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Article

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Targeting G-quadruplexes in the rhinovirus genome by Pyridostatin inhibits uncoating and highlights a critical role for sodium ions.

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ABSTRACT

The ~2.4 μm long rhinovirus ss(+)RNA genome consists of roughly 7,200 nucleotides. It is tightly folded to fit into the ~22 nm diameter void in the protein capsid. In addition to previously predicted secondary structural elements in the RNA, using the QGRS mapper, we revealed the presence of multiple quadruplex forming G-rich sequences (QGRS) in the RV-A, B, and C clades, with four of them being exquisitely conserved. The biophysical analyses of ribooligonucleotides corresponding to selected QGRS demonstrate G-quadruplex (GQ) formation in each instance and resulted in discovering another example of an unconventional, two-layer zero-nucleotide loop RNA GQ stable at physiological conditions. By exploiting the temperature-dependent viral breathing to allow diffusion of small compounds into the virion, we demonstrate that the GQ-binding compounds PhenDC3 and pyridostatin (PDS) uniquely interfere with viral uncoating. Remarkably, this inhibition was entirely prevented in the presence of K⁺ but not Na⁺, despite the higher GQ stabilising effect of K⁺. Based on virus thermostability studies combined with ultrastructural imaging of isolated viral RNA, we propose a mechanism where Na⁺ keeps the encapsidated genome loose, allowing its penetration by PDS to promote the transition of QGRS sequestered in alternative metastable structures into GQs. The resulting conformational change then materialises in a severely compromised RNA release from the proteinaceous shell. Targeting extracellularly
circulating RVs with GQ-stabilisers might thus become a novel way of combating the common cold.

INTRODUCTION

Rhinoviruses (RVs) are the etiologic agents of the common cold, a usually mild upper respiratory illness. Nevertheless, RVs are increasingly found also to cause severe lower respiratory tract diseases \(^1\) \(^2\). Due to the lack of vaccines and effective treatment options, their socio-economic impact through lost working days and cost of symptom-alleviating medication is in the order of billions per year in the USA alone \(^3\) \(^4\). Belonging to the family \textit{picornaviridae}, RVs are built from 60 copies of each four capsid proteins (VP1 through VP4) that enclose a single-stranded ~7.2 kb long (+) RNA genome. VPg, a 21 amino acid long peptide, is covalently linked to its 5’-end via a tyrosine phosphodiester bond, and a genome-encoded poly-(A) tail extends its 3’-end. Infection initiates upon recognition of cell-surface receptors. These include the low-density-lipoprotein receptor (LDLR) and related proteins, intercellular adhesion molecule 1 (ICAM-1), and cadherin-related family member 3 (CDHR3), depending on the RV-receptor group (minor or major) and the RV species (A, B, or C). Currently, more than 170 RV types are known [https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/picornavirales/w/picornaviridae/681/genus-enterovirus].

Upon uptake into the host cell by receptor-mediated endocytosis, the native RV particle (~30 nm diameter) converts into the expanded (i.e. altered) A-particle (~31.2 nm diameter) with holes at the 2- and 3-fold axes of icosahedral symmetry. This conformational change is triggered by the endosomal acidic pH and/or the viral receptor. Concomitant with expansion, VP4 is lost, N-terminal amphipathic sequences of VP1 are externalised, and contacts between the RNA and the inner wall of the capsid become modified \(^5\) \(^6\). Data on the closely related poliovirus and enterovirus 71 \(^7\) \(^8\) suggest that the viral genome, in the form of a single strand, exits through a hole in the capsid, which is tightly connected to a pore in the endosomal membrane, thereby forming an RNase-inaccessible channel \(^9\) \(^10\). On the other hand, \textit{in vitro} studies with acidified echovirus 18, another member of the \textit{Enterovirus} genus, have shown that entire pentamers of coat protein detach from the capsid, giving rise to large holes allowing for the exit of incompletely unfolded RNA \(^11\). The same group also observed about 7 % of such open particles in a very recent cryo-EM analysis of ICAM-1 triggered uncoating of RV-B14 in combination with a low pH \(^12\).

On arrival of the RV genome in the cytosol, the VPg is removed by a cellular unlinkase activity, and the RNA used as a template for an internal ribosomal entry site (IRES) directed translation by host cell ribosomes. The resulting polyprotein is co-translationally and autocatalytically processed by the viral proteinases \(2A^{\text{pro}}\) and \(3C(D)^{\text{pro}}\) into structural and
non-structural proteins\textsuperscript{13}. Viral proteins such as 2C and 3A together with recruited host cell proteins including lipid kinases build the replication complex (RC), which is intimately associated with the outer surface of virus-induced single and double-membrane vesicles. These are the sites for the production of (−) ssRNA, in turn serving as a template for the production of multiple copies of (+) ssRNA in separate RCs, both mediated by the viral RNA-dependent RNA polymerase 3D\textsuperscript{pol}. The newly produced (+) ssRNAs are used for further translation, the synthesis of additional (−) ss RNAs, or for packaging into provirions, followed by a rapid maturation cleavage of their immature capsid protein VP0 into VP2 and VP4. The resulting progeny virions are then released by lytic and non-lytic mechanism from the infected host cell (reviewed in\textsuperscript{14,15}).

Up to date, no vaccines are available against RVs and in the absence of approved antiviral drugs the therapeutic treatment is centred on the relief of the common cold symptoms. One prominent route in developing new antiviral drugs is targeting viral proteins or host factors vital for the virus replication (e.g. Baggen, Thibaut\textsuperscript{16}). However, targeting viral polypeptides often results in the rapid emergence of drug-resistant mutants, while compounds acting against host factors may give rise to substantial cellular toxicity. An alternative approach targets essential domains in the highly structured genomes of RNA viruses, limiting the development of resistance\textsuperscript{17}. In that context, G-quadruplexes (GQs) have recently emerged as promising antiviral drug targets\textsuperscript{18}.

GQs are 4-stranded higher-order nucleic acid structures formed intra- or intermolecularly by guanine (G) rich stretches of DNA or RNA. The basic unit consists of a square, co-planar arrangement of four guanines (a tetrad or G-quartet) connected via Hogsteen hydrogen bonding. Their self-stacking via π–π interactions results in a GQ with minimally two layers. A cation inserted between or in the centre of the tetrads, dependent on its ionic radius, further stabilises the structure. The core guanines are linked by three nucleic acid sequences (loops) of varying composition, topology, and size. The overall stability of individual GQs is governed by the number of layers, length and identity of the loops, the flanking nucleotides and the concentration and type of bound cation. In contrast to DNA GQs, RNA GQs are practically monomorphic, displaying (with a few exceptions\textsuperscript{19,20}) all strands oriented in parallel, and are preferentially stabilised by potassium ions (K\textsuperscript{+}) binding between adjacent tetrads\textsuperscript{21}. Higher-order structures may further result from 5´–5´or 3´–3´ stacking of the terminal quartets of individual GQs.

GQs have been identified in all domains of life and have been implicated in a large number of critical biological processes, including transcription, translation, epigenetic regulation, DNA recombination, splicing, and mRNA transport\textsuperscript{22,23}. These unconventional secondary structures are also found in the genome of DNA and RNA viruses such as Ebola virus, herpes simplex virus, human papillomavirus, human immunodeficiency virus, Zika
virus, Influenza virus, human coronaviruses, and hepatitis C virus, where they control various steps in the virus life cycle, ranging from the expression and replication of their genetic material to the assembly into protective nucleocapsids. In an increasing number of examples, it was found that GQ-stabilising compounds such as pyridostatin (PDS), PhenDC3, Braco19 and others inhibited viral replication at the level of transcription, translation, and/or genome replication. This has been related to a stabilisation of certain GQs in the genome or transcripts of these pathogens acting e.g., as physical road blocks for virus- and/or host cell-encoded enzymes such as DNA and RNA polymerases or the ribosomes. Alternatively, these compounds were also found to compete with the host cell or viral proteins for binding to these structures with frequently inhibitory but occasionally also stimulatory effects on their reproduction.

Up to date, nothing is known about a possible role of these unconventional secondary structures in picornaviruses. We have therefore initiated such study with RV-A2 as a model. In infectivity studies, we find that the GQ ligands PDS and PhenDC3 specifically inhibit the uncoating of the viral RNA, which has not yet been described for any virus. Using a broad combination of biophysical and ultrastructural analysis, we demonstrate the conversion of metastable alternative structures retained in the viral RNA into GQ promoted by PDS. This, in turn, results in a conformational change most likely underlying the defective RNA release from the capsid. Strikingly, the process is critically governed at the level of the genomic RNA compaction by the prevalent alkaline cation (K⁺, Na⁺) present in the capsid, with K⁺ drastically limiting PDS access. These results show that targeting of GQs might open up an entirely new avenue for combating the common cold viruses during their dissemination in the sodium-rich extracellular fluids.

RESULTS

QGRS-mapper identifies potential GQ-forming sequences in all RV genomes.

Employing extensive bioinformatics analysis, Lavezzo and colleagues recently predicted GQs in viral genomes of human virus species, including RVs. We independently confirmed and extended their findings for all RV genome sequences available in Genebank by using a local copy of the same program that runs on the quadruplex-forming G-rich sequence (QGRS) mapper web-based server. This software has been originally developed to identify putative QGRS in DNA, where the G-score reflects the stability of a predicted GQ, which usually increases with the number of G-tetrades and decreases with the loop size. The respective search motif G_{3}L_{1-7}G_{3}L_{1-7}G_{3}L_{1-7}G_{3} takes into account that most experimentally identified DNA GQs conformed to short-looped (1 to 7 nucleotides) structures with three and occasionally more G-tetrades. However, RNA GQs are generally more stable than the
corresponding DNA GQs; two-layer RNA GQs are therefore not uncommon, and the overall higher stability of RNA GQs allows the insertion of larger loops compared to DNA GQs. Recently, an atypical RNA GQ with no first loop has been described. Therefore, we adjusted the search parameters of the QGRS mapper to allow the identification of putative unconventional long-loop and zero-nucleotide loop G-quartets, including those featuring only two layers. We then plotted the respective QGRS prediction G-scores ≥ 10 against their positions in the genomic RNA sequences (Fig. 1a). Strikingly, the vast majority of rhinoviral genomes feature only QGRS predicted to form just two-layered GQs. RV-A41 and RV-B4 are singled out by the additional presence of one and two three-layer GQs, respectively.

Furthermore, most RV-ABC genomes feature at least one and up to seven putative zero-nucleotide loop GQs without marked conservation. The number of potential QGRS varies from 6 to 19 (mean 12) for the RV-As, from 9 to 19 (mean 13) for the RV-Bs, and from 10 to 23 (mean 15) for the RV-Cs. The augmented prevalence of QGRS for the RV-C species is likely attributable to their genomes' distinctly higher GC content (43 % for RV-C, 38 % for RV-A, and 38 % for RV-B). The analysis further revealed four highly conserved QGRS (Fig. 1a - asterisk), all located in the open reading frame. The slight differences in their position between the genera (RV-A, RV-B, and RV-C) result from clade-specific insertions and deletions. An additional conserved QGRS is uniquely present in the RV-Bs, upstream and close to the second highly conserved QGRS motif. By contrast, only moderately conserved QGRS were predicted in the 5' untranslated region (UTR), which comprises essential regulatory elements such as the 5' cloverleaf structure and the IRES, required for replication and cap-independent translation. No single QGRS was predicted within the short 3' UTR, featuring a highly conserved hairpin involved in virus replication.

