Published in final edited form as:

J Chem Inf Model. 2020 December 28; 60(12): 6566–6578. doi:10.1021/acs.jcim.0c00742.

Application of Molecular Dynamics Simulations to the Design of Nucleotide Inhibitors Binding to Norovirus Polymerase

Holly Freedman,

Li Ka Shing Applied Virology Institute, Li Ka Shing Institute of Virology, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

Juthika Kundu,

Li Ka Shing Applied Virology Institute, Li Ka Shing Institute of Virology, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

Egor Petrovitch Tchesnokov,

Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

John Lok Man Law,

Li Ka Shing Applied Virology Institute, Li Ka Shing Institute of Virology, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

James A. Nieman,

Li Ka Shing Applied Virology Institute, Li Ka Shing Institute of Virology, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

Raymond F. Schinazi,

Center for AIDS Research, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30322, United States

D. Lorne Tyrrell,

Li Ka Shing Applied Virology Institute, Li Ka Shing Institute of Virology, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

Matthias Gotte,

Li Ka Shing Institute of Virology and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

Michael Houghton

Corresponding Authors Holly Freedman hfreedma@ualberta.ca; Michael Houghton mhoughto@ualberta.ca.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

Li Ka Shing Applied Virology Institute, Li Ka Shing Institute of Virology, and Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta T6G 2E1, Canada

Abstract

The RNA-dependent RNA polymerase (RdRp) of norovirus is an attractive target of antiviral agents aimed at providing protection against norovirus-associated gastroenteritis. Here, we perform molecular dynamics simulations of the crystal structure of norovirus RdRp in complex with several known binders, as well as free-energy simulations by free-energy perturbation (FEP) to determine binding free energies of these molecules relative to the natural nucleotide substrates. We determine experimental EC₅₀ values and nucleotide incorporation efficiencies for several of these compounds. Moreover, we investigate the mechanism of inhibition of some of these ligands. Using FEP, we screened a virtual nucleotide library with 121 elements for binding to the polymerase and successfully identified two novel chain terminators.

Graphical Abstract

INTRODUCTION

The discovery of sofosbuvir and its tremendous success at curing HCV infection with, so far, very few cases of resistance¹ sets precedence for the use of nucleoside antiviral agents to target viral RNA-dependent RNA polymerases (RdRps). Gastroenteritis associated with infection by human norovirus (NoV), a positive-sense single-strand RNA virus belonging to the *Caliciviridae* family, has been estimated to kill 50 000 children worldwide yearly,² and cases of noroviral illness, especially in immunocompromised persons, can be severe. It poses a serious health risk in situations where people are in close contact such as elderly care homes, dormitories, hospitals, and cruise ships. Currently, no FDA-approved antiviral agents are available to treat NoV-induced gastroenteritis, and no vaccine is available for prevention of this illness.³⁻⁶ NoV depends upon its RNA-dependent RNA polymerase (RdRp) for both genome replication and transcription. NoV RdRp was first crystallized in 2004 in the

apoform.⁷ In 2008, it was crystallized in complex with active site metal ions, an RNA transcript, an RNA template, and an incoming cytidine triphosphate (CTP) molecule,⁸ and other crystal structures have since followed.^{6,9-15} The enzymatic mechanism of viral RdRps is highly conserved and occurs in a five-step reaction cycle. Specifically, an NTP first binds to the open complex, there is a conformational change to the closed complex, and then nucleotidyl transfer occurs, prior to pyrophosphate release and translocation. In the crystal, NoV RdRp is trapped in the substrate-bound, closed complex form by the restricted environment, which leaves no space for primer extension, thus providing a valuable structure of this substrate-bound complex.⁸ NoV RdRp, like all other crystallized RdRps, is shaped like a cupped right hand (see Figure 1).¹⁶ Among other polymerases with known crystal structures, it is most structurally related to DNA polymerase β .⁸ Thumb, fingers, and palm domains make up the right-hand-like structure, and the NTP binding pocket occupies the center of the palm domain. In NoV RdRp, the amino terminal domain bridges the fingers and thumb domains. The availability of this crystal structure makes it possible to apply molecular modeling to investigate structural aspects of the binding of inhibitors to NoV RdRp. NoV RdRp was previously the subject of molecular dynamics (MD) simulations, which examined structural differences between the polymerase bound by CTP, dCTP, and 5' phosphonate analogues.¹⁷ Herein, we perform simulations focusing on NoV RdRp bound by base-modified and sugar-modified NTP analogues.

Binding free energies calculated by free-energy perturbation (FEP) have been found to give excellent agreement with experimental data for numerous protein/inhibitor systems, usually with errors less than 1.0 kcal/mol.¹⁸ Free-energy simulation was previously applied in our group to determine relative binding free energies of NTP analogues to mitochondrial RNA polymerase (POLRMT), yielding average errors of about 1.0 kcal/mol.¹⁹ Free-energy simulation has also been applied to NTP molecules binding to HCV RdRp.²⁰ Viral inhibitors binding to NoV RdRp that have been identified so far include 2'-C-methyl-CTP (2'-Me-CTP),²¹⁻²³ 2'-amino-2'-deoxy-CTP (ACT),⁹ 5-nitro-CTP (NCT),⁸ and recently 4-amino-7-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-2-methyl-7H-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide (2Me7Am-ATP)²⁴ (Figure 2). Here, we calculate the binding free energies relative to the natural NTP molecules in complex with NV RdRp by applying FEP and compare these to experimentally determined EC₅₀ values, determined by real-time quantitative polymerase chain reaction (real-time qPCR), and to nucleotide incorporation efficiencies. The highly charged and flexible phosphate groups on nucleotide ligands demand lengthy free-energy simulations, for which we make use of our Blue Gene Q supercomputer. Using the same workflow, we screened a virtual library of nucleotides for binding to NoV RdRp and further evaluated two of the top hits experimentally.

Additionally, we studied the mechanisms of inhibition of these compounds. With regard to the two ribose-modified compounds, 2'-Me-CTP was found to be a nonobligate chain terminator.²⁵ On the other hand, the base-modified compound, NCT, most likely acts as a competitive inhibitor of natural substrate binding without being added to the growing oligonucleotide chain, as has been shown concerning its inhibition of the poliovirus RdRp.²⁶ Ng and co-workers⁸ previously suggested a few potential mechanisms of inhibition of NCT, based upon comparison of the crystal structures of the NCT-bound and CTP-bound

complexes. That is, structural changes to the enzyme/NCT complex were identified, which might allow NCT to act as an inhibitor. We examined whether these structural differences are preserved in our molecular dynamics simulations of the solvated complexes compared to the structures of other NTP-bound complexes to illuminate the structural changes that most likely contribute to inhibition. We also examined structural differences likely to cause 2Me7Am-ATP to act as an inhibitor.

As an aside, we note that the relatively short time scale of our simulations is a limitation of our approach since they only reliably sample the conformational space around the crystal structure of the closed catalytic complex but not other important conformational substates.

RESULTS

Molecular Dynamics Simulations.

We created a solvated molecular model of NoV RdRp in complex with CTP by running a 117 ns MD simulation of the system and clustering the resulting trajectory. CTP was then replaced with ATP in the model, simulations were continued until the root-mean-square deviation (RMSD) relative to the initial structure had stabilized for at least 80 ns (Figure 3), and a representative structure of the ATP-bound polymerase was selected after clustering.

We then replaced the natural nucleotide with each of 2'-Me-CTP, ACT, NCT, or 2Me7Am-ATP in our models of CTP- and ATP-bound NoV RdRp for pyrimidine or purine NTPs, respectively. We performed 300 ns simulations of each complex. The structures of the active sites of each of the NTP-bound complexes obtained by averaging over all trajectory frames are shown in Figure 4. Several notable differences were observed among the six structures. First, differences are seen in the interactions formed between the bound NTP molecule and Arg182, which is critical in stabilizing the pyrophosphate leaving group during nucleotidyl transfer. Different NTP phosphate atoms are involved in this interaction for the different complexes. Moreover, π -stacking between the Arg182 guanidino group and the nucleobase of the incoming NTP was observed in the NCT-bound complex only. In particular, the angle between the normal to the plane formed by the Arg182 side chain and the normal to the plane formed by the nucleic acid base belonging to the ligand was found to take on an average value of 14.7° with a standard deviation of 7.8° for the NCT complex. The small value of this angle corresponds to π -stacking, whereas for the CTP-bound complex, this angle was computed to be 33.8° with a standard deviation of 13.7° .

