteroids with a panel of small-molecule pharmacological inhibitors of the ATP synthase diminished RV yields, indicating that ATP5B positively regulates RV genome assembly and particle formation (113).

RV MORPHOGENESIS INHIBITORS

RV morphogenesis starts with the packaging of genome segments in newly formed viral cores, followed by the addition of a second layer of VP6 trimers to form DLPs, which subsequently bud into endoplasmic reticulum (ER) membranes for TLP assembly, although the specific mechanism for the exit of DLPs from VPs remains unknown. The TLP assembly is a unique morphogenetic process that requires NSP4, which is synthesized as an ER transmembrane glycoprotein and with which the glycoprotein VP7 associates within the luminal ER membrane (114–117). The C-terminal cytoplasmic domain of NSP4 hooks the DLPs through binding to VP6, triggering the budding of the DLP through the NSP4-containing membranes, where the particles become transiently enveloped (118). The transient lipid envelope is then removed by an unknown mechanism, and the outer capsid proteins VP7 and VP4 are assembled onto the particle, giving rise to the mature infectious TLP. The intracellular membranes used for particle budding were long thought to be those of the ER because NSP4 is synthesized as an ER transmembrane protein. However, a recent study has shown that NSP4 exits the ER by hijacking ER exit sites in coat complex protein II (COPII) vesicles and that the NSP4-containing COPII vesicles are encountered by the autophagy machinery in the cytoplasm; together, the COPII vesicles and the autophagy machinery mediate the trafficking of NSP4 to VPs. It has been described that NSP4 interacts with Sec24 to be incorporated into COPII vesicles that are released into the cytoplasm. GDP/GTP exchange on Sar1 induces the insertion of Sar1 into the ER and the subsequent recruitment of Sec23/Sec24. Sar1 interacts with Sec23, which allows Sec24 to bind the cargo protein NSP4 and concentrates these proteins into the nascent COPII vesicle. LC3 interacts with NSP4 and inserts itself into the NSP4-containing, COPII-derived membranes. The LC3/NSP4-containing membranes traffic to VPs. These findings support a change in the paradigm as regards membrane origin for RV morphogenesis (119). Although the innate immune response is crucial in viral infections (45), the role of autophagy, an innate immune mechanism, in RV infection is still controversial (120–122).

Many immunosuppressive agents have been tested as possible anti-RV drugs. In this sense, rapamycin is an FDA-approved immunosuppressant used to prevent allograft rejection because of its capacity to inhibit the mammalian target of rapamycin (mTOR), leading to the activation of autophagy (123). Autophagy is modulated by the phosphatidylinositol 3 kinase (PI3K)-protein kinase B (Akt)-mTOR pathway, in which the ultimate phosphorylation/activation of mTOR inhibits the autophagy cascade by the activation of 4E-binding protein 1 (4E-BP1) (124). Yin et al. observed that the use of rapamycin inhibits RV infection by activating the autophagy cascade. These authors also observed that the PI3K inhibitor LY294002 and the PI3K-mTOR inhibitor BEZ235, as well as silencing the

mTOR protein expression, effectively reduced RV replication. The authors proposed rapamycin as a feasible anti-RV treatment, especially in transplant patients (125). 18- β -Glycyrrhetic acid (18 β GRA) is an aglycone and the active metabolite of glycyrrhizin, which is present in the licorice root (126). Hardy et al. (127) and Hendricks et al. (128) observed that 18 β GRA presents anti-RV activity both *in vitro* and *in vivo*. The antiviral mechanism is not totally clear, but the authors hypothesized that the effects of 18 β GRA might be due to its capacity to modulate the PI3K/Akt pathway, as previously demonstrated by Kao et al. (129, 130). The induction of the synthesis of chemokines and the expression of chemokine receptor genes facilitate the recruitment of B cell populations to the gut mucosa, then ameliorating RV-caused diarrhea (127–130).

The cell metabolism produces multiple reactive oxygen species (ROS). As these molecules are harmful when accumulated in great amounts, cells have developed multiple mechanisms to maintain metabolic homeostasis (131). Among them, nuclear factor 2 (Nrf2) is a transcription factor that targets multiple antioxidant response element (ARE) genes involved in the restoring of the reduced cellular environment. Under basal conditions, Nrf2 interacts with the cytosolic Keap1 protein, which prevents Nrf2 translocation to the nucleus. The RV infection is known to cause ER stress, with the subsequent release of ROS from the mitochondria and the induction of the unfolded protein response (UPR) (131). RA839 is a small molecule that specifically binds Keap1, causing Nrf2 to translocate to the nucleus. The treatment of cell cultures with RA839 resulted in an increased expression of antioxidant genes. Patra et al. have demonstrated that RA839 effectively inhibits RV replication, as evidenced by a decrease in the number of VPs, a lower degree of accumulation of structural RV proteins, and lower viral yields. Interestingly, RA839 exerts a potent anti-RV activity mediated by the activation of the Nrf2/ARE pathway at noncytotoxic concentrations *in vitro*. The authors hypothesized that the anti-RV effect of RA839 might be due to the modulation of the redox stress response (132). Antioxidants such as *N*-acetyl cysteine and some anti-inflammatory drugs have also been described as effective treatments in RV-induced AGE, highlighting the importance of the stress response against RV infections (131).

RV RELEASE INHIBITORS

The final step of the RV life cycle is the TLP release from the infected cell. It has traditionally been thought that RVs are released from nonpolarized cells through a lytic process, with the loss of cell integrity (133–135). Studies using differentiated Caco-2 cells have shown that new virions are almost exclusively released from the apical pole (136–138). Recent studies have additionally demonstrated a crucial role of the actin cytoskeleton in efficient RV exit (139–142). Trejo-Cerro et al. used jasplakinolide, a well-known actin cytoskeleton inhibitor, to show a loss of the preferential apical release of RV from MA104 cells, suggesting a major role for actin in virus release (142). The authors showed that the fraction of extracellular virus was small compared to that of virus that remains associated with the cell; however, based on the notion that this egress mechanism seems to operate in nonpolarized as well as polarized cells, the authors claimed that the actin-dependent viral release might have an important role in the dissemination of the virions *in vitro* and probably *in vivo* (142).

CONCLUSIONS

Despite the availability of four oral, live, attenuated anti-RV vaccines, RV remains one of the main causes of morbidity and mortality in young children. Several authors have demonstrated the antiviral activities of multiple compounds disrupting the RV replication cycle at different stages (Table 1). Only nitazoxanide has been evaluated in a human double-blind placebo trial in which a significant amelioration of RV AGE was observed. None of the studied compounds so far is currently used as an effective anti-RV treatment.

The host response to viral infections has been an important object of study to find antiviral targets and to develop drugs or gene silencers as potential anti-RV therapies. Some metabolic pathways are highly conserved among cell lines, and since these

pathways are used by many viruses, they might serve as targets for broad-spectrum antiviral treatments, as is the case for lipid metabolism. The host provision of LDs is indispensable to establishing a productive RV infection. Then, the use of lipid modulators is an attractive therapeutic approach and constitutes our rational base to study the potential anti-RV effect of UA. Members of the *Flaviviridae* family, namely, dengue virus, hepatitis C virus, West Nile virus, and Zika virus, interact with LDs to usurp the host lipid metabolism to carry out their own viral replication and pathogenesis (143). Therefore, compounds interfering with LD metabolism might be used as extended-spectrum antiviral drugs, including those against viruses infecting humans.

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