NMR analysis demonstrates the folding of synthetic ribooligonucleotides representing selected QGRS of RV-A2 RNA into GQs, which is differentially affected by PDS.

For RV-A2, QGRS-mapper predicted 11 QGRS with G-score ≥ 10 (Supplementary Fig. S1). We chose to study the candidates with the lowest and highest G-scores, G11 and G20, the latter also representing one of the four highly conserved GQs (see above). Assuming the almost invariable adoption of parallel GQs by RNA, G11 would give rise to an unusual monomeric two-layer GQ bearing a zero-nucleotide loop 3 in combination with a long loop 1 (Table 1; a schematic illustration of the likely G11 and G20 RNA GQ structure is displayed in Supplementary Fig. S1). To confirm their GQ-forming propensity, we collected $^1$H NMR spectra using 250 µM of the derived synthetic ribooligonucleotides in 10 mM sodium phosphate (pH 7.4), 100 mM KCl, similarly as described elsewhere. Each ribonucleotide was denatured at 95 °C for 10 min, followed by cooling to 4 °C for 10 min to favour
intramolecular annealing into GQ and possibly other secondary structure(s) in the presence of monovalent cations. A high concentration of K$^+$ usually stabilises GQs, as shown in other instances$^{36}$. G11 and G20 displayed $^1$H signals of the bulk of the imino proton in the region between 10.4 – 12.4 ppm, which is the typical NMR signature of GQs (Fig. 1b – salmon-coloured area). The lack of sharp signals can be explained by G-register exchange dynamics$^{44}$ since certain G runs have more than two Gs, indicating the possible presence of alternative conformations.

Peaks in the canonical Watson-Crick base pair region at $^1$H NMR shifts larger ~12.4 ppm (olive-coloured area) are observable at low (4 °C) but not at high temperatures (34 °C, the optimal temperature for RV-A2 replication), indicating that these secondary structure elements (presumably hairpin(s)) are less stable than the GQs. Within the range assigned to GQs, the second peak (11.9 – 12.4 ppm) observed only for G11 (Fig. 1b) exhibited unaltered intensity in an HDX experiment performed for 7 h at 37 °C. The solvent inaccessibility of these imino protons indicated that they were most likely also part of the GQ core (Supplementary Fig. S2a). In the G20 spectrum, three distinct peaks (one within and two next to the upfield broader of the GQ region) completely disappeared with increasing temperature, indicating that the corresponding imino protons are conceivably not involved in the G-tetrad core formation. The addition of 500 µM pyridostatin (PDS), which specifically binds with high affinity to DNA and RNA GQs but not to other nucleic acid secondary structures except i-motif forming DNA$^{45}$, resulted in substantial changes of the imino $^1$H peaks for both synthetic RNAs (Fig. 1b and Supplementary Fig. S2b), indicative of a specific interaction.

The only other $^1$H-NMR study we are aware of using PDS and a GQ was performed with a three-layer DNA GQ derived from the src kinase gene (SRC). This showed an upfield-shifting of GQ imino proton signals stemming from PDS binding to the top G-quartet in a stacking mode$^{46}$. However, the differential effect observed by us suggested that the binding mode of PDS may vary for G11 vs G20. We hence used molecular docking to assess this possibility further. For lack of detailed structural information about the G11/G20 system, we instead in silico evaluated the interaction of a crystallographic model of a pseudorabies virus RNA GQ molecule composed of two G-quartets (PQS18-1, GGCUCGGCGGCGGA) (PDB 6JJH$^{47}$) and PDS. Using increasing concentrations of PDS in this in silico analysis, we unexpectedly discovered that it could form defined dimers mostly stabilised by π-π interactions. This was then experimentally substantiated by UV-absorption spectroscopy, which clearly demonstrated a concentration-dependent self-association of PDS ($K_a$ ~42 µM) in the buffer chosen for NMR spectroscopy (Supplementary Fig. S3).

The analysis of the best docking poses for the monomeric and dimeric PDS and PQS18-1 revealed two distinct classes of binding modes (Supplementary Fig. S4): i) end-
stack (a and c), and ii) groove/loop-binding (b and d). In both instances, the 2-(quinolin-4-oxo)ethanamine moiety of PDS interacts with the nucleobases via Van der Waals forces and an additional contribution of electrostatic interactions between the charged amino groups of PDS structure and the phosphate groups of the GQs. These binding modes may be variably exploited for the interaction with G11 and G20, presumably resulting in the idiosyncratic PDS-induced peak shifts.

Altogether, these results provided the initial bioinformatics analysis demonstrating the likely formation of two-layered GQs by both ribooligonucleotides under favourable conditions for the folding of such structures.

| Ribooligonucleotides          | Sequence                      |
|-------------------------------|-------------------------------|
| Negative Control              | 5'- UUA CCC UUA CCC UUA CCC UUA CCC UUA -3' |
| miniTERRA (G-score = 42)      | 5'- UUA GGG UUA GGG UUA GGG UUA GGG UUA -3' |
| G11 (position 2048 – 2074)    | 5'- GGC ACU CAU GUU AUA UGG GAU GUG GGG -3' |
| G20 (position 1038 – 1064)    | 5'- CCU CAA AGG GUU GGU GGU GGA AAC UAC -3' |

Table 1 – Sequences of the synthetic ribooligonucleotides used in this study. Note that in the negative control, all Gs of miniTERRA were replaced by Cs.

Circular dichroism spectroscopy of the synthetic ribooligonucleotides G11 and G20 reveals a parallel GQ folding topology.

We next verified the tentatively assigned folding topology of G11 and G20 by circular dichroism (CD) spectroscopy. CD is a gold standard to determine the strand orientations of GQs. These might be parallel, as commonly adopted by RNA and DNA, or anti-parallel and hybrid, which, with a few exceptions, are observed only for DNA. Telomeric-repeat-containing RNA (miniTERRA; Table 1), which adopts a three-layer parallel conformation, served as a positive control.

The CD profiles of miniTERRA, G11, and G20 taken at 25 °C in either 100 mM sodium or 100 mM potassium phosphate buffer (pH 7.4) in each instance displayed the signature of an all-parallel topology (ellipticity shows a negative band at 240 nm and a positive band at 265 nm). The monovalent Na⁺ and K⁺ cations differentially stabilise GQs owing to their different ionic radius and hydration free energies. Generally, potassium promotes folding and stabilises (ribo)oligonucleotide GQs to a larger extent than sodium, though some exceptions have been reported. In accordance with the latter, the degree of GQ formation of G20 was noticeably the same irrespective of the presence of Na⁺ or K⁺ as indicated by the practically unchanged CD spectrum (Fig. 1c). By contrast, as found for the vast majority of GQ forming RNAs, the miniTERRA control and the G11 ribooligonucleotide
showed a higher proportion of GQ structure in K\(^+\) compared to Na\(^+\). The trough at 210 nm
only evident for G11 and G20 is likely attributable to the additional presence of structural
elements comprising A-type duplex RNA regions as also revealed in the above NMR
analysis at low temperature.

**Na\(^+\) and K\(^+\) differently impact the physicochemical parameters of G11, G20, and
miniTERRA ribooligonucleotides.**

As seen in Figure 2a, the CD melting and annealing spectra are almost
superimposable for the G11 and G20 ribooligonucleotides and the miniTERRA control when
dissolved in a sodium-containing buffer. The lack of a marked hysteresis indicates
thermodynamic equilibrium between denaturation and refolding in the examined temperature
range and the used ramp rate and is typical for intramolecular GQs. The situation for
miniTERRA was strikingly different in the potassium buffer, where the unfolding/annealing
profiles exhibited a marked hysteresis (Fig. 2a); this is typically observed for intermolecular
GQ formation, where the shape of the melting and refolding curves depends on the speed of
temperature ramping. Another possibility is a slow conformational change of an
intramolecular GQ from one topology to another. However, according to CD spectroscopy,
miniTERRA in the presence of either cation (Na\(^+\), K\(^+\)) formed a parallel GQ (Fig 1c)
exclusively. Since the multimerisation of miniTERRA could be ruled out by a subsequent
electrophoretic analysis (see below), the relatively slow ramp rate of 1 °C/min apparently still
did not allow the reversible refolding of this three-layer intramolecular GQ. This is in line with
findings by others that multiple layers of tetrades are particularly prone to hysteresis. By
contrast, in the presence of K\(^+\), the CD melting/cooling profiles of both two-layered GQs
exhibited just a rather subtle (G11) to negligible hysteresis (G20). The latter's overlapping
heating and cooling curves, as also seen with Na\(^+\) buffer, reinforces the notion that G20
forms a monomeric (see below) intramolecular GQ in presence of each of these cations.

Others have shown that K\(^+\)-containing solutions stabilise the parallel topologies of
two- and three-quartet RNA GQ much more than Na\(^+\)-containing solutions, with the
difference in the melting temperatures \(\Delta T_m\) ranging from 15 to more than 30 °C. Such
drastic change in \(T_m\) (determined at the intersection of the second derivative with the x-axis
in Fig 2a) was also evident for the three-layer miniTERRA GQ in K\(^+\) compared to Na\(^+\). By
contrast, the melting temperatures of G11 and G20 in the K\(^+\) containing buffer were just ~ 4
°C higher than those determined in Na\(^+\) buffer.

The limited hysteresis of G11 melting/cooling might indicate the formation of a dimeric
GQ resulting from end-to-end stacking of two intramolecular GQs. This gains additional solid
support from native gel electrophoresis, which clearly shows the occurrence of dimers in the
presence of potassium ions for G11. Under these conditions, G20 and the miniTERRA
control gave rise to just a single band, indicating that they existed primarily as monomers.
Based on these results, the slight hysteresis of the G11 melting can be best interpreted by assuming a fast monomer refolding, followed by slower oligomerisation.

The adoption of a GQ conformation by the two RV-A2 derived RNA sequences (G11 and G20) and the positive control (miniTERRA) was then confirmed with a Thioflavin T (ThT) light-up assay with all measurements done at room temperature. ThT end-stacks to RNA GQs, which strongly increases its fluorescence. As seen in Fig. 2b and 2c, no significant fluorescence was detected upon incubation with the negative control (C for G substituted miniTERRA) ribooligonucleotide (see Table 1). In contrast, a clear signal was obtained for all three tested GQs, whose magnitude was quite comparable for G11 in Na⁺ (left panel of Fig. 2b) and K⁺ buffer and about 1.5 and 2-fold higher in the presence of K⁺ for miniTERRA and G20, respectively (left panel of Fig. 2c). The ThT fluorescence intensity was strongest for miniTERRA and approximately 3 to 4-fold lower for G11 and G20 (but still 30 to 40 times higher than for the negative control), roughly correlating with their respective G-scores. Since the same amounts of oligonucleotides were employed, this indicated that a fraction of G11 and G20 adopted a non-GQ conformation as already observed in the 1H-NMR analysis. The coexistence of A-type RNA (hairpin) structure and a two-layer GQ structure has been recently described by Lightfoot and others. Based on the ThT light-up probe, G11, but not G20 would be almost indifferent to the choice of the two cations, which is opposite to the results of the label-free CD analysis. Most likely, the conformation of the respective GQs subtly differs when bound to Na⁺ (which coordinates with the Gs in the middle of a tetrade) or K⁺ (residing between G-tetrads), which may variably affect the binding affinity for ThT giving e.g. the impression of an apparent higher stability of G20 in K⁺ buffer. However, this does not change our overall conclusion that GQ structures are formed with both cations in this assay.