Another notable difference between the structures shown in Figure 4 is that the NTP γ -phosphoryl group may interact with either Lys174 or Lys180 in the complexes. Strong electrostatic interactions with positively charged residues Lys174 and/or Lys180 are important for anchoring the negatively charged NTP molecule in the active site, as well as for stabilizing the pyrophosphate leaving group during catalysis.

In our pyrimidine-bound models, the coordination of active site metal ions is the same as in the 3BSO crystal structure,⁸ with one exception for the CTP-bound complex. Namely, metal A is coordinated by the side-chain carboxylate groups of Asp242 from motif A, of Asp343 and of Asp344, by the primer 3' hydroxyl O atom, by an α -phosphoryl nonbridging O atom,

and by one water molecule. Metal B is coordinated by the side-chain carboxylate groups of Asp242 and Asp343, by the main-chain carbonyl O atom of Tyr243, and by α , β , and γ nonbridging phosphoryl O atoms. In the CTP-bound complex, a water molecule is in between the α -phosphoryl nonbridging O atom and metal A, replacing it in the coordination shell. In our models of the polymerase bound by a purine (ATP or 2Me7Am-ATP), the coordination of metal atom A involves the same atoms as for most pyrimidines, and the coordination of metal B involves the same ones except that a water molecule replaces the α -phosphoryl group in the coordination shell.

NoV RdRp Enzyme Assay.

An inhibitor can block polymerization by one of at least two means. Either it may be added to the growing oligonucleotide and terminate the chain by preventing the addition of the next NTP or it may act as a competitive inhibitor by occupying the nucleotide-binding site without reacting. In Figure 5A, we show the transition state for nucleotidyl addition by poliovirus RdRp calculated by Shen et al.,²⁷ who performed quantum chemical calculations on a molecular cluster representing the poliovirus RdRp active site. The two nucleotides shown in the figure exemplify the positions of an immediate chain-terminating inhibitor, which is incorporated into the primer but blocks the addition of the next NTP molecule, and an inhibitor that binds but does not react, corresponding to an incoming NTP molecule, respectively.²⁸⁻³¹

2'-Me-CTP acts as a chain terminator of NoV RdRp,²⁵ probably through steric hindrance of the 2'-Me group during nucleotidyl addition.³² On the other hand, in poliovirus RdRp, NCT has been shown to occupy the NTP binding site, blocking nucleotidyl addition.²⁶

We monitored NoV RdRp nucleotide incorporation along a symmetrical RNA primer/template designed such that [α -³²P]-GTP is utilized as the first nucleotide to be incorporated, which results in a radioactively labeled growing primer strand (Figure 6, second lane from the left). Incubation with specific NTP mixes resulted in limited RNA synthesis yielding expected products (Figure 6, left panel). Low levels of incorrect (mismatched) nucleotide additions were present, and we controlled for these products with lane ("ATP + UTP, 1 μ M"). Product 17 illustrates the migration pattern of product with incorporated cytidine monophosphate (CMP), cytosine arabinoside monophosphate (ara-CMP, the C2' epimer of CMP), and incorporated ACT (labeled as 2'-NH₂-CTP). Note that products containing incorporated CMP analogues migrate differently from one another, which reflects their structural differences. In the presence of ATP and UTP, there is no formation of products longer than product 17, which points to chain termination. This is not the case if ATP, 2'-dCTP, and UTP are present in the reaction mixture; product is extended to product 20 at levels comparable to reaction mixture containing ATP, CTP, and UTP (55 versus 77%, respectively). This suggests that the incorporation of 2'-dCTP does not cause chain termination. Incorporation of ACT also does not cause chain termination, and the extent of the products longer than product 17 is also comparable with the condition where CTP is present instead of the analogue (68 versus 77%, respectively).

Free-Energy Simulations.

The representative structures of CTP and ATP bound to NoV RdRp were used as starting conformations for free-energy simulations, for pyrimidine and purine bound complexes, respectively. We calculated values of -0.95 and -0.63 kcal/mol for the perturbation of CTP to the 2'-modified analogues 2'-Me-CTP and ACT, respectively (Table 1). These values were consistent with both NTPs being inhibitors of NoV RdRp and indicated that 2'-Me-CTP is the better inhibitor of the two. Using replicon assays, we measured experimental EC_{50} values for 2'-Me-CTP and ACT of 3.3 and 50 μ M, respectively. Thus, the simulations qualitatively capture the preferred binding by the former inhibitor. With our enzyme assays, we measured an incorporation efficiency relative to CTP of 216 for ACT and an incorporation efficiency relative to CTP of 63 for 2'-Me-CTP. A larger value of the incorporation efficiency denotes slower incorporation relative to the maximum level, with an efficiency value of 1 assigned to the incorporation of CTP, and thus the order of these numbers follows the same trend as the computational values and the measured EC_{50} values. However, the measured incorporation efficiency of ara-CTP is 198, and the decrease in this value compared to that of ACT does not correlate with the increase in our computed relative binding free energy of ara-CTP compared to that of ACT (0.0 kcal/mol compared to -0.63 kcal/mol, respectively).

Our calculated G values for the base-modified analogues NCT and 2Me7Am-ATP binding to NoV RdRp compared to those for CTP and ATP, respectively, are 0.82 and -0.40 kcal/mol (Table 1). The latter value is consistent with 2Me7Am-ATP being an inhibitor. Our experimentally determined EC_{50} value for 2Me7Am-ATP is 6.2 μ M. In our calculations, ATP was converted to 2Me7Am-ATP in the NoV RdRp active site in two steps, corresponding to first the addition of the 7-amide group, and second the addition of the 2-methyl group. Interestingly, the free-energy difference originated almost entirely from the second step, stressing the important thermodynamic contribution of the 2-methyl group to the ability of 2Me7Am-ATP to compete with ATP for binding.

On the other hand, the value we calculate for NCT binding indicated that NCT binds less well to NoV RdRp than the other inhibitors studied here. NCT has been crystallized bound to NoV RdRp⁸ and has been reported to be a potent inhibitor of picornaviral polymerases in general, associated with antiviral activity.²⁶ Because an average error of ~ 1.0 kcal/mol was associated with our previous calculations of binding free energies of POLRMT inhibitors by comparison to experimental values,¹⁹ experimental testing would be needed to assess the validity of this computational prediction.

For further confirmation, we also performed FEP calculations of the relative binding free energies of 2'-deoxy-2'-fluoro-2'-C-methyl-CTP (2'-F-2'-C-Me-CTP) and of 2',3'-dideoxy-CTP to NoV RdRp compared to CTP binding. We obtained values of -0.21 kcal/mol and 0.86 kcal/mol, respectively, relative to CTP binding (Table 1). Experimentally, it was found by Costantini et al.²² that 2'-F-2'-C-Me-CTP inhibits synthesis of NoV RNA at an EC_{50} value of 3.2 μ M. 2',3'-Dideoxy-CTP is a reverse transcriptase inhibitor used to treat HIV infection, which is absorbed by cells and phosphorylated intracellularly,³³ but does not inhibit NoV replication in our assays (Table 1), consistent with the positive value we calculated for the relative binding free energy.

Despite the qualitative agreement of some of our computed relative binding free energies with the experimental EC₅₀ values or incorporation efficiencies, we note that the magnitudes of the relative binding free energies that we have calculated in Table 1 are all close in value to one another, associated with estimated errors of up to 2 kcal/mol, and less than the estimated accuracy of the FEP method of about 1 kcal/mol. Thus, while some agreement with experiment may be expected, accurate computational predictions cannot be expected for this set of nucleotides. However, predicted relative binding free energies greater than 2 kcal/mol in magnitude should be more dependable, and we now describe a virtual screen to search for compounds with such binding free energies.