We then examined whether the tetrade-associated cation type (K⁺ or Na⁺) impacts the interaction of the respective GQs with PDS by conducting a fluorescent indicator displacement (FiD) assay, similar to the one described in refs. FID allows evaluating the relative affinity and selectivity of compounds binding to a GQ. Expectedly, ThT was displaced by PDS in each instance (right panels in Fig. 2b/c). However, while the dose-response curve for miniTERRA was comparable in Na⁺ and K⁺ containing buffers, the reduction in fluorescence determined for G11 and G20 was about 2-fold more efficient in the presence of Na⁺ as indicated by the respective IC₅₀ values (Table 2). Notably, with the lower G-scoring G11, a sharp decrease of the ThT fluorescent signal was already evident at low PDS concentrations. Since ThT was shown not to markedly alter the stability of GQs, this might indicate a higher affinity of PDS for the noncanonical GQ-forming G11 in comparison to miniTERRA and G20 as reflected by the correspondingly lowest IC₅₀ values (Table 2).
Table 2 – IC<sub>50</sub> values for the displacement of ThT by PDS. Significance determined by using ANOVA with Tukey’s multiple comparison test.

| Ribooligonucleotides | IC<sub>50</sub> (µM) | Na<sup>+</sup> | K<sup>+</sup> | Significance |
|----------------------|---------------------|-----------|-----------|--------------|
| mini TERRA           | 19.3 ± 0.5          | 17.5 ± 0.7| NS        |              |
| G11 (position 2048 – 2074) | 2.8 ± 1.4         | 6.1 ± 1.1  | p<0.0001  |              |
| G20 (position 1038 – 1064) | 10.5 ± 5.2        | 20.3 ± 1.0 | p<0.001   |              |

**Pyridostatin (PDS) and PhenDC3 reduce RV-A2 infectivity.**

Taken together, employing several orthogonal assays, we could demonstrate the intrinsic ability of two selected RV-A2-derived QGRS to form RNA GQs. However, within the context of the viral RNA, these and the other predicted candidate sequences may fail to fold into such scaffolds if embedded into or overlapping with alternative secondary structures of higher stability or more rapid formation (and trapped in a metastable state). Furthermore, the G-quartet fold of some untested putative QGRS may be too unstable at the temperature of virus propagation. PDS and other so-called GQ-stabilising compounds can selectively enhance the mechanical and thermal stability of DNA and RNA GQs over the level achieved by K<sup>+</sup> alone. Thereby, they can also force alternative secondary structures to transform into the compound-stabilised quadruplex conformation. The latter was evident for G20 and, less pronounced, for G11 by the loss of Watson-Crick peaks in the <sup>1</sup>H-NMR spectrum on incubation with PDS (Supplementary Fig. S2b).

It is presently believed that the uncoating of RV-A2 and several other enteroviruses requires a structural switch of the genomic RNA and the transient unfolding of secondary structures for transit as a single-strand through one of the narrow pores formed in the A-particles. Therefore, we reasoned that the exposure of the encapsidated viral RNA to PDS by stabilising preexisting GQs and promoting the transition of unstructured and/or alternatively folded QGRS into such unconventional secondary structures might interfere with its *in vitro* and *in vivo* uncoating. RVs, in contrast to other enteroviruses such as poliovirus, are readily permeable for monovalent cations such as Cs<sup>+</sup> already in the cold and also for small organic compounds such as dansylazidine and ribogreen when incubated at breathing conditions. Capsid breathing describes a transitory expansion of the protein shell with temporary exposure of normally internal amino acid sequences through reversibly formed small holes, commencing at room temperature to around physiological temperatures, dependent on the RV serotype. We thus attempted to deliver PDS to the viral RNA genome within the native virion by exploiting this phenomenon. All incubations were done in...
phosphate-buffered saline (PBS) to prevent PDS aggregation into long fibrils, as we have recently observed for Tris- but not phosphate-based buffers.

First, we confirmed the dependence of PDS delivery to the inside of the capsid as a function of temperature. Purified RV-A2 was incubated with PDS under conditions of strongly diminished capsid breathing (at 4 °C) and a capsid breathing-promoting temperature (at 34 °C). Unbound PDS was removed, and a Particle Stability Thermal Release Assay (PaSTRy) was performed to determine a possible impact of PDS on temperature-dependent uncoating, a commonly used model for in vitro uncoating of picornaviruses. Fig. 3a shows the temperatures where the genomic RNA becomes accessible to SYTO 82 as deduced from the temperature vs fluorescent emission curves (Supplementary Fig. S6). Virus pre-incubated with PDS at 34 °C exhibited a striking 4.7 °C earlier onset of genomic RNA accessibility to SYTO 82 compared to the control condition, i.e., incubation with PDS at 4 °C (to prevent diffusion of this compound through the protein shell) (TON of 37.7 °C vs. 42.4 °C). However, the temperatures Tmax at the peak of the SYTO 82 signal (indicative of the complete conversion of all native virions into permanently porous A-particles) and the peak of the first derivative (at T50, corresponding to 50 % RNA accessibility) remained practically unaltered.

We interpret this finding as that PDS, by interacting with (potential) QGRS in the genome of RV-A2, directly or indirectly affected RNA contacts with the capsid, thereby resulting in enhanced mobility of the protein shell of the native virus. This allowed appreciable uptake of the SYTO 82 dye into the virion for binding the viral RNA already at a lower temperature compared to the control. However, the PDS-induced effect did not critically impact the rate of the temperature-dependent conversion of native to A-particle as inferred from the unchanged T50 for this sigmoidal conversion. The heat-triggered RNA release starting at Tmax (resulting in the subsequent drop of the SYTO 82 signal) was also not affected. As thermal unzipping of secondary structures is required for the exit of the RNA from the capsid in this in vitro uncoating model, the bound PDS evidently did not increase the melting temperature of the viral GQs above one of the most stable non-GQ secondary structures (presumably mostly hairpin loops) inside the capsid.

We then assessed the effect of PDS on the in vivo uncoating of RV. HeLa cells were infected with RV-A2 pre-incubated with PDS and without PDS (control), both at 34 °C for 4 h. This treatment time sufficed for the entry of appreciable amounts of RiboGreen into the capsid of RV-A2. Unbound PDS was removed by centrifugation in an Amicon ultrafilter unit, followed by repeated washing with PBS at 25 °C. The viral samples were then transferred to HeLa cells grown in 10 cm diameter dishes and incubated for 30 min to allow for viral uptake and uncoating in the absence of inhibitory compounds. Supernatant and cells were collected by scraping, and internalised viral material was recovered by five times
freezing and thawing. Cell debris was removed, and aliquots of the supernatants were subjected to immunoprecipitation with the subviral (A- and B-particle) specific monoclonal antibody 2G2. From equal aliquots of the precipitated material, protein and RNA were recovered and quantified in Western blots (Fig. 3b) and by RT-qPCR, respectively (Fig. 3c). Comparing these results, it becomes clear that regardless of whether the pre-incubation was carried out in the presence or absence of PDS, the same amount of viral proteins were detected. This excludes any influence of PDS on cell attachment, e.g. via forming virus-trapping filaments as observed in Tris-based buffers (see above and ref. 71) or on the overall rate of native to subviral (A + B) particle conversion. However, quantification of the viral RNA contained in the 2G2 precipitates showed that the virus incubated in the presence of PDS and recovered from cells 30 min post-infection (pi) in the form of subviral A- and B-particles contained about 70 % more viral RNA than virus pre-incubated without PDS. This can be taken to indicate that significantly less RNA was released from the endocytosed PDS-treated virus during the first 30 min following the challenge of the cells.

Thirty min pi aliquots from similarly infected cells (as above) were also analysed by sedimentation through preformed sucrose density gradients. Ultracentrifugation under the specified conditions allows for separating native virus, A-particles, and empty B-particles. The profile in Fig. 3d verifies the presence of mostly B-particles sedimenting at 80S for the virus subject to control conditions (– PDS). In stark contrast, a substantial fraction of the virus treated with PDS shifted towards higher sedimentation rates with a peak in-between 150S (where the native virus sediments) and 80S, corresponding to (viral RNA containing) A-particles (hash). These results again point to a substantial impairment of in vivo RNA release by the incorporated PDS.

The observed effect of PDS in the PaSTRY assay and on the in vivo uncoating of RV-A2 was most likely due to the trapping/stabilisation of GQs located in the encapsidated RNA rather than an unspecific binding to other components of the virion. However, in one report, PDS was found to act as a weak inhibitor of the C5 convertase, a component of the complement system. Therefore, to further furnish our hypothesis, we repeated the in vivo analysis at capsid breathing conditions with another frequently used GQ-binding compound, Phen-DC3. RV-A2 was incubated as described above with PDS at 20 µM and 200 µM and in parallel with PhenDC3 at 1 µM and 5 µM, respectively. A mock-treated virus was used as a control. All samples were subjected to repeated centrifugal ultrafiltration to remove the excess of the compounds maximally. HeLa cells were then challenged with these samples and incubated for 9 h to allow for a one-cycle infection. RV-A2 positive cells were determined by fluorescence-activated cell sorting (FACS), using the intracellularly produced VP2 as a readout. Fig. 3e demonstrates a concentration-dependent decrease in the number of cells containing replicating RV-A2 upon pretreatment with PDS. Notably, a significant reduction
was also observed with 5 µM of Phen-DC3. This result further strengthened our hypothesis that PDS triggers a structural change in the encapsidated RNA as postulated from the PaSTRY analysis, preventing its orderly egress from the capsid under *in vivo* conditions.

**PDS affects the conformation of the free RV genome and reduces viral infectivity in the presence of Na\(^+\) but not in the presence of K\(^+\).**

We next embarked on an ultrastructural analysis of gently extracted rhinoviral RNA for directly visualising the proposed PDS-induced structural change. The protein shell of RV-A2 was proteolytically removed with proteinase K. To additionally examine a possible differential impact of the prevalent extracellular (Na\(^+\)) and intracellular (K\(^+\)) monovalent cation, the digestion was performed with the purified virus in sodium- as well as in potassium-only containing phosphate buffer.