Virtual Screening for Novel NoV RdRp Nucleoside Inhibitors.

Relative binding free energies to NoV RdRp were calculated for a virtual library of nucleotide analogues in a search for prospective drug candidates. A library of substituted derivatives of CTP, ATP, or UTP was built by associating chosen connection points of the natural nucleotide with elements of a fragment library containing 69 entries, by using the combinatorial builder belonging to the MOE (Molecular Operating Environment) program.³⁴ Fragments were added at chosen connection points if allowed by spatial constraints. We then ran FEP simulations to find G values, relative to native substrates, for binding of the derivatives suggested by MOE to NoV RdRp, on our Blue Gene Q supercomputer. A few suggestions from MOE had to be discarded because these contained atom types that could not be parameterized with the MATCH program.³⁵ In all, FEP calculations were used to consider 32 fragment additions at the C5 position of CTP, 31 at the N4 position of CTP, 6 at the C4' position of CTP, 24 at the C7 position of ATP, and 28 at the C5 position of UTP, for a total of 121 compounds. We chose to focus more intensely on CTP derivatives compared to ATP derivatives because physiologically ATP is present at a much higher cellular concentration than CTP and thus higher dosages of potential ATP derivative drug molecules would be required compared to those of CTP derivatives.

The top-scoring compounds from our screen are shown in Table 2. All are CTP derivatives. One ATP derivative, substituted by a 2-pyridyl group at the C7 position, was found to be associated with a fairly negative G value of -2.20 kcal/mol, but we discounted this compound since its associated estimated error was quite large (2.92 kcal/mol). Among the top-scoring compounds, synthetic routes were successfully determined for the compounds that we have labeled as aa101 and aa102. These compounds were predicted to have improved binding relative to CTP as a substrate to NoV RdRp by -4.2 and -1.7 kcal/mol, respectively. Compound aa101 has been previously investigated for possible inhibition of the hepatitis C virus and of yellow fever virus in replicon assays and was found to be ineffective and also to have low toxicity.³⁶ While neither of the corresponding nucleosides was active in our NoV RdRp replicon assay, both were found to be chain terminators of NoV RdRp in the triphosphate form (Figure 6). A lack of either phosphorylation within the cells or cellular uptake is most likely responsible for the inactivity of the compounds in the replicon assay. This emphasizes the importance of performing enzyme assays on candidate nucleotide inhibitors since prodrugs can be designed to overcome difficulties with cellular phosphorylation or uptake. However, both compounds aa101 and aa102 were found to have poor experimental incorporation efficiencies, with values of 2100 and 4600, respectively.

Proposed Mechanisms of Inhibition of NCT and 2Me7Am-ATP.

In all of our simulations, as well as in available crystal structures,^{8,9} the NTP analogues occupy the position of the incoming nucleotide. The reaction mechanism of nucleic acid polymerases involves two proton transfers.³⁷⁻³⁹ Shen et al.²⁷ proposed that in poliovirus RdRp, a water molecule accepts a proton from Lys359 and transfers it to a γ -phosphoryl nonbridging O atom. A second water molecule accepts a proton from the 3'OH group and Asp328 stabilizes this transfer. The transition state is then formed as the negatively charged 3' O atom undergoes nucleophilic attack on the α -phosphoryl P atom, and the bond between this P atom and the α - β bridging O atom starts to break.²⁷ In Figure 5B–D, we show the CTP-, NCT-, and 2Me7Am-ATP-bound NoV RdRp complexes viewed in a similar orientation to how the poliovirus RdRp is shown in panel (a). It may be noted in panels (b–d) that Lys374 or Lys174, and Asp344 in NoV RdRp are positioned to substitute for the roles of Lys359 and Asp328, respectively, in poliovirus RdRp.

NCT.—Several suggestions were made by Zamyatkin et al.⁸ concerning the mechanism of inhibition of NCT, based upon comparison between the crystal structure of NCT-bound NoV RdRp and the CTP-bound complex. One suggestion was that the catalytic site might be restructured by nucleotide binding so that Arg182 cannot stabilize the cleavage of the bond between the P_{α} atom and the α - β bridging O atom. An essential role of an enzyme in catalysis is to supply an environment polarized in the directions that facilitate the transfer of electrons and/or atomic nuclei during reaction. Accordingly, the presence of the positively charged residue Arg182 near the α - β bridging O atom is thought to be essential for stabilizing the transfer of negative charge to this O atom during cleavage of the bond between this O atom and the P_{α} atom. A second possible factor contributing to the mechanism of inhibition mentioned was the proximity of the negatively charged NCT nitro O atoms to the P_{α} atom, which would destabilize the nucleophilic attack by the negatively charged 3'-hydroxyl O atom of the primer on this P atom. Third, it was proposed that the coordination of active site metal ions might differ in the presence of the inhibitor and in particular that Asp344 might not be positioned to coordinate metal A.⁸

We thus analyzed our trajectories of the NCT- and CTP-bound complexes to check whether the structural differences seen in the crystal structures that were proposed to possibly contribute to the reaction mechanism are preserved during simulations of the solvated system.

Regarding differences between interactions of Arg182 with the NTP molecule, it was noted that in the crystal structure of NCT-bound NoV pol, NH1 but not NH2, belonging to Arg182, forms a hydrogen bond with the α , β -bridging O atom, while in the CTP-bound complex, both do form. Zamyatkin et al.⁸ proposed that the π -stacking of the nitro group of NCT with Arg182, observed in their crystal structure of the NCT-bound complex (as well as in our simulations), might be responsible for Arg182 becoming more distant from the β phosphate, thus reducing the ability of Arg182 to stabilize the pyrophosphate leaving group. Therefore, we measured the respective distances between each of NH1 and NH2 belonging to Arg182 and the α , β -bridging O atom, for each of our average NTP-bound complexes (Table 3). On the one hand, in the average structure of NCT-bound NoV pol, the distance between NH2

and the α , β -bridging O atom is 3.7 Å, too large to correspond to a stable hydrogen bond. However, the same may be said for the ACT and 2'-Me-CTP-bound complexes for which this distance is 4.1 and 4.7 Å, respectively, for which reaction occurs.²⁵ Moreover, for the ATP-bound complex, the average distance to the α , β -bridging O atom is large for both NH1 and NH2 (5.0 and 4.1 Å, respectively). Therefore, it is unlikely that differences in the position of Arg182 are responsible for NCT being an inhibitor.

We next focused on the possibility that the NCT nitro group might disrupt the nucleophilic attack from the primer 3'-hydroxyl O on the P $_{\alpha}$ atom, by its proximity to these two atoms. In Figure 7A,B, we plot the closest nitro O distance to the 3'-hydroxyl O atom and to the P $_{\alpha}$ atom, respectively. The nitro O/P $_{\alpha}$ distance is 4.3 Å in the average structure, and it fluctuates from about 3.4–4.8 Å. The nitro O/3'-hydroxyl O distance is slightly larger, namely, 5.1 Å in the average structure, and fluctuates from about 3.8 to 5.6 Å. Based upon these distances, it seems reasonable that the positions of the nitro O atoms might inhibit the nucleotidyl addition reaction.

The last potential important structural difference proposed by Zamyatkin et al.⁸ was a difference in the distance of Asp344 to metal A in the NCT-bound complex leading to a disruption in metal ion coordination. By contrast, in Table 3, this distance (1.8 Å) corresponds to a strong coordinate bond for the NCT-bound complex, as for each of the other complexes. In addition, all of the coordination distances of the catalytic Asp residues are small for NCT, as for all of the complexes (Table 3).

Although our data does not support the two alternative explanations for the mechanism of inhibition of NCT, namely, differences in the metal ion coordination distances and the change in H-bonding of the Arg182 side chain with the triphosphate moiety of NCT compared to CTP, it is insufficient to discount these two secondary effects as possibly contributing to the mechanism of inhibition. It is problematic to describe ion binding using a molecular mechanics force field, and this may be the cause for the difference between the simulations and the crystal structures. For example, polarization and charge-transfer effects, which are neglected in force field approximations, can modulate ion-ligand binding. Moreover, it is impossible to obtain proper sampling of ion positions on the time scale of our simulations since Mg²⁺ ions chelated to phosphate are trapped for the duration of the simulations due to the strength of the binding affinity.⁴⁰

2Me7Am-ATP.—2Me7Am-ATP is another base-modified NTP inhibitor²⁴ having a base substituent positioned between the primer 3' hydroxyl O and the P $_{\alpha}$ atom, when bound to the NoV RdRp active site. In the average structure of the complex, the distance between the NH₂ moiety of the amide group and the 3' hydroxyl O is 4.9 Å, and the distance between this NH₂ group and the P $_{\alpha}$ atom is 3.8 Å. The latter distance is plotted as a function of time in Figure 7. However, the NH₂ group in this amide group is electropositive and thus would be expected to stabilize, rather than destabilize, the nucleophilic attack of the primer 3' hydroxyl O on the P $_{\alpha}$ atom essential for catalysis. That is, this effect would not be expected to contribute to the inhibitory mechanism of 2Me7Am-ATP.

We observe in our model of the 2Me7Am-ATP-bound NoV RdRp enzyme that the ϵ -amino groups on the positively charged residues Lys374, Lys174, and Lys180 are considerably more distant from the γ -phosphoryl group than in the other NTP-bound complexes studied here. The corresponding distances are given in Table 3. This structural difference for the 2Me7Am-ATP-bound enzyme seems to be brought about by the fact that the 2-methyl group slightly offsets the base-pairing between 2Me7Am-ATP and the RNA template, partially raising the 2Me7Am-adenine base above what would normally be the plane of the base-pair (compare Figure 4F to the other parts of Figure 4). This effect is propagated to the position of the γ -phosphoryl group of 2Me7Am-ATP, distancing it from the positively charged catalytic lysine residues. As described above, catalysis requires the transfer of a proton from a charged residue via an intermediary water molecule to the γ -phosphoryl group.^{27,37,38} We therefore propose that this change in the relative position of the γ -phosphoryl group relative to the active site lysine residues might be responsible for 2Me7Am-ATP's mechanism of inhibition.

DISCUSSION

In summary, we have studied the binding of the four inhibitors 2'-Me-CTP, ACT, NCT, and 2Me7Am-ATP to NoV RdRp. We have computed the binding free energies relative to the natural substrates of these four inhibitors, and for three of these, namely, 2'-Me-CTP, ACT, and 2Me7Am-ATP, we have also measured experimental EC₅₀ values and/or nucleotide incorporation efficiencies. Among these inhibitors, our calculations correctly identify 2'-Me-CTP as the most potent inhibitor.

A virtual screen of a library of 121 nucleotide analogues uncovered two compounds, aa101 and aa102, which were shown to act as chain terminators in our experimental nucleotide incorporation assay. However, these two compounds showed very poor incorporation efficiencies as substrates to NoV RdRp, despite the computational prediction by FEP of favorable binding by -4.2 and -1.7 kcal/mol, respectively, compared to CTP. This may be due to the influence of reaction kinetics on incorporation efficiencies, so that efficiencies do not correlate with free energies of binding, which are thermodynamic quantities.

We show that ACT is not a chain terminator to NoV RdRp, although its detailed mechanism of inhibition remains to be elucidated. We have also considered possible causes for the inhibition of NoV RdRp by NCT and 2Me7Am-ATP, by analysis of molecular dynamics simulations of the complexes. Our simulations concur with ref 8 that in the case of NCT, the proximity of the negatively charged nitro O atoms to the P _{α} atom could block the nucleophilic attack of the 3'-O of the nucleotide previously added to the primer on this P _{α} atom, thus impeding catalysis. Our analysis does not support that a change in the position of Arg182 can explain the inability of an NTP inhibitor such as NCT to react with the primer since this position varies widely among all of the inhibitors, including those NTP molecules that react. In the case of 2Me7Am-ATP, the catalytic site is restructured by nucleotide binding so that there is no close contact between a catalytic lysine residue and the γ -phosphoryl group, required for proton transfer. This is a result of a shift of the 2Me7Am-adenine's interaction with the template RNA from a planar base-pair geometry, caused by the 2-methyl group on the base interfering with the base-pairing. However, we caution that

the differences between structural parameters measured in our simulations and those in the crystal structures, along with the presence of multiple steps in the catalytic cycle, suggest that different conformational substates exist, and so conformational changes larger than what we observed in our simulations may in fact be associated with the presence of different nucleotides. Our predictions await experimental validation and could potentiate the future design of novel nucleoside inhibitors.

MATERIALS AND METHODS

Molecular Dynamics Simulation of CTP- and ATP-Bound NoV RdRp.

The structures of all of our models of NTP-bound NoV RdRp complexes are based upon the 3BSO crystal structure.⁸ 3BSO has a resolution of 1.8 Å resolution and includes three Mn²⁺ ions and the self-complementary oligonucleotide UGCCCCGGG, representing both the RNA transcript and RNA template. In our model, Mn²⁺ ions were replaced with Mg²⁺ ions for a better representation of the cellular environment. This change is partially justified by the observation by Shaik et al.⁴¹ that structural distortions associated with changes in the metal ion present are small.

NoV RdRp is a 510-residue protein, and most residues are present in the 3BSO crystal structure, with the exception of residues 1–4, 467–471, and 489–510, which are unstructured.⁸ We therefore included residues 5–488 in our model based upon the crystal structure and filled in the missing loop residues from 467 to 471, using the homology-modeling feature in MOE.³⁴ Residue protonation states were assigned at a pH of 7.0 by the MOE program.³⁴

We first equilibrated our model of the CTP-bound NoV RdRp complex in AMBER. The AMBER ff12SB force field^{42–45} was used to parameterize the protein and nucleic acid, and parameters for CTP were taken from the RED database.⁴⁶ For Mg²⁺, we used the Allnér et al.⁴⁷ parameter set, which is a newer model focused on yielding experimentally observed Mg²⁺–water exchange rates, and improved interactions with nonbridging O atoms belonging to the RNA backbone. Simulation setup was performed in the leap module of the AMBER 14 program package.⁴⁸ A cubic box of preequilibrated explicit TIP3P water was added extending to a distance of 15 Å from the protein. The total charge on the system was neutralized by the addition of Na⁺ ions, and afterward, an additional 71 Na⁺ ions and the same number of Cl[−] ions were added to bring the ionic concentration to 150 mM. Molecular dynamics simulations were performed with periodic boundary conditions using the pmemd.MPI module of AMBER 14.⁴⁸ The water surrounding the molecular system was first relaxed, and then the entire system was relaxed. Next, restraining all solute atoms by a force constant of 25 kcal/mol, the system was heated to a final temperature of 300 K at a constant volume over a period of 50 ps using a Langevin thermostat. Restraints were then gradually relaxed to zero over 100 ps with a time step of 2 fs at constant pressure, and then a 2 ns equilibration was performed also at constant pressure. We then ran a 117 ns MD simulations in NAMD.⁴⁹ The plot of active site RMSD (root-mean-square deviation) is shown in Figure 3. We chose a representative structure to be used as the initial configuration in FEP calculations by clustering trajectory snapshots with respect to positions of active site residues using 28 sets. The number of clusters used was chosen so that the SSR/SST value,

which represents the percentage by which the configurational variance is improved by the introduction of clusters, levels out and the DBI value, representing the ratio of the within-cluster scatter to the separation between clusters, is at a local minimum. A representative was chosen from the largest cluster, which should represent the free-energy minimum, and the representative structure was the one that has the lowest cumulative RMSD compared to all other frames in the cluster. This representative structure was used for the depiction in Figure 1, which was created using the Pymol molecular graphics program.⁵⁰ Starting with our model of the CTP-bound NoV RdRp complex, the CTP residue and its base-pairing G residue in the template RNA strand were manually mutated to the ATP-U pair. We then performed an MD simulation of the ATP-bound NoV RdRp for 120 ns.