Initially, we evaluated the released naked (ex virion) viral RNA for the presence of GQs by mixing with ThT. Importantly, due to its moderate affinity, ThT binds only to already established GQs\(^{56}\). At 30 °C, this resulted in a significant increase in the fluorescent signal compared to the background (Fig 4a, upper panel). However, the formation of some GQs in the viral RNA might be at least partially prevented by sequestration of the G-rich regions into kinetically trapped competing structures. In order to assess this possibility quantitatively, we heated the ex virion RNA for 10 min to 60 °C (the temperature where it unwinds to escape from the RV-A2 capsid\(^{73}\)) in the presence of ThT followed by its slow refolding at room temperature, thereby promoting the formation of the thermodynamically most stable secondary structures. Under these conditions, the fluorescent emission intensity of the light-up probe substantially raised by about 20-fold in both samples (Figure 4a, lower panel). This indicated that distinctly more GQs were now available for ThT binding, likely resulting from a conformational transition of kinetically trapped, metastable alternative (e.g. hairpin) structures to the more stable quadruplex structure triggered by the elevated temperature. A similar result was recently obtained in a study examining the kinetic vs thermodynamic control of a sequence within an mRNA able to switch from a hairpin to a GQ, using N-methyl mesoporphyrin (NMM) as light-up indicator\(^{81}\). The recorded ThT signal was consistently higher for the Na\(^+\) compared to the K\(^+\)-containing samples, despite the greater GQ stabilising propensity of the latter cation, amounting to a ~40 % difference at 30 °C, which diminished to just 9 % for the 60 °C heated samples. It suggests that the viral RNA molecule with intact 3D structure is possibly more compact in the presence of the charge-neutralising K\(^+\), leading to reduced accessibility of the already existing GQs for the ThT probe. Binding of ThT to GQs forming during refolding of the heated viral RNA was expectedly much less affected by the monovalent cation type when the probe was already present before full compaction of the nucleic acid molecule.
We next incubated the ex virion RNA with 20 µM PDS, again in a Na⁺ or K⁺-containing phosphate buffer, and submitted it to rotary shadowing. The platinum replicas were then observed by transmission electron microscopy (TEM) (Fig. 4b). In the absence of PDS, the ex virion RNA remained compact and approximately spherical though slightly deformed in Na⁺ (insets), indicating that compaction Mg²⁺ and polyamines bound to the encapsidated genome were not removed by our extraction procedure. A PaSTRY analysis with these samples demonstrates the maintenance of a tertiary structure organisation under these conditions (see below). Strikingly and entirely in line with the proposed pyridostatin-induced RNA reorganisation deduced from the PaSTRy experiment (performed in PBS), PDS in the Na⁺-containing buffer led to substantial elongation of these RNA cores, making them appear irregular rods (left panel). Unexpectedly, no such effect of PDS was seen in the K⁺-containing buffer, where the RNA remained roughly spherical (right panel).

We then employed atomic force microscopy (AFM) imaging to independently confirm the TEM analysis outcome. AFM scans of identically prepared samples indeed yielded very similar images (Fig. 4c and Supplementary Fig. S7). They show mostly irregular rods in Na⁺-containing buffer + PDS with lengths of up to 200 nm and a diameter of about 10 nm (Supplementary Fig. S7, upper panels) and spherical RNA in K⁺-containing buffer + PDS with a diameter of roughly 25 nm (lower panels), compatible with the dimension of the capsid internal cavity. This confirms that the structures shown in Fig. 4b are specific for the viral RNA and not artefacts of the platinum contrasting and/or the drying process.

The drastic shape change only observed in Na⁺ containing buffer likely results from the rescuing of unstable GQs and/or the shifting of long-lived metastable structures with alternative G-quartet forming ability to the more stable GQ conformation by PDS. This presumably disturbs short and long-range interactions determining the global structure of the RNA genome, similarly as described for certain small molecules on binding to tRNA and riboswitches. Possibly, K⁺ binding pockets in the viral RNA as e.g. found in ribosomal RNA further constrain and condense its native tertiary structure compared to Na⁺, limiting the access of the compound to these regions to account for the observed different effect of PDS. To substantiate this hypothesis, we assessed the stability of the protein-free (ex virion) RNA by carrying out a differential scanning fluorimetry (DSF) similarly as described by Silvers, Keller. Inspection of the DSF traces obtained in Na⁺ and K⁺ buffer indicated similarly low accessibility of the viral RNA for SYTO 82 at 25 °C up to about 40 °C, attesting that the RNA molecule stays compact and extensively folded within this temperature range (Supplementary Fig. S8). Here, SYTO 82 intercalates mostly into solvent-accessible double-stranded regions located at the periphery, which seems less affected by choice of these cations compared to the accessibility of GQs for ThT under similar conditions (Fig. 4). The subsequent relatively rapid fluorescence increase from the lower baseline (at 44.5 °C and 51
°C for Na⁺ and K⁺, respectively) to the upper base line (= maximal response; at 54.3 °C and
57.1 °C for Na⁺ and K⁺, respectively) relates to the disruption of (mostly) the RNA tertiary
structure, now allowing SYTO 82 binding also to internally localised stem regions. The rise
was ~2-fold (Na⁺) and ~1.2-fold (K⁺), indicating a more extensive unfolding in the sodium-
containing buffer. The higher stability of the tertiary contacts of the viral genome in the
presence of K⁺ is illustrated by the around 4 °C higher Tₘ1. The following progressive drop in
emission intensity is due to melting of the more stable secondary structures with the release
of SYTO 82, resulting in somewhat closer spaced higher melting temperatures Tₘ2 (60.5 °C
for Na⁺ and 63 °C for K⁺) for this transition.

The above *in vitro* data derived with the ex virion RNA altogether implies that its
distinctly more compact tertiary structure imposed by K⁺ hinders the PDS molecules from
arriving at internal regions able to transform into GQs on the binding of this compound. If
correct, this should materialise in a distinctly smaller number of PDS molecules associated
with the RV-A2 genome in potassium compared to sodium buffer. As assessing this figure is
technically less demanding with encapsidated RNA, we quantified the amount of PDS that
remained associated with the virions after incubation (20 μM) at 4 °C or at 34 °C for 4 h in
Na⁺ or K⁺ containing buffers and subsequent extensive washing to remove any externally
deposited traces of the compound. Table 3 shows that the number of PDS molecules, which
penetrated the capsid upon incubation at 34 °C in Na⁺-containing buffer was by far higher
than in all the other incubation conditions.

| Treatment | PDS bound (moles/mole virus) | Relative to maximum |
|-----------|-----------------------------|---------------------|
| 34 °C / Na⁺ | 10                          | 100 %               |
| 4 °C / Na⁺  | 0.9                         | 8.8 %               |
| 34 °C / K⁺  | 0.2                         | 1.4 %               |
| 4 °C / K⁺   | 0.4                         | 3.7 %               |

Table 3 – Mass spectrometry quantification of PDS content per virion.

The result further corroborated our hypothesis that K⁺, while commonly cooperating
with PDS in GQ stabilisation, unexpectedly protects the rhinoviral RNA from binding of this
compound not only ex virion but also inside authentic viral particles (*in virion*). The magnitude
of the difference of virion-incorporated PDS was quite impressive, as the encased
polymerotide of picornaviruses is already very compact. This might have conceivably
mitigated the tertiary structure changes in the presence of Na⁺ vs K⁺, believed to govern the
extent of pyridostatin binding to viral QGRS when free in solution. A monovalent cation-
dependent breathing activity as an additional source for that difference was ruled out by a
nanoDSF analysis of purified virions diluted in 100 mM sodium or potassium phosphate buffer (Supplementary Fig. S9).

We then assessed whether the different PDS uptake by RV-A2 as found by MS resulted in consequences on its infectivity by determining the TCID<sub>50</sub> of virus exposed to the compound diluted in the respective monovalent cation-containing phosphate buffer. To avoid any unspecific loss of infectivity due to thermal inactivation, we reduced the incubation temperature to 25 °C and compensated for the resulting diminished breathing by extending the treatment time to 20 h. As shown in Fig. 4d, the virus sample treated with PDS in the presence of Na<sup>+</sup> exhibited a titer reduction by 2 logs compared to control conditions (the same buffer without PDS). By contrast, PDS treatment in K<sup>+</sup> buffer did not affect the virus titer as expected from the small amount of the compound detected by MS in the virion of similarly treated virus (Table 3). Note that the slight reduction of infectivity on changing the internal monovalent cation environment from Na<sup>+</sup> to K<sup>+</sup> in the absence of the PDS was not significant.

Finally, we explored at what step of the infection cycle PDS might act on RV-A2 in vivo (i.e. without pre-incubation) by a time-of-addition experiment. The virus was bound to the cells for 30 min at 4 °C, PDS was added, and the cells shifted to 34 °C (T0). The same experiment was conducted in parallel, except that PDS was added at T180 and T300, respectively. This roughly corresponds to RV entry and uncoating (T0), RNA synthesis (T180), and assembly (T300) <sup>94, 95, 96</sup>. The cells were maintained for 9 h pi (one full cycle of infection), and viral synthesis was measured by fluorescence-activated cell sorting (FACS).

As can be seen in Fig. 4e, the infection rate and viral synthesis was only significantly impacted upon the addition of PDS at T0.

**DISCUSSION**

GQs have recently become a focal point as promising antiviral targets <sup>18</sup>. Despite this surge in interest, the possible importance of this unconventional secondary structure remains unexplored in picornaviruses. In this report, we fill this gap by focusing on RVs of the genus *Enteroviruses* within the *Picornaviridae* family, which are responsible for more than 50 % of common cold cases <sup>4</sup>. Using QGRS mapper <sup>35</sup>, we identified putative intramolecular QGRS for all completely sequenced rhinoviruses of clade A, B, and C. Altogether, RVs comprised between 6 and up to 23 such QGRS motifs, which, with the exceptions of RV-A41 and RV-B4, would give rise to only two-layer GQs. In most genomes, a variable fraction of the predicted GQs would furthermore lack one G-quartet connecting loop. Most of the putative QGRS are not or just weakly conserved, making it unlikely that they play an important role in the virus life cycle. Nonetheless, four of these putative QGRS, all located in the ORF and predicted to fold into conventional two-layer GQs, are highly conserved across all A, B and C
types (Fig. 1a); they were also identified in an independent analysis of all human virus families by Lavuzzo et al.\textsuperscript{34} using a proprietary software. While suggesting a functional relevance for these QGRS, they might instead encode a critical sequence in the corresponding polyprotein as an alternative reason for their strict maintenance, which remains to be investigated. Atypical RNA GQs such as those with a bulged out nucleotide or a vacancy will escape our analysis, but there are currently only a few reports on their existence and possible role\textsuperscript{48}.

The bioinformatics analysis indicated that targeting of GQs might be a promising new approach for combating RV infections, with the strongly conserved GQs conceivably presenting a high genetic barrier to drug resistance. For this purpose, we chose RV-A2 as one of the best-characterised representative\textsuperscript{97}. The 11 putative QGRS within its genomic sequence would all give rise to just two-layer G-quartets (Supplementary Fig. S1). Using various orthogonal biophysical assays, we demonstrated that the synthetic ribooligonucleotides G11 and G20, representing the QGRS with the lowest and highest G-score, respectively, formed all-parallel GQs in the presence of mM concentrations of K\textsuperscript{+} as well as of Na\textsuperscript{+}. This is also the most common conformation of naturally occurring RNA GQs\textsuperscript{98}. However, in contrast to many other examples\textsuperscript{36}, their thermal stability was only moderately (by ~4 to 5 °C) enhanced by K\textsuperscript{+} vs Na\textsuperscript{+}. Also, G11 and G20 differed only little in their stability with respect to the same coordinating alkaline cation, despite the considerably higher G-score of G20. At lower temperatures (0 to 25 °C), a fraction of each ribooligonucleotide folds into an A-type RNA structure (presumably hairpins). These alternative conformers were not further explored as they were unstable at 34 °C, the optimal temperature for RV-A2 replication\textsuperscript{99}. This analysis is, to our knowledge, now the second showing that an unconventional, two-layer zero-nucleotide loop RNA GQ as represented by G11 is stable at physiological conditions. Interestingly, while no first loop was present in the example of the previous report\textsuperscript{37}, G11 lacks the third loop.

The \textsuperscript{1}H-NMR analysis evidenced that both, G11 and G20, bound the GQ stabiliser pyridostatin (PDS;\textsuperscript{91,100}). In all experiments with this compound, we specifically avoided Tris-buffer as a solvent since we recently found it to promote aggregation of PDS into variably-sized fibres\textsuperscript{71}. Using phosphate buffer instead, we noted the formation of PDS dimers, which were the dominant species at concentrations \(\geq 200\ \mu\text{M}\). Molecular docking indicated that this association was largely driven by a \(\pi-\pi\) stacking interaction between two PDS molecules. An \textit{in silico} modelling with the structurally well-characterised pseudorabies virus RNA-derived two-quartet GQ molecule PQS18-1 showed that both monomeric and dimeric PDS bind to the exposed G-tetrades on the top or bottom of a GQ and share a second binding mode involving the bases in the groove and a loop. (Supplementary Fig. S4). This is entirely in line with a \textsuperscript{19}F-NMR study at a similar ligand (PDS) to RNA ratio\textsuperscript{101} as used by us, which besides
π-π end-stacking, indicated additional (uncharacterised) binding site(s) for PDS on the employed RNA GQ. Our analysis corroborates and extends previous reports on modelling the GQ-PDS interaction, which proposes either a π-π end-stacking mode or an exclusive binding of PDS to the loop/groove interface, being furthermore restricted to docking of just the monomeric compound. While our findings for PDS are novel, they are not entirely unexpected, as stacking-mediated dimerisation and further aggregation of flat polycyclic aromatic nucleic-acid binding dyes are already known for long (e.g. Bradley and Wolf). Further, at least one other GQ-ligand (DMSB, a cyanine dye) was shown to bind as a dimeric associate to the terminal G-tetrad and the groove of a DNA GQ.

Prompted by the evident GQ forming ability of two predicted putative QGRS sequences in the RV-A2 genome, we exploited the virus breathing phenomenon to investigate the impact of PDS on an encapsidated RNA. We found that infection of cells with RV-A2 loaded with PDS in PBS buffer, which is rich in sodium and low in potassium, resulted in substantial accumulation of subviral A- (or 135S) particles at 30 min pi, whereas most native virus particles of the untreated control have already converted into empty B-particles. This result strongly indicated that PDS compromised the uncoating of the viral genome. Since PhenDC3, a chemically different GQ-interacting compound, had the same effect, the targets were in all likelihood putative QGRS sequences in the genomic RNA that already formed GQs within the capsid or could fold into such GQs on stabilisation by these compounds. A PaSTRY analysis furthermore showed a markedly enhanced capsid mobility of PDS-loaded virions compared to the untreated control. This strikingly resembled the results of a low-level covalent modification of the encapsidated RNA of flock house virus (FHV), a small icosahedral (+)ssRNA virus, by an aziridine derivative, which also led to enhanced capsid mobility, suggested to originate from disrupted capsid protein-RNA interactions in the native particle. By analogy, we concluded that PDS similarly triggered a rearrangement of the encapsidated rhinoviral RNA affecting contacts with the inner surface of the shell, including those mediated by the recently identified enterovirus packaging signals, leading to enhanced capsid breathing.

The proposed conformational change induced by PDS was directly verified by ultrastructural analysis of rhinoviral RNA gently freed from the surrounding capsid by proteinase K digestion. Instead of PBS, the incubation was done in a phosphate buffer containing exclusively Na⁺ or K⁺ as the major extra- and intracellular monovalent cation to assess their potential role. Remarkably, the GQ-specific light-up probe ThT revealed that in both conditions, only a few GQs were already established in the ex virion RNA. Most putative QGRS were apparently sequestered into alternative, long-lived (kinetically trapped), metastable secondary structures, which transformed into thermodynamically more stable GQs on heating and slow cooling of the viral RNA, resulting in the observed massively...
increased ThT fluorescence emission. Presumably, during positive-strand RNA synthesis in
the infected cells, sequences of the nascent (+) strand comprising these QGRS fold much
faster into alternative, metastable conformations such as RNA hairpins than into the more
stable GQ (tens of microseconds vs hundreds of milliseconds \(^{110,111}\)) as soon as they emerge
from the active centre of the viral RNA replicase. If separated from each other by an
appreciable activation energy barrier, as indicated by the ThT analysis, these metastable
structures, following their rapid encapsidation (which is tightly coupled with replication \(^{112}\)),
will persist in the progeny virions. The kinetically favoured formation of a metastable hairpin-
like structure instead of an alternative, more stable, GQ during co-transcriptional folding of a
nascent mRNA has been recently reported \(^{81}\).

Rotary shadowing and AFM demonstrated a profound PDS-induced shape change of
the ex virion RNA, in full agreement with our prediction. However, to our surprise, this
occurred only in the presence of Na\(^+\) but not the typically more strongly GQ-stabilising K\(^+\).
Then, the DSF analysis provided a plausible explanation for this initially puzzling
phenomenon, showing that potassium ions considerably strengthened the tertiary structure
of the ex virion RNA compared to sodium ions. We speculate that this is due to specific
chelation sites for K\(^+\) in the RV-A2 genomic RNA as described in certain ribozymes \(^{113,114}\)
and ribosomal RNA \(^{89}\), which reinforce their tertiary structure. The viral RNA will likewise
further compact and rigidify when the postulated pockets are occupied by K\(^+\), which we
believe renders internally located, potential GQ-forming sequences inaccessible for
pyridostatin. In support of this, Favre and coworkers have previously shown that tight folding
of various RNA species substantially restricted the intercalation of ethidium bromide \(^{115}\).

Conversely, these GQ-forming sequences must remain accessible for PDS in the less
tightly packed ex virion RNA exposed to Na\(^+\) (at the same concentration as K\(^+\)) to explain the
observed drastic structural reorganisation, which we attribute to the transition of QGRS
sequestered in the alternative, metastable conformations into GQs promoted by PDS. Apart
from its GQ-stabilising effect, this compound likely accelerates the process by its direct
participation in the GQ folding, thereby lowering its activation energy, as shown in an optical
tweezer study \(^{61}\). An analogous refolding of stem-loop structures into GQs induced by the G-
quadruplex ligand PDP (a PDS derivative) was recently proposed for an RNA of hepatitis C
virus \(^{116}\), and a profound effect on RNA long-range folding due to extensive differences in
secondary structures acquired with GQ formation was predicted in a bioinformatics analysis
\(^{91}\). The unexpected “protective” effect of K\(^+\) was also clearly evident when RV-A2 was
incubated with PDS at breathing conditions, which did not result in a change in infectivity,
while the virus titer dropped by two logs on its replacement by Na\(^+\) in the buffer. Accordingly,
mass spectrometry revealed a marked accumulation of PDS inside the capsid only in the
presence of sodium and not potassium. It must be emphasised that the mere exchange of
Na\(^+\) for K\(^+\) in the capsid did not significantly impact the infectivity despite the different level of compactness of the genomic RNA believed to regulate the compound access.

How may the binding of PDS to the QGRS of the viral genome under permissive monovalent cation conditions affect its release from the capsid? In the currently favoured model of enterovirus endosomal uncoating, the viral RNA must transiently unfold in order to pass with its 3´ end first through one of the in total 30 small pores (\(~1.5\) nm diameter \(^7\)) opening permanently at each of the two-fold icosahedral symmetry axis of the subviral A-particle. The emerging RNA reaches the cytosol without contact with the endosomal contents by passing through a connecting channel in the endosomal lipid bilayer formed by 5–6 copies of expelled VP4 with a lumen diameter of between approximately 4.6 nm and 12 nm. Based on preliminary data, host factor(s), in coordination with released VP4 acting as chaperone, may pull the genome from the capsid once its unstructured poly(A) tract at the 3´ end appears outside of the endosome \(^5\), \(^7\), \(^64\), \(^65\), \(^66\), \(^117\), \(^118\). The forced transfer through the narrow capsid opening will result in the reversible unzipping of secondary and tertiary structure elements akin to the electrophoretically driven transport of structured RNA through the \(\alpha\)-hemolysin nanopore \(^119\). The average number of PDS molecules (10) incorporated into the capsid of RV-A2 in the presence of Na\(^+\) is comparable to the number of putative QGRS (11). Assuming that they become folded into GQs as described for the ex virion RNA, it is tempting to speculate that the stabilising effect of PDS prevents their unwinding by the above mechanism. With an effective size of \(~2.4\) nm, the GQ-compound complex would sterically block the ejection of the genomic RNA through the 2-fold related pore. This scenario closely resembles the one suggested for compound-stabilised GQs located in the ORF of mRNAs on encountering a translating ribosome, believed to plug its entry site featuring a diameter of \(~1.5\) nm \(^120\), thereby obstructing further entry of the mRNA. However, though attractive, we consider this rather unlikely, as our PaSTRY experiment with RV-A2 showed that incorporation of PDS did not increase the temperature \(T_{\text{max}}\), where RNA release starts (Supplementary Fig. 6). This implies that PDS-binding by rhinoviral GQs did not raise their stability above the one attributable to the most stable non-GQ secondary structure(s) formed in the encapsidated rhinoviral RNA. We consequently favour an alternative mode of action based on the likely structural reorganisation of the encapsidated genome triggered by PDS, as indicated by PaSTRY and directly visualised with the ex virion RNA. This might conceivably compromise the formation of the well-ordered RNA layer beneath the protein shell of the A particle proposed to guide its ordered egress \(^5\). In addition or alternatively, it could dislodge the viral RNA’s 3´ end found to exit first \(^65\) from a position believed to reside in the vicinity of one of the pores opening at the two-fold axis to allow its facile ejection, perhaps directed by electrostatic focusing \(^121\). Its PDS-driven relocation would result in a high entropic penalty for finding such holes via thermal fluctuation (e.g. Polson and McLure \(^122\)), critically...
diminishing the successful vectorial traversal of the viral RNA through the capsid. This problem is presently little appreciated, though one report already highlighted its role in a coarse-grained model of RV-A2 uncoating, and the results with PDS now provide first experimental cues that it might matter. Worth mentioning, the icosahedral (+) ssRNA phages MS2 has remarkably solved this problem by the strong binding of a hairpin at the 3’ end of the viral genome to a single copy of a maturation protein, which is directly incorporated into the capsid, replacing a coat protein dimer at one of the icosahedral two-fold axes, being pulled out alongside the RNA by attachment to and subsequent retraction of a bacterial F-pilus. Our reasoning does not contradict the fact that the heat-triggered uncoating of RV-A2 is unaffected by PDS, as the substantially increased thermal motion will restore the RNA’s chances of finding a suitable exit pore. We note that several other RNA-binding compounds incorporated into the encapsidated genomic RNA of rhino- and other enteroviruses, such as RiboGreen, SYTO 82, neutral red, proflavine, and acridine orange did not markedly affect their infectivity (the latter three when examined in the dark as they render viruses photosensitive). These molecules intercalate into double-stranded secondary structures (up to 200 molecules for proflavine or about 1 per 23 nucleotides, assuming 60 % of the ~7,500 nucleotides long encapsidated RNA being involved in secondary structures as found for the related encephalomyocarditis virus). While this leads to partial unwinding of dsRNA segments, it apparently had little impact on the uncoating of the viral RNA (e.g. Danthi, Tosteson), which is not entirely surprising, given the different interaction of GQ-stabilisers with their RNA target. An alternative model of enterovirus uncoating proposes the dissociation of pentamers from the capsid at low pH, enabling exit of the viral RNA as a bulk without the need for transient unfolding. The proposed mechanism invokes protonation of bases of the RNA and dissociation of polyamines leading to a close apposition of viral genome to positively charged regions of the capsid followed by its cracking due to an increased pressure exerted on the capsid from the inside. This is thought to lead to the expulsion of pentamer(s) followed by genome release. While PDS might also interfere with such a process, we consider this mode of uncoating less important for RV-A2, as cryo-EM analysis of low pH treated RV-A2 did not reveal the presence of significant amounts of open particles.