Molecular Dynamics and FEP Simulations of NTP-Bound Complexes.

Starting with the representative model of CTP-bound NoV RdRp or the final structure from the simulation of ATP-bound NoV RdRp, we ran free-energy perturbation simulations, and standard molecular dynamics simulations of complexes bound by nucleotide analogues, in the NAMD molecular dynamics program.⁴⁹ Simulations were performed on our IBM Blue Gene Q supercomputer. Ligands were parameterized with the MATCH program, while protein and nucleic acid residues were parameterized using CHARMM22⁵¹ and CHARMM27,^{52,53} respectively. CHARMM27 force field parameters, which were contributed by B. Roux and co-workers, were also used on Mg^{2+} . For free-energy simulations, dual topology files and coordinates were prepared. Systems were solvated in an aqueous 0.150 M NaCl solution in a cubic box of solvent extending to a distance of 15 Å from the complex. For FEP simulations, an additional set of solvated input files was created for simulation of the free ligand in an aqueous 0.150 M NaCl solution, so that binding free energies could then be determined using a thermodynamic cycle. All simulated systems were equilibrated by first minimizing for 2000 steps and then gradually heating to 300 K (to match experimental conditions) using a Langevin thermostat over 60 ps with harmonic restraints. Restraints were then gradually released over a period of 250 ps, and afterward, systems were equilibrated for 1 ns, using a 0.5 fs time step. Standard molecular dynamics simulations were then run for 300 ns each.

In FEP simulations, the coupling parameter, λ , which describes the fractional potential energy character of the first molecule associated with each window, was scaled from 0 to 1 by increments of 0.02, i.e., over 50 simulation windows. In each window, equilibration was performed over 200 000 steps followed by 200 000 steps of data collection, with a time step of 2 fs. We made use of a “soft core” potential, i.e., van der Waals and electrostatic potentials were scaled separately to prevent atom clashes, which might occur if atoms appear or disappear in a highly charged state. The particle mesh Ewald (PME) method was used to treat long-range electrostatics and SHAKE⁵⁴ was used to constrain bonds involving hydrogen atoms. The system energy was sampled every 10 steps. After having performed 40 ns simulations in the forward direction from CTP to the nucleotide analogue, simulations were also run in the backward direction for 40 ns for each system, and the two results were averaged. For each alchemical transformation, the free-energy difference G is found as^{49,55-57}

$$\Delta\Delta G = -k_B T \sum_{i=1}^N \ln \langle \exp[-(\mathcal{H}(\mathbf{x}, \mathbf{p}, \lambda_{i+1}) - \mathcal{H}(\mathbf{x}, \mathbf{p}, \lambda_i)) / k_B T] \rangle$$

where $\mathcal{H}$ is the system Hamiltonian, N is the number of windows, in our case 50, k_B is the Boltzmann constant, T is the absolute temperature, and the bracket $\langle \rangle$ denotes averaging over all sampled phase space points $(\mathbf{x}, \mathbf{p})$. Errors shown in Tables 1 and 2 were estimated to equal one-half of the difference between the results corresponding to forward and backward simulations. As another measure of the statistical component of the associated errors, we applied the ParseFEP program⁵⁸ to the forward simulation data set for 2'-C-methyl-CTP to determine the variance of the free-energy estimates for the forward free-energy perturbations in the complex and in water. Based on this analysis, the error in the binding free-energy estimate for the forward perturbation for this molecule was 0.21 kcal/mol.

Trajectory Analysis.

RMSD values of the active site relative to the initial configuration were calculated using the Amber analysis tool *cpptraj* for the 117 ns MD simulation of the CTP-bound NoV RdRp complex and the 120 ns MD simulation of the ATP-bound NoV RdRp complex. The active site was defined as all residues, not including the bound NTP, within 6 Å of the bound NTP.

Our depictions in Figures 4 and 5 use structures averaged over the last 200 ns of 300 ns trajectories using *cpptraj*⁵⁹ and were created using the MOE program.³⁴ The distances reported in Table 3 use these same time-averaged structures. Selected distances shown in Figure 7 were calculated using the Gromacs⁶⁰⁻⁶³ analysis tool *gmx_mindist* along the entire 300 ns trajectories of the NCT-bound and 2Me7Am-ATP-bound complexes, after converting the NAMD trajectories to Gromacs format using *catdcd*.

NTP Incorporation Assays.

All nucleotide triphosphates of the compounds shown in Figure 1 were ordered as lithium salts (TriLink BioTechnologies), and the triphosphates of aa101 and aa102 were synthesized by Chemcyte.

The original sequence coding for human NoV RNA-dependent RNA polymerase (NoV RdRp) was kindly provided by Dr. Kenneth Ng (University of Calgary).⁷ The coding sequence was cloned into pPR-IBA1 plasmid (IBA, Germany) using the InFusion Cloning Kit (Takara Bio) according to the manufacturer's protocol. NoV RdRp was induced in BL21(DE3)pLysS cells (Novagen) and purified using an N-terminal Strep-tag according to the manufacturer's protocol (IBA, Germany) with the following modifications. BL21(DE3)pLysS cell pellets with induced NoV RdRp were lysed by the addition of 1% Tween 20, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 M NaCl, 10% glycerol, and 100 mM Tris-HCl pH = 8. Lysed BL21(DE3)pLysS cells were mixed with polyethyleneimine (1% w/v) to remove nucleic acids. Purified protein was buffer-exchanged (PD10, GE Healthcare) into Tris-HCl pH = 8 buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM TCEP) supplemented with 10% glycerol. Protein was concentrated on 30 kDa MWCO

Amicon-Ultra (Millipore) centrifugal membranes. Protein concentration was determined using an extinction coefficient of $86\,560\text{ M}^{-1}\text{cm}^{-1}$ calculated by GPMW-lite free software (Alphalyse). Protein preparations were supplemented with glycerol to 40% and stored at -20°C .

NoV RdRp activity was monitored on a symmetrical RNA primer/template ($10\text{ }\mu\text{M}$, $5'\text{pAAAGUUCGCGCGC}$) in the presence of Tris-HCl (pH 8, 25 mM), [$\alpha\text{-}^{32}\text{P}$]-GTP ($0.1\text{ }\mu\text{M}$), various combinations of NTP ($1\text{ }\mu\text{M}$) and NTP analogues ($100\text{ }\mu\text{M}$), and NoV RdRp ($0.75\text{ }\mu\text{M}$). Reaction mixtures ($10\text{ }\mu\text{L}$) were incubated for 10 min at 30°C followed by the addition of $5\text{ }\mu\text{L}$ of MgCl_2 (2.5 mM). Reactions were stopped after 30 min by the addition of $15\text{ }\mu\text{L}$ of formamide/EDTA (25 mM) mixture and incubated at 95°C for 10 min. Three microliters of reaction samples was subjected to denaturing 8 M urea of 15% polyacrylamide gel electrophoresis to resolve products of RNA synthesis followed by signal quantification (ImageQuant 5.2, GE Healthcare Bio-Sciences, Sweden) through phosphorimaging (Typhoon TRIO variable mode imager, GE Healthcare Bio-Sciences, Sweden). In the case of single nucleotide incorporation assays, the incorporated nucleotide product fraction was plotted versus nucleotide substrate concentrations and fitted to the Michaelis-Menten equation using GraphPad Prism 7.0 (GraphPad Software, Inc., CA).

Relative efficiency of nucleotide incorporation by NoV RdRp for a nucleotide substrate analogue was calculated as the ratio of the $V_{\text{max}}/K_{\text{m}}$ values for CTP and the CTP analogue, respectively. The V_{max} and K_{m} values were determined on the basis of an 8-data point experiment repeated three times for CTP and CTP analogues.