Time-of-drug-addition showed that PDS had little consequence for RV-A2 protein production when added after the uncoating stage. All subsequent events required for viral reproduction occur in a high K+ and low Na+ cytosolic environment, limiting the access of the viral RNA for the compound as found for the ex virion RNA. However, Lu and coworkers have recently shown that the enteroviral RNA within infected host cells is represented by an ensemble of 3D structures, which substantially differed depend on the stage of engagement (translation, replication) or when prevented from being packaged. It is, therefore, reasonable...
to assume that even at the high intracellular K$^+$ level, in certain more open conformations of
these ensembles, the QGRS will become accessible for the compound, likely leading to
stabilisation of the respective GQs. A complete block of PDS binding by intracellular K$^+$ is
also unlikely based on recent life cell imaging of RNA GQ in the absence or presence of a
variant of PDS. Previous experiments with GQs featuring ≥ four G-tetrades showed that
they could be unwound by the DHX36 helicase even when bound by GQ-stabilising ligands
such as PDS and PhenDC3, with a rate dependent on the thermal stability of the GQ-
compound complex. We thus believe that the battery of intracellular host cell-derived
helicases together with the virus-encoded helicase 2C efficiently disrupt any
intracellularly formed, intrinsically weak two-layer rhinoviral GQ even when bound by PDS.

In summary, we have shown that targeting QGRS by GQ-stabilising compounds
specifically inhibits the uncoating of a common cold virus and provides a mechanistic
explanation based on biophysical and ultrastructural analysis. Strikingly, PDS-uptake into the
virus occurs in the presence of physiological concentrations of Na$^+$ but not K$^+$ due to their
differential impact on viral RNA compaction rather than GQ formation. Finally, apart from its
potential as a new anti-rhinoviral compound, PDS did not interfere with the binding of RV-A2
to the host cell and likely preserved the immunogenic epitopes, making these low-infectious
particles attractive candidates in the development of attenuated vaccines for this group of
viruses.

MATERIALS AND METHODS

Bioinformatic analysis of rhinoviral genomes for identification of putative GQs

The QGRS mapping software was kindly made available by Paramjeet S. Bagga from
Ramapo College, New Jersey. The QGRS Mapper algorithm predicts the ability of a
sequence containing G-repeats to fold into one to several distinct intramolecular
quadruplexes (termed Quadruplex-forming G-rich Sequences, QGRS) and assigns them a
stability score (G-score) based on published biophysical data. We performed our search
using the preset (default) search options: maximal length of 45, minimum G-group as 2 and
loop size from 0 to 36; allowing a loop size of 0 (zero loop) included non-canonical QGRS as
reported in yeast microsatellite DNA and mRNA involved in polyamine biosynthesis. The
complete RV A (75), B (26) and C (19) sequences (120 sequences in total, available at
the time of writing and accessible through The Pirbright Institute) were used. The
corresponding GenBank accession numbers are listed in the Supplementary Table 1. Non-
overlapping sequences with a G-score ≥ 10 within the sliding window were rendered as a
2D-line plot presenting the QGRS length and a heat-map showing the individual G-scores.
**Oligonucleotides and reagents**

Synthetic 27-mer RNA oligonucleotides (the positive control = human telomeric repeat-containing RNA miniTERRA \(^{135}\)), G20, and G11 (for sequences see Table 1) and the negative control (G20 with all Gs replaced by Cs), real-time PCR primers (RV-A2 Fw: 5’ gcacctgtgtcagagttttc 3’; Rv: 5’ aggtgtcagtgtattttattgactaggctg 3’ and Aichi virus A (AiV) Fw: 5’ tgtacaacacccactccatgtg 3’; Rv: 5’ tccacagagggagttcctg 3’) were purchased from Microsynth. Protein molecular weight markers, DNA ladder, ethidium bromide, Dulbecco’s Modified Eagle’s Medium (DMEM), trypsin-EDTA solution, Pen-Strep antibiotics, bovine serum albumin (BSA), thioflavin T (250 µM stock solution prepared in ultra-pure water and kept at 4 °C), pyridostatin (4-(2-Aminoethoxy)-N2,N6-bis[4-(2-aminoethoxy)-2-quinolinyl]-2,6-pyridinedicarboxamide trifluoroacetate salt) (20 mM stock solution prepared in ultra-pure water, aliquoted and kept at – 80 °C and thawed immediately before use), and Phen-DC3 (3,3′-[1,10-Phenanthroline-2,9-diylbis(carbonylimino)]bis[1-methylquinolinium] 1,1,1-trifluoromethanesulfonate (1:2)) (1 mM stock solution prepared in DMSO, aliquoted and kept at – 80 °C and thawed immediately before use) were purchased from Merck. TRIzol reagent, SYTO 82 orange, Hoechst 33342 solution, and goat anti-mouse AlexaFluor 488, goat anti-mouse IgG HRP-conjugated secondary antibody, and SuperSignal West Pico chemiluminescent substrate were purchased from Thermo Fisher. Foetal bovine serum (FBS) was purchased from Life Technologies. IRDye 680RD Goat anti-Mouse IgG secondary antibody was purchased from LI-COR.

**Cells and virus**

HeLa Ohio cells were originally obtained from ATCC and maintained in DMEM, supplemented with 10 % FBS, 1 % penicillin and streptomycin. Cells were kept in a humidified 5 % CO\(_2\)-containing atmosphere at 37 °C. In infection assays, the serum concentration was reduced to 2 % FBS and cells incubated at 34 °C, the optimal growth temperature of RV-A2. For virus infection, we used RV-A2, initially acquired from ATCC and propagated and purified following the protocol detailed in \(^{99}\).

**ThT assay for detection of GQ**

Ribooligonucleotides were diluted in 100 mM potassium or sodium phosphate buffer (pH 7.4) to a final concentration of 5 µM, incubated for 10 min at 90 °C followed by 10 min at 4 °C, and mixed with ThT (final concentration 5 µM). Samples were excited at 440 nm, and emission was measured at 490 nm using a PerkinElmer VICTOR Nivo Multimode Plate Reader.

For examination of viral RNA, purified RV-A2 (~2 µg) was suspended in 100 mM sodium phosphate buffer or 100 mM potassium phosphate buffer, both at pH 7.4, and the protein shell was digested with 5 µg of proteinase K at 4 °C overnight. The following day, the
ex virion RNA samples were ultrafiltered using 100 K Merck Amicon Ultra Filter units according to the manufacturer’s protocol, followed by 4 X 400 µl washes with the respective buffers. Samples were mixed with ThT (final concentration 5 µM), and the volume was adjusted to 100 µl. The ThT fluorescence signal was acquired as described above. The sample labelled at 30 °C was maintained at room temperature all the time. The 60 °C labelled sample was incubated at this higher temperature for 10 min and cooled to room temperature on the bench for 30 min, followed by the acquisition of the fluorescence signal.

**Fluorescent indicator Displacement assay (FiD)**

ThT-containing ribooligonucleotide samples prepared as above (100 µl) were dispensed into wells of a 96-well plate. PDS was then added in 2 µM steps up to 50 µM, and the respective fluorescence was recorded at 34 °C. Signal loss due to ThT dilution by the solvent control (water) was negligible. A titration curve was constructed from three independent experiments by plotting the percentage fluorescence drop (obtained by dividing the mean fluorescence intensity at each PDS concentration by the mean initial signal ((no PDS) x 100) against the concentration of PDS.

**Particle Stability Thermal Release Assay (PaSTRy)**

PaSTRy-related experiments were performed according to Real-Hohn, Groznica with minor adaptations. RNA exposure was monitored with SYTO 82 in a Bio-Rad CFX Connect Real-Time PCR instrument. Purified RV-A2 (~3.5 µg) was pre-incubated ± PDS at 200 µM final concentration and with PBS (volume adjusted to 70 µl) at 4 °C (no virus breathing) and 34 °C (permitting virus breathing) for 4 h. Unbound PDS was removed by centrifugation in 100 K Merck Amicon Ultra Filter units according to the manufactures’ protocol, followed by 4 X 400 µl washes with PBS at 25 °C to eliminate any remaining unbound PDS. SYTO 82 was added to a final concentration of 5 µM, and the volumes were adjusted to 70 µl with PBS. Three 20 µl aliquots from each of these samples were dispensed into the wells of a thin-walled PCR plate, and the temperature was ramped from 25 - 95 °C at 1.5 °C / min, and SYTO 82 light-up fluorescence was recorded. Six independent measurements were done for each condition. Data were rendered as a dot plot revealing the onset of access of SYTO 82 to the RNA (Real-Hohn, Groznica and Supplementary Fig. S1).

**Differential Scanning Fluorimetry (DSF) analysis of RV-A2 ex virion RNA**

Purified RV-A2 (~2 µg) was suspended in 100 mM sodium phosphate buffer or 100 mM potassium phosphate buffer, both at pH 7.4, and the protein shell was digested with 5 µg of proteinase K at 4 °C overnight. On the following day, the ex virion RNA samples were ultrafiltered using 100 K Merck Amicon Ultra Filter units according to the manufactures’ protocol, followed by 4 X 400 µl washes with the respective buffers. SYTO 82 was added to a
final concentration of 5 µM, and the volumes were adjusted to 70 µl with the respective
buffers. Three 20 µl aliquots from each of these samples were dispensed into the wells of a
thin-walled PCR plate, and the temperature was ramped from 25 - 95°C at 1.5 °C / min, and
SYTO 82 light-up fluorescence was recorded. Three independent measurements were
performed for each condition, and the fluorescence signal means for each condition were
displayed.