Replicon Assays.

The quantity of NV genome in the replicon-bearing cells (HG23) was measured by real-time qRT-PCR with the One-step qScript XLT RT-PCR kit (Quanta Biosciences) following an established protocol using primer Neo-F ($5'\text{-CCGGCTACCTGCCCCATTC-3'}$) and Neo-R ($5'\text{-CCAGATCATCCTGATCGACAAG-3'}$). The PCR thermal profile was 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min followed by a cooling step at 40°C for 30 s. For qRT-PCR, the total RNA in cells (in 12-well plates) was extracted with the RNeasy kit (Qiagen, Valencia, CA). Each RNA sample was prepared from the same standardized number of cells ($\sim 2 \times 10^5$) to calculate the NV genomic RNA level per cell. For relative quantification, the cycle threshold (C_t) value of Neo was subtracted from that of β -actin, and comparative method (2^{-C_t}) values were then normalized using results from the control cell group.

Inhibitory activity of each compound was determined by at least five steps in serial at 3-fold dilution. Nonlinear curve regression with variable slope was used to calculate the 50% inhibitory concentration (IC_{50}) values, which were selected based on what appeared to fit the data in the most biologically relevant way (top value set at 100 and bottom value set at 0; GraphPad Prism, v7.0D (GraphPad Software, Inc., CA)).

ACKNOWLEDGMENTS

We are grateful to Kenneth Ng for providing us with plasmid encoding the NoV RdRp sequence. We thank Darren Hockman for obtaining materials and Philip Winter for computer assistance. This research has been enabled by the

use of computing resources provided by the Southern Ontario Smart Computing Innovation Platform (SOSCIP), Scinet, Compute Canada, and WestGrid.

Funding

We are grateful for funding support from Albert Innovates to the Li Ka Shing Applied Virology Institute (LKSAVI), from a Canadian Excellence Research Chair (CERC) grant held by M.H., and from a Canadian Institute for Health Research grant (401067) to M.G. R.F.S. is supported in part by the NIH sponsored Center for AIDS Research at Emory University (P30AI050409).

ABBREVIATIONS

ATP	adenosine triphosphate
CTP	cytidine triphosphate
FEP	free-energy perturbation
MOE	molecular operating environment
qPCR	quantitative polymerase chain reaction
RMSD	root-mean-square deviation

REFERENCES

- (1). Svarovskaia ES; Dvory-Sobol H; Parkin N; Hebner C; Gontcharova V; Martin R; Ouyang W; Han B; Xu S; Ku K; Chiu S; Gane E; Jacobson IM; Nelson DR; Lawitz E; Wyles DL; Bekele N; Brainard D; Symonds WT; McHutchison JG; Miller MD; Mo H Infrequent Development of Resistance in Genotype 1-6 Hepatitis C Virus-Infected Subjects Treated with Sofosbuvir in Phase 2 and 3 Clinical Trials. *Clin. Infect. Dis* 2014, 59, 1666–1674. [PubMed: 25266287]
- (2). Norovirus Worldwide, 2020 <https://www.cdc.gov/norovirus/trends-outbreaks/worldwide.html>.
- (3). Rocha-Pereira J; Nascimento MSJ Targeting Norovirus: Strategies for the Discovery of New Antiviral Drugs; Arbuthnot P, Ed.; InTech: Rijeka, Croatia, 2012; pp 121–150.
- (4). Rocha-Pereira J; Neyts J; Jochmans D Norovirus: Targets and Tools in Antiviral Drug Discovery. *Biochem. Pharmacol* 2014, 91, 1–11. [PubMed: 24893351]
- (5). Weerasekara S; Prior AM; Hua DH Current Tools for Norovirus Drug Discovery. *Expert Opin. Drug Discovery* 2016, 11, 529–541.
- (6). Deval J; Jin Z; Chuang YC; Kao CC Structure(s), Function(s), and Inhibition of the RNA-Dependent RNA Polymerase of Noroviruses. *Virus Res.* 2017, 234, 21–33. [PubMed: 28041960]
- (7). Ng KK; Pendas-Franco N; Rojo J; Boga JA; Machin A; Alonso JM; Parra F Crystal Structure of Norwalk Virus Polymerase Reveals the Carboxyl Terminus in the Active Site Cleft. *J. Biol. Chem* 2004, 279, 16638–16645. [PubMed: 14764591]
- (8). Zamyatkin DF; Parra F; Alonso JM; Harki DA; Peterson BR; Grochulski P; Ng KK Structural Insights into Mechanisms of Catalysis and Inhibition in Norwalk Virus Polymerase. *J. Biol. Chem* 2008, 283, 7705–7712. [PubMed: 18184655]
- (9). Zamyatkin DF; Parra F; Machin A; Grochulski P; Ng KK Binding of 2'-Amino-2'-Deoxycytidine-5'-Triphosphate to Norovirus Polymerase Induces Rearrangement of the Active Site. *J. Mol. Biol* 2009, 390, 10–16. [PubMed: 19426741]
- (10). Hogbom M; Jager K; Robel I; Unge T; Rohayem J The Active Form of the Norovirus RNA-Dependent RNA Polymerase is a Homodimer with Cooperative Activity. *J. Gen. Virol* 2009, 90, 281–291. [PubMed: 19141436]
- (11). Alam I; Lee JH; Cho KJ; Han KR; Yang JM; Chung MS; Kim KH Crystal Structures of Murine Norovirus-1 RNA-Dependent RNA Polymerase in Complex with 2-Thiouridine Or Ribavirin. *Virology* 2012, 426, 143–151. [PubMed: 22341781]

- (12). Mastrangelo E; Pezzullo M; Tarantino D; Petazzi R; Germani F; Kramer D; Robel I; Rohayem J; Bolognesi M; Milani M Structure-Based Inhibition of Norovirus RNA-Dependent RNA Polymerases. *J. Mol. Biol* 2012, 419, 198–210. [PubMed: 22446684]
- (13). Tarantino D; Pezzullo M; Mastrangelo E; Croci R; Rohayem J; Robel I; Bolognesi M; Milani M Naphthalene-Sulfonate Inhibitors of Human Norovirus RNA-Dependent RNAPolymerase. *Antiviral Res.* 2014, 102, 23–28. [PubMed: 24316032]
- (14). Zamyatkin D; Rao C; Hoffarth E; Jurca G; Rho H; Parra F; Grochulski P; Ng KK Structure of a Backtracked State Reveals Conformational Changes Similar to the State Following Nucleotide Incorporation in Human Norovirus Polymerase. *Acta Crystallogr., Sect. D: Biol. Crystallogr* 2014, 70, 3099–3109. [PubMed: 25478829]
- (15). Croci R; Pezzullo M; Tarantino D; Milani M; Tsay SC; Sureshbabu R; Tsai YJ; Mastrangelo E; Rohayem J; Bolognesi M; Hwu JR Structural Bases of Norovirus RNA Dependent RNA Polymerase Inhibition by Novel Suramin-Related Compounds. *PLoS One* 2014, 9, No. e91765. [PubMed: 24622391]
- (16). Ng KK; Arnold JJ; Cameron CE Structure-Function Relationships among RNA-Dependent RNA Polymerases. *Curr. Top. Microbiol. Immunol* 2008, 320, 137–156. [PubMed: 18268843]
- (17). Malá K; Barvik I Substrate Recognition by Norovirus Polymerase: Microsecond Molecular Dynamics Study. *J. Comput.-Aided Mol. Des* 2013, 27, 373–388. [PubMed: 23619980]
- (18). Wang L; Wu Y; Deng Y; Kim B; Pierce L; Krilov G; Lupyan D; Robinson S; Dahlgren MK; Greenwood J; Romero DL; Masse C; Knight JL; Steinbrecher T; Beuming T; Damm W; Harder E; Sherman W; Brewer M; Wester R; Murcko M; Frye L; Farid R; Lin T; Mobley DL; Jorgensen WL; Berne BJ; Friesner RA; Abel R Accurate and Reliable Prediction of Relative Ligand Binding Potency in Prospective Drug Discovery by Way of a Modern Free-Energy Calculation Protocol and Force Field. *J. Am. Chem. Soc* 2015, 137, 2695–2703. [PubMed: 25625324]
- (19). Freedman H; Winter P; Tuszyński J; Tyrrell DL; Houghton M A Computational Approach for Predicting Off-Target Toxicity of Antiviral Ribonucleoside Analogues to Mitochondrial RNA Polymerase. *J. Biol. Chem* 2018, 293, 9696–9705. [PubMed: 29739852]
- (20). Chen Z; Cox BD; Garnier-Amblard EC; McBrayer TR; Coats SJ; Schinazi RF; Amblard F Synthesis and Anti-HCV Activity of a Series of Beta-D-2'-Deoxy-2'-Dibromo Nucleosides and their Corresponding Phosphoramidate Prodrugs. *Bioorg. Med. Chem. Lett* 2017, 27, 5296–5299. [PubMed: 29066308]
- (21). Rocha-Pereira J; Jochmans D; Dallmeier K; Leyssen P; Cunha R; Costa I; Nascimento MS; Neyts J Inhibition of Norovirus Replication by the Nucleoside Analogue 2'-C-Methyl-cytidine. *Biochem. Biophys. Res. Commun* 2012, 427, 796–800. [PubMed: 23063849]
- (22). Costantini VP; Whitaker T; Barclay L; Lee D; McBrayer TR; Schinazi RF; Vinje J Antiviral Activity of Nucleoside Analogues Against Norovirus. *Antiviral Ther.* 2012, 17, 981–991.
- (23). Rocha-Pereira J; Jochmans D; Debing Y; Verbeken E; Nascimento MS; Neyts J The Viral Polymerase Inhibitor 2'-C-Methylcytidine Inhibits Norwalk Virus Replication and Protects Against Norovirus-Induced Diarrhea and Mortality in a Mouse Model. *J. Virol* 2013, 87, 11798–11805. [PubMed: 23986582]
- (24). Bougher JH III; Chagalvala RV; Downey AL; Drach JC ; Lanier ER Jr; McIver AL; Robertson BD; Selleseth DW; Sethna PB; Townsend L; Ware RW Pyrrolopyrimidine nucleosides and analogs thereof. U.S. Patent US104074572019.
- (25). Jin Z; Tucker K; Lin X; Kao CC; Shaw K; Tan H; Symons J; Behera I; Rajwanshi VK; Dyatkina N; Wang G; Beigelman L; Deval J Biochemical Evaluation of the Inhibition Properties of Favipiravir and 2'-C-Methyl-Cytidine Triphosphates Against Human and Mouse Norovirus RNA Polymerases. *Antimicrob. Agents Chemother* 2015, 59, 7504–7516. [PubMed: 26392512]
- (26). Harki DA; Graci JD; Galarraga JE; Chain WJ; Cameron CE; Peterson BR Synthesis and Antiviral Activity of 5-Substituted Cytidine Analogues: Identification of a Potent Inhibitor of Viral RNA-Dependent RNA Polymerases. *J. Med. Chem* 2006, 49, 6166–6169. [PubMed: 17034123]
- (27). Shen H; Sun H; Li G What is the Role of Motif D in the Nucleotide Incorporation Catalyzed by the RNA-Dependent RNA Polymerase from Poliovirus? *PLoS Comput. Biol* 2012, 8, No. e1002851. [PubMed: 23300428]

- (28). Balzarini J; Das K; Bernatchez JA; Martinez SE; Ngure M; Keane S; Ford A; Maguire N; Mullins N; John J; Kim Y; Dehaen W; Vande Voorde J; Liekens S; Naesens L; Gotte M; Maguire AR; Arnold E Alpha-Carboxy Nucleoside Phosphonates as Universal Nucleoside Triphosphate Mimics. *Proc. Natl. Acad. Sci. U.S.A* 2015, 112, 3475–3480. [PubMed: 25733891]
- (29). Balzarini J; Menni M; Das K; van Berckelaer L; Ford A; Maguire NM; Liekens S; Boehmer PE; Arnold E; Gotte M; Maguire AR Guanine Alpha-Carboxy Nucleoside Phosphonate (G-Alpha-CNP) shows a Different Inhibitory Kinetic Profile Against the DNA Polymerases of Human Immunodeficiency Virus (HIV) and Herpes Viruses. *Biochem. Pharmacol* 2017, 136, 51–61. [PubMed: 28390939]
- (30). Jochmans D; Deval J; Kesteleyn B; Van Marck H; Bettens E; De Baere I; Dehertogh P; Ivens T; Van Ginderen M; Van Schoubroeck B; Ehteshami M; Wigerinck P; Gotte M; Hertogs K Indolopyridones Inhibit Human Immunodeficiency Virus Reverse Transcriptase with a Novel Mechanism of Action. *J. Virol* 2006, 80, 12283–12292. [PubMed: 17020946]
- (31). Jochmans D Novel HIV-1 Reverse Transcriptase Inhibitors. *Virus Res.* 2008, 134, 171–185. [PubMed: 18308412]
- (32). Migliaccio G; Tomassini JE; Carroll SS; Tomei L; Altamura S; Bhat B; Bartholomew L; Bosserman MR; Ceccacci A; Colwell LF; Cortese R; De Francesco R; Eldrup AB; Getty KL; Hou XS; LaFemina RL; Ludmerer SW; MacCoss M; McMasters DR; Stahlhut MW; Olsen DB; Hazuda DJ; Flores OA Characterization of Resistance to Non-Obligate Chain-Terminating Ribonucleoside Analogs that Inhibit Hepatitis C Virus Replication in Vitro. *J. Biol. Chem* 2003, 278, 49164–49170. [PubMed: 12966103]
- (33). Cihlar T; Ray AS Nucleoside and Nucleotide HIV Reverse Transcriptase Inhibitors: 25 Years After Zidovudine. *Antiviral Res.* 85, 39–58. [PubMed: 19887088]
- (34). Molecular Operating Environment (MOE), version 2018.1, 1010 Sherbrooke St. West, Suite #910; Chemical Computing Group Inc.: Montreal, QC, Canada, 2018.
- (35). Yesselman JD; Price DJ; Knight JL; Brooks CL 3rd MATCH: An Atom-Typing Toolset for Molecular Mechanics Force Fields. *J. Comput. Chem* 2012, 33, 189–202. [PubMed: 22042689]
- (36). Fogt J; Januszczuk P; Framski G; Onishi T; Izawa K; De Clercq E; Neyts J; Boryski J Synthesis and Antiviral Activity of Novel Derivatives of 2'-Beta-C-Methylcytidine. *Nucleic Acids Symp. Ser* 2008, 605–606.
- (37). Steitz TA A Mechanism for all Polymerases. *Nature* 1998, 391, 231–232. [PubMed: 9440683]
- (38). Castro C; Smidansky ED; Arnold JJ; Maksimchuk KR; Moustafa I; Uchida A; Gotte M; Konigsberg W; Cameron CE Nucleic Acid Polymerases use a General Acid for Nucleotidyl Transfer. *Nat. Struct. Mol. Biol* 2009, 16, 212–218. [PubMed: 19151724]
- (39). McDonald SM RNA Synthetic Mechanisms Employed by Diverse Families of RNA Viruses. *Wiley Interdiscip. Rev.: RNA* 2013, 4, 351–367. [PubMed: 23606593]
- (40). Šponer J; Bussi G; Krepl M; Banas P; Bottaro S; Cunha RA; Gil-Ley A; Pinamonti G; Poblete S; Jurecka P; Walter NG; Otyepka M RNA Structural Dynamics as Captured by Molecular Simulations: A Comprehensive Overview. *Chem. Rev* 2018, 118, 4177–4338. [PubMed: 29297679]
- (41). Shaik MM; Bhattacharjee N; Feliks M; Ng KK; Field MJ Norovirus RNA-Dependent RNA Polymerase: A Computational Study of Metal-Binding Preferences. *Proteins* 2017, 85, 1435–1445. [PubMed: 28383118]
- (42). Wang JM; Cieplak P; Kollman PA How Well does a Restrained Electrostatic Potential (RESP) Model Perform in Calculating Conformational Energies of Organic and Biological Molecules? *J. Comput. Chem* 2000, 21, 1049–1074.
- (43). Pérez A; Marchan I; Svozil D; Sponer J; Cheatham TE 3rd; Loughton CA; Orozco M Refinement of the AMBER Force Field for Nucleic Acids: Improving the Description of Alpha/Gamma Conformers. *Biophys. J* 2007, 92, 3817–3829. [PubMed: 17351000]
- (44). Zgarbová M; Otyepka M; Sponer J; Mladek A; Banas P; Cheatham TE 3rd; Jurecka P Refinement of the Cornell et al. Nucleic Acids Force Field Based on Reference Quantum Chemical Calculations of Glycosidic Torsion Profiles. *J. Chem. Theory Comput* 2010, 7, 2886–2902.