**Virus yield reduction assay**

Purified RV-A2 (∼1 µg) ± PDS (20 µM final concentration, or ultra-pure water as
control), was adjusted to 100 µl in 100 mM sodium or potassium phosphate buffer (pH 7.4)
and incubated overnight at 25 °C. Unbound PDS was removed by centrifugal ultrafiltration as
above. The virus titers (TCID<sub>50</sub>) were determined as described elsewhere.<sup>136</sup>

**Immunocytochemistry and flow cytometry**

Cells grown in a 6-well plate until 90 % confluent were infected with 0.1 µg RV-A2
either untreated (corresponding to an MOI of 1) or pretreated with PDS (at 20 or 200 µM final
concentration) diluted in PBS and incubated for 4 h at 34 °C. The unbound PDS was
removed by centrifugal ultrafiltration. The same experiment was carried out with Phen-DC3
at 1 µM and 5 µM. At 9 h pi the infection medium was aspirated, cells were washed once with
PBS and detached with 0.1 % trypsin in 0.05 % EDTA. The trypsin was inactivated with 10 %
FBS in DMEM. Cells were harvested by low-speed centrifugation at 300 g for 3 min at 4 °C.
The pellet was resuspended in 500 µl ice-cold PBS, followed by the addition of 500 µl 4 %
formaldehyde in PBS and incubation for 10 min at 4 °C. This and all subsequent steps were
done with gentle rocking. Cells were subsequently washed 3 times with 1 ml of ice-cold
PBST (PBS plus 0.1 % Tween-20, pH 7.4) at 4 °C, resuspended in PBS + 0.1 % Triton X-
100, and incubated for 10 min at 4 °C. The cells were then incubated in blocking buffer (1 %
BSA, 0.1 % Tween-20 PBS (pH 7.4)) for 30 min at 4 °C, followed by incubation with 10 µg/ml
8F5, a monoclonal antibody specific for VP2 of RV-A2<sup>137</sup> in blocking buffer for 1 h at 4 °C.
Cells were again washed 3 times with PBST and incubated for 1 h with goat anti-mouse
AlexaFluor 488 antibody diluted (1 : 1,000) in blocking buffer at 4 °C. Samples were then
incubated with Hoechst dye solution in PBS (1 : 2,000) for 10 min for staining nuclei, followed
by 3 times washing with PBST. They were finally resuspended in PBS and analysed with a
BD Bioscience FACSARia III flow cytometer; more than 10<sup>4</sup> events were acquired for each
sample. Forward scattering (FSC) vs VP2 (FITC-A) plots were generated by Tree Star
FlowJo X v10.0.7 software.

**Immunoprecipitation**

Cells grown in a 10 cm culture plate until ~80 % confluent were infected with ~1 µg
RV-A2 pretreated ± 200 µM PDS (as for flow cytometry, see above) at 34 °C; 30 min pi the
medium was removed and replaced with 1 ml PBS. The cells were gently detached with a
cell scraper (Corning) and subjected to 3 freeze/thaw cycles. Cell debris was removed by
low-speed centrifugation, and the supernatant was divided into 2 aliquots. From one aliquot,
viral uncoating intermediates (i.e. subviral A- and B-particles) were immunoprecipitated using
MAb 2G2 bound to protein G magnetic beads (Dynabeads-Protein G; Life Technologies).
The second aliquot was taken as a negative control by omitting MAb 2G2 but otherwise
processing it identically. As a positive control, ∼1 µg of heated RV-A2 (10 min at 56 °C
resulting in an almost ~100 % conversion into subviral B-particles) was processed identically.
After extensive washing in PBS, the immunoprecipitates were resuspended in 100 µl PBS.
Two µl of 5X protein sample buffer were added to 18 µl of each sample and the mixture
heated to 95 °C for 10 min. The proteins were separated by SDS-PAGE (10 %) followed by
transfer to an Immobilon-P membrane. The Western blot was done essentially as described
in 138. In brief, the viral protein (VP2) was detected with mouse monoclonal antibody (8F5),
anti-mouse-horseradish peroxidase and SuperSignal West Pico PLUS chemiluminescent
substrate (Thermo Fisher). The signal was quantified using a ChemiDoc Gel Imaging System
(Bio-Rad). To investigate the proportion of A- and B-particles, the viral RNA was also
quantified in 50 µl of the remaining respective resuspended immunoprecipitates. As internal
control and for normalisation, RNA obtained from 100 µl Aichi virus (AiV; a member of the
kobuvirus species of the *Picornaviridae* family), corresponding to 2 X 10⁷ TCID₅₀, was added
to each sample. RNA was recovered by TRIzol (1 ml; Invitrogen) extraction and precipitation
(together with GlycoBlue from Invitrogen) following the manufacturer’s protocol. First strand
cDNA synthesis was carried out with the NEBNext reagent kit (New England Biolabs, UK)
using random primers and the samples quantified by qPCR using primers specific for RV-A2
or AiV, using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

**Sucrose gradient sedimentation**

Cells were infected with RV-A2 pretreated ± 200 µM PDS for 30 min at 34 °C as
described above (immunoprecipitation method), the medium was removed, and 1 ml PBS
was added, followed by gently dislodging the cells with a cell scraper (Corning). The
resuspended cells were subjected to 3 freeze/thaw cycles. Cell debris was removed by low-
speed centrifugation. Five hundred µl of the resulting supernatants containing the (sub)viral
particles were deposited onto preformed 10–40 % (w/v) sucrose density gradients made in
virus buffer (5 ml of 50 mM NaCl, 20 mM Tris-HCl, pH 7.4) and centrifuged at 4 °C for 30 min
in an SW55 Ti rotor (Beckman) at 286,794 g. Aliquots (250 µl) were collected from top to
bottom and frozen at −80 °C until further use. Twenty µl of each fraction were deposited onto
a methanol-activated Immobilon-P membrane (Millipore) placed in a 96-well Bio-Dot
Microfiltration Apparatus (Bio-Rad) for the dot blot analysis. Viral protein VP2 was detected
using the VP2-specific MAb 8F5 and IRDye 680RD Goat anti-mouse IgG secondary antibody
essentially as described for the development of the Western blot further above. The fluorescent signal acquisition was performed in an Odyssey Infrared Imager (LI-COR).

**Time-of-drug addition experiment**

HeLa cells grown in 6-well tissue culture plates to roughly 80% confluency were challenged with RV-A2 at MOI = 10 for 30 min in infection medium at 4 °C with steady rocking allowing the virus to attach to its receptor while preventing its internalisation for achieving a synchronised infection. Then, the inoculum was removed, cells were washed three times with PBS, a fresh infection medium was added, and incubation continued at 34 °C to trigger virus internalisation (T = 0 min pi). Immediately (T0) or after 60, 180 or 300 minutes (T60, T180 and T300 respectively), PDS was added to a final concentration of 20 µM. At 9 h pi, cells were processed for flow cytometry and immunocytochemistry as described above. Using Tree Star FlowJo X v10.0.7 software, a FITC-A histogram showing the mean fluorescence intensity (MFI) from more than 10^6 events corresponding to de novo produced VP2 was generated upon gating based on FSC and SSC properties.

**Nuclear magnetic resonance**

NMR experiments were performed with a 600 MHz Bruker Avance 3HD+ spectrometer at 4, 25 and 34 °C. The concentration of the ribooligonucleotides was ~0.25 mM. The solution contained 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 10% D_2O. ^1H NMR shifts were referenced relative to (external) DSS (Sodium trimethylsilylpropanesulfonate). The typical sample volume was 500 µl. Water suppression for ^1H NMR spectra was performed using a double WATERGATE echo with extra water flip-back pulse to avoid saturation of exchangeable hydrogens due to hydrogen exchange. Typically ca 1000 scans each were required to obtain a ^1H NMR spectrum with sufficient signal to noise ratio. For the hydrogen-deuterium exchange (DXH) assay, 0.3 mM G11 RNA was prepared in 10 mM sodium phosphate (pH 7.4), 100 mM KCl and diluted 1:3 in D_2O to 0.1 mM G11 RNA (final concentration) immediately before acquisition. The signal attenuation of the imino hydrogens due to hydrogen-deuterium exchange was observed in a series of ^1H NMR spectra throughout 9 h.

**Circular Dichroism (CD) and melting profile**

Ribooligonucleotides were used at 20 µM in 100 mM potassium phosphate (pH 7.4) or 100 mM sodium phosphate (pH 7.4), as indicated in the figure. Unfolding and refolding experiments were performed with temperature ramping from 25 - 90 °C or 90 - 25 °C, respectively, at 1 °C/min using a Chirascan plus spectropolarimeter equipped with a Peltier temperature control system from Applied Photobiophysics. The buffer's CD spectrum was recorded identically and subtracted from the spectrum obtained for the RNA-containing solution. Data were zero-corrected at 400 nm.
Ribooligonucleotide electrophoresis

The synthetic ribooligonucleotides (miniTERRA, G11, and G20) in 20 µl 100 mM potassium phosphate buffer (pH 7.4) at a final concentration of 25 µM were heated to 95 °C for 10 min, transferred to 4 °C and incubated for an additional 10 min. Samples were then mixed with Gel Loading Dye (Blue 6X; New England Biolabs) and loaded onto a non-denaturing 12 % polyacrylamide gel containing 150 mM KCl. The gel was run in TBE buffer supplemented with KCl at 150 mM. Electrophoresis was performed in a fume hood with the chamber placed in an ice bucket for 3 h at 50 V. Ribooligonucleotides and DNA ladder (Low Molecular Weight DNA Ladder, New England Biolabs) were stained by incubation for 30 min in Gel-Red 3X solution and scanned in a Typhoon fluorescent scanner (General Electric, USA).

Electron Microscopy and Rotary Shadowing

Purified RV-A2 (~2 µg) was suspended in 100 mM sodium phosphate buffer or 100 mM potassium phosphate buffer, both at pH 7.4, and the protein shell was digested with 5 µg proteinase K at 4 °C overnight. The ex virion RV-A2 RNA was first diluted to a concentration of approximately 0.1 mg/ml in the same buffers used for digestion and subsequently diluted 1:1 in spraying buffer, containing 200 mM ammonium acetate and 60 % (v/v) glycerol, pH adjusted to 7.4. Immediately after dilution, the samples were sprayed onto freshly cleaved mica chips (Agar Scientific, UK) and quickly transferred into a BAL-TEC MED020 high vacuum evaporator (BAL-TEC, Liechtenstein) equipped with electron guns. While rotating, samples were coated with 0.6 nm platinum (BALTIC, Germany) at an angle of 7°, followed by 6 nm carbon (Balzers, Liechtenstein) at 90°. The obtained replicas were floated off from the mica chips, picked up on 400 mesh Cu/Pd grids (Agar Scientific), and inspected in an FEI Morgagni 268D TEM (Thermo Fisher Scientific, The Netherlands) operated at 80kV. Images were acquired using an 11 megapixel Morada CCD camera (Olympus-SIS, Germany).

Atomic force microscopy (AFM)

AFM imaging of the ex virion RV-A2 genomic RNA was done as follows: approximately 2 µg of purified RV-A2 were suspended in 100 mM sodium phosphate buffer or 100 mM potassium phosphate buffer, and its protein shell was digested with 10 U of proteinase K at 4 °C overnight. The samples were incubated with 20 µM PDS for 10 min, then deposition onto freshly cleaved mica and immediately imaged in a Pico-SPM atomic force microscope (Molecular Imaging, Phoenix, AZ, USA) equipped with a fluidic cell. The AFM images were acquired with acoustic AC (Tapping) mode using the MSNL (Bruker) cantilever E (with the nominal spring constant of 0.1 N/m) at 15 kHz. The scanning speed was 3000 nm/s, and the number of pixels per line was 256.
Molecular docking procedure

The chemical structure for PDS in the monomer and dimer forms at pH = 7.4 was built and minimised in terms of energy by Density Functional Theory (DFT), with the Becke-3-Lee Yang Parr (B3LYP) method and standard 6-31G* basis set, available in Spartan'18 software (Wavefunction, Inc., Irvine, USA) [https://www.wavefun.com/ accessed in January 2021]. The physicochemical properties, logP and dipolar moment, were also calculated using the energy parameters described above. The parallel RNA GQ's crystallographic structure was obtained from the Protein Data Bank (PDB) [https://www.rcsb.org/ accessed in January 2021] with access code 6JJH. This structure was used as a 3D model for molecular docking calculations to gain insights into the mode(s) of interaction between GQ RNAs and PDS due to its structural similarity with the RNA GQs used in the experimental assays and its high crystallographic resolution to about 1.74 Å. The molecular docking calculations were performed using GOLD 5.7 software (Cambridge Crystallographic Data Centre, Cambridge, UK) [https://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold/ accessed in January 2021]. Hydrogen atoms were added to the RNA GQ structure according to ionisation and tautomeric states inferred by the software. For methodology validation, redocking studies with the heterocyclic porphyrin TMPyP4 were carried out to evaluate the best scoring function to be used in the in silico studies (ChemPLP, Goldscore, ChemScore or ASP). The root mean square deviation (RMSD) value was 1.0039, 1.1222, 1.1999, and 0.9305 Å for ChemPLP, Goldscore, ChemScore and ASP, respectively. Since the lowest RMSD value was obtained with ASP, this function was then used in all further in silico studies. For each search, a 10 Å radius spherical volume around the RNA GQ structure was selected. The best docking poses were identified through the best docking score value, and figures were generated with PyMOL Delano Scientific LLC software (Schrödinger, New York, USA) [https://www.pymol.org/2/ accessed in December 2020].

Metabolomic analysis

Purified RV-A2 (12 µg) was incubated with PDS at 20 µM final concentration in 100 mM potassium phosphate (pH 7.4) or 100 mM sodium phosphate (pH 7.4) for 4 h at 4 °C (no virus breathing) or 34 °C (permitting virus breathing). Unbound PDS was removed from virus samples by centrifugation in 100 K Merck Amicon Ultra Filter units according to the manufactures' protocol, followed by 4 X 400 µl washes with the respective buffers to eliminate any remaining unbound PDS. Subsequently, virus samples were incubated with acidified methanol (1 % formic acid) for denaturing the capsid to allow extraction of PDS captured inside the virion. After centrifugation, the supernatant was analysed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). One µl of the extract was injected in an RSLC ultimate 3000 (Thermo Fisher Scientific) directly coupled to a TSQ Vantage mass spectrometer (Thermo Fisher Scientific) via electrospray ionisation in the positive ion
mode. A Kinetex C18 column was used (100 Å, 150 x 2.1 mm) beforehand to separate PDS from other components of the extract, employing a flow rate of 80 µl/min. A 10-minute linear gradient was used from 95 % A (1 % acetonitrile, 0.1 % formic acid in water) to 80 % B (0.1 % formic acid in acetonitrile). LC-MS/MS was performed by employing the selected reaction monitoring (SRM) mode of the instrument using the transitions 597.4 m/z → 511.3 m/z (CE 20) and 597.4 m/z → 468.3 m/z (CE 25). Data were interpreted manually, and the absolute amount of PDS quantified with a calibration curve obtained with external pyridostatin standard solutions.

Quantification and statistical analysis

All experiments were done at least in duplicate for a total of n ≥ 2 biological replicates. Data are displayed as mean ± standard deviation (SD) (for n > 2). Statistical significance was determined using the unpaired one-tailed Student's t-test. The p-value and sample size n of each experimental group are provided in the respective figure legends.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

AR-H, DB, and HK designed the study and wrote the manuscript. AR-H, MG, GK, RZ, OAC, and LV conducted experiments, analysed and interpreted the data. PH provided advice in the design of the experiments and analysis of the data. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY

All datasets presented in this study are included in the article/supplementary material.
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**FIGURES LEGENDS**

**Figure 1.** The genomic RNA of all RVs harbours sequences with the propensity to form GQs. This is confirmed for those two with the highest and lowest G-score from RV-A2 via nuclear magnetic resonance and circular dichroism. (a) RNA sequences of 120 RVs available in the NCBI database were analysed for putative GQ forming sequences by using a local copy of the QGRS mapper. G-scores \( \geq 10 \) are shown as rectangles with their length corresponding to the length of the putative GQ. The colour corresponds to the respective G-score as indicated with the colour key bar. Note that the sequences were used without alignment optimisation, i.e. neither deletions nor insertions were taken into account. Asterisks denote highly conserved GQs. Species RV-A, RV-B, and RV-C are specified. (b) One-dimensional \(^1\)H-NMR spectra of ribonucleotides representing putative two-layered GQ-forming sequences of RV-A2 with the lowest G-score (G11) and the highest G-score (G20),
both at 0.25 mM in 10 mM sodium phosphate (pH 7.4), 100 mM KCl. In addition, the
chemical shift region characteristic of imino protons involved in Hoogsteen base pairing
(salmon coloured box) and the Watson-Crick base pair region (olive coloured box) are
indicated. NMR spectra were measured at different temperatures (277 K and 307 K) and
after adding PDS to 0.5 mM (i.e. a stoichiometry of 1:2) final concentration. (c) CD
spectroscopy of miniTERRA, G11, and G20 diluted in 100 mM sodium phosphate buffer (pH
7.4) or 100 mM potassium phosphate buffer (pH 7.4) to 20 µM reveals an all-parallel RNA
strand orientation, irrespective of the cation present in the buffer.

Figure 2. Biophysical characterisation of miniTERRA (control GQ), RV-A2 GQ G11 and
G20 and impact of K⁺, Na⁺ and PDS. Ribooligonucleotides dissolved at 5 µM either in 100
mM sodium phosphate buffer (pH 7.4) (a - left panels) and (b) or in 100 mM potassium
phosphate buffer (pH 7.4) (a - right panels) and (c). (a) CD melting profiles of G11, G20, and
miniTERRA measured at 265 nm; the trace for unfolding is red and refolding in blue. The
temperature was ramped up or decreased at a rate of 1 °C per min and the corresponding
unfolded state fraction is shown in the left subpanels for each cation (Na⁺, K⁺) as normalised
ejellipticity (ᶿ). The second-order derivative of the respective curves is presented in the right
subpanels. (b) Left panel: The same ribooligonucleotides dissolved in 100 mM sodium
phosphate buffer (pH 7.4) were incubated with ThT, and the fluorescence was measured at 490 nm (n = 3). Right panel: Ribooligonucleotides identically pre-incubated with ThT were
titrated with PDS, the fluorescence was measured after each addition and normalised to the
initial fluorescence signal (i.e. without PDS = 100 %; n = 3). (c) The same experiment as in
(b) with ribooligonucleotides dissolved in 100 mM potassium phosphate buffer (pH 7.4).

Figure 3. GQ-binding compounds increase RV-A2 capsid permeability, impair
uncoating, and decrease infectivity. (a) Scatter plot of the temperature of onset (T_on) of
capsid permeability for SYTO 82 determined in a PaSTRy with purified RV-A2 that had been
pre-incubated ± 200 µM PDS at 34 °C for 4 h (for the raw data, see Supplementary Fig. S1; n
= 6). (b and c) HeLa cells were infected with purified RV-A2 that had been pre-incubated ±
200 µM PDS at 34 °C for 4 h. Thirty min pi, cells were harvested, and the intracellular virus
was released by three cycles of freezing and thawing and separated from cellular debris by
low-speed centrifugation. The cleared native and subviral particle-containing supernatant
was subjected to immunoprecipitation with mAb 2G2 specific for A- and B-particles (b and c).
(b) Proteins in the samples were separated by SDS-PAGE (10 %) followed by Western
blotting using anti-VP2 mAb 8F5 and a goat anti-mouse IgG HRP-conjugated secondary
antibody, and bands were quantified by densitometry. (c) RNA in the samples was isolated,
reverse transcribed and subjected to qPCR using specific primers. The quantity of RV-A2
RNA was normalised by relating the Ct value to the one obtained for a known amount of AiV seed virus that had been added to all cleared supernatant samples prior to the RNA isolation.

(d) Cleared supernatants prepared as above were separated by 10 – 40 % (w/v) sucrose density gradient centrifugation. Fifteen fractions were collected from top to bottom, proteins were heat-denatured, dot blots were prepared, and viral material was quantified with mAb 8F5 and IRDye 680RD goat anti-mouse IgG secondary antibody (n = 3). The obtained signal intensity was plotted against the gradient fraction (250 µl each of a total of 5 ml) from top to bottom. Note that VP2 is present in all (sub)viral particles. Native virus (150S) and subviral B-particles (80S) generated in vitro by heating of RV-A2 to 56 °C for 10 min were used as sedimentation controls and run on separate gradients. Their position is indicated in the plot; the position of the 14S pentamers was inferred from the literature. (e) Purified RV-A2 was incubated ± PDS or Phen-DC3 at the concentrations indicated for 4 h at 34 °C. The compounds, which have not entered the virion were removed from the samples by centrifugal ultrafiltration followed by multiple washing steps to eliminate any external traces of the compound; the (un)treated virus material was then used to infect HeLa cells. The percentage of infected cells was determined at 9 h pi by FACS analysis of the intracellularly produced VP2 with the mAb 8F5 and a secondary anti-mouse AlexaFluor 488 conjugated secondary antibody and is indicated as bars (n = 3; * p ≤ 0.05; ** p ≤ 0.01; NS, not significant).

Figure 4. PDS treatment of ex virion RNA results in a drastic PDS-induced reorganisation in the presence of sodium but not potassium ions. (a) RV-A2 RNA was gently released into solution by proteolysis of the capsid protein shell in 100 mM sodium phosphate buffer (Na+) or 100 mM potassium phosphate buffer (K+). The ex virion RNA samples were mixed with ThT (final concentration 5 µM), and the ThT fluorescence signal acquired at 30 °C. Afterwards, samples were incubated for 10 min at 60 °C and cooled to room temperature on the bench for 30 min, followed by ThT fluorescence acquisition. (b) RV-A2 RNA (prepared identically as in (a)) was incubated with 20 µM PDS for 10 min at room temperature and subjected to rotary shadowing followed by TEM. Inserts depicting representative images of ex virion RNA in sodium or potassium phosphate buffers non-treated with PDS are displayed (50 nm scale bar). (c) Samples similarly treated as in (b) were analysed by AFM in the presence of 100 mM sodium or potassium phosphate buffer. (d) Purified RV-A2 was diluted in the same buffers as above and incubated overnight with or without 20 µM PDS at room temperature. Unbound PDS was removed by centrifugal ultrafiltration and the infectivity of the samples was determined by end-point titration. The viral titer measured as TCID_{50} is rendered by a bar graph (n = 3). The significance of the differences was evaluated by two-way ANOVA; NS – statistically not significant (p ≥ 0.05). (e) HeLa cells were seeded in six-well plates and cultivated at 37 °C until 70 - 80 % confluent on
the day of the experiment. Cells were then challenged with RV-A2 (MOI = 10) for 30 min at 4°C, allowing virus attachment. Synchronised virus entry was triggered by transfer into a 34°C, 5% CO₂ tissue culture incubator. Immediately before (T0), 180 min (T180) and 300 min (T300) after the temperature shift, the medium in the respective well was adjusted to 20 µM PDS and incubation of cells continued for 9 h to allow for one cycle of infection. Non-infected cells cultivated in the absence of PDS were examined in parallel. Cells were harvested, fixed, permeabilised and immunostained with the VP2-specific mAb 8F5, followed by secondary antibody fluorescently labelled with Alexa Fluor 488 and analysed by flow cytometry. Non-infected and infected populations are displayed at the left and right, respectively, in the fluorescence intensity histogram, and the corresponding percentage is provided on top. MFI is the mean value of fluorescence intensity calculated for each sample.
Supplementary Files

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