- (45). Cheatham TE 3rd; Cieplak P; Kollman PA A Modified Version of the Cornell et al. Force Field with Improved Sugar Pucker Phases and Helical Repeat. *J. Biomol. Struct. Dyn* 1999, 16, 845–862. [PubMed: 10217454]
- (46). Vanquelef E; Simon S; Marquant G; Garcia E; Klimerak G; Delepine JC; Cieplak P; Dupradeau FYRED Server: A Web Service for Deriving RESP and ESP Charges and Building Force Field Libraries for New Molecules and Molecular Fragments. *Nucleic Acids Res.* 2011, 39, W511–W517. [PubMed: 21609950]
- (47). Allnér O; Nilsson L; Villa A Magnesium Ion-Water Coordination and Exchange in Biomolecular Simulations. *J. Chem. Theory Comput* 2012, 8, 1493–1502. [PubMed: 26596759]
- (48). Case DA; Babin V; Berryman J; Betz R; Cai Q; Cerutti D ; Cheatham T III; Darden T; Duke R; Gohlke H Amber 14; University of California, 2014.
- (49). Phillips JC; Braun R; Wang W; Gumbart J; Tajkhorshid E ; Villa E; Chipot C; Skeel RD ; Kale, L.; Schulten, K. Scalable Molecular Dynamics with NAMD. *J. Comput. Chem* 2005, 26, 1781–1802. [PubMed: 16222654]
- (50). Delano WL The PyMOL Molecular Graphics System, 2002 <http://www.pymol.org>.
- (51). MacKerell AD; Bashford D; Bellott M; Dunbrack RL; Evanseck JD; Field MJ; Fischer S; Gao J; Guo H; Ha S; Joseph-McCarthy D; Kuchnir L; Kuczera K; Lau FT; Mattos C; Michnick S; Ngo T; Nguyen DT; Prodhom B; Reiher WE; Roux B; Schlenkrich M; Smith JC; Stote R; Straub J; Watanabe M; Wiorkiewicz-Kuczera J; Yin D; Karplus M All-Atom Empirical Potential for Molecular Modeling and Dynamics Studies of Proteins. *J. Phys. Chem. B* 1998, 102, 3586–3616. [PubMed: 24889800]
- (52). Foloppe N; MacKerell JAD All-Atom Empirical Force Field for Nucleic Acids: 1) Parameter Optimization Based on Small Molecule and Condensed Phase Macromolecular Target Data. *J. Comput. Chem* 2000, 21, 86–104.
- (53). MacKerell JAD; Banavali N All-Atom Empirical Force Field for Nucleic Acids: 2) Application to Molecular Dynamics Simulations of DNA and RNA in Solution. *J. Comput. Chem* 2000, 21, 105–120.
- (54). Ryckaert JP; Ciccotti G; Berendsen HJC Numerical Integration of Cartesian Equations of Motion of a System with Constraints: Molecular-Dynamics of N-Alkanes. *J. Comput. Phys* 1977, 23, 327–341.
- (55). Zwanzig RW High-Temperature Equation of State by a Perturbation Method. i. Nonpolar Gases. *J. Chem. Phys* 1954, 22, 1420–1426.
- (56). Gilson MK; Given JA; Bush BL; McCammon JA The Statistical-Thermodynamic Basis for Computation of Binding Affinities: A Critical Review. *Biophys. J* 1997, 72, 1047–1069. [PubMed: 9138555]
- (57). Beveridge DL; DiCapua FM Free Energy Via Molecular Simulation: Applications to Chemical and Biomolecular Systems. *Annu. Rev. Biophys. Biophys. Chem* 1989, 18, 431–492. [PubMed: 2660832]
- (58). Liu P; Dehez F; Cai W; Chipot C A Toolkit for the Analysis of Free-Energy Perturbation Calculations. *J. Chem. Theory Comput* 2012, 8, 2606–2616. [PubMed: 26592106]
- (59). Roe DR; Cheatham TE 3rd PTRAJ and CPPTRAJ: Software for Processing and Analysis of Molecular Dynamics Trajectory Data. *J. Chem. Theory Comput* 2013, 9, 3084–3095. [PubMed: 26583988]
- (60). Hess B; Kutzner C; van der Spoel D; Lindahl E GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. *J. Chem. Theory Comput* 2008, 4, 435–447. [PubMed: 26620784]
- (61). Van Der Spoel D; Lindahl E; Hess B; Groenhof G; Mark AE; Berendsen HJ GROMACS: Fast, Flexible, and Free. *J. Comput. Chem* 2005, 26, 1701–1718. [PubMed: 16211538]
- (62). Pronk S; Pall S; Schulz R; Larsson P; Bjelkmar P; Apostolov R; Shirts MR; Smith JC; Kasson PM; van der Spoel D; Hess B; Lindahl E GROMACS 4.5: A High-Throughput and Highly Parallel Open Source Molecular Simulation Toolkit. *Bioinformatics* 2013, 29, 845–854. [PubMed: 23407358]
- (63). Burgess RR Protein Precipitation Techniques. *Methods Enzymol.* 2009, 463, 331–342. [PubMed: 19892180]

Figure 1.

(A) Cartoon representation of NoV RdRp, from our model based on the 3BSO crystal structure. A CTP molecule bound to the active site is shown in ball and stick representation. The positions of the domains and subdomains are outlined in part (B). The thumb domain is depicted in gray. The palm is in green, except for motif A, which is in dark blue, motif B in cyan, motif C in pale blue, and motif D in yellow. The index, middle, ring, and pinky fingers are colored in red, violet, purple, and light pink, respectively.

Figure 2. Structures of the known NoV RdRp inhibitors that are the focus of our study. Nucleoside precursors of (A) ACT; (B) 2'-Me-CTP; (C) NCT; and (D) 2Me7Am-ATP.

Figure 3.

RMSD, relative to the starting structure, of the active site of our computational model of the NoV RdRp complex bound by CTP, (A) as a function of simulation time. The RMSD plot for our simulations initiated after replacing the CTP in the active site by ATP at the end of the MD simulation of the CTP-bound complex is shown in panel (B).

Figure 4.

Cartoon representation of the active site of NoV RdRp bound by an incoming NTP molecule, with residues labeled and shown in stick mode. Mg ions are in dark red and coordinating water O atoms are shown as red spheres. The bound NTP molecule is CTP in panel (A) ACT in panel (B) 2'-Me-CTP in panel (C) NCT in panel (D) ATP in panel (E) and 2Me7Am-ATP in panel (F). Residues are colored as in Figure 1.

Figure 5.

(A) Transition state of poliovirus, reproduced with permission from ref 27. Copyright 2012 Shen et al. The active site of NoV RdRp, viewed in a similar orientation as used in (A) is shown bound by (B) CTP, (C) NCT, and (D) 2Me7Am-ATP. Catalytic residues and residues coordinating metal ions are shown in stick mode. In (B–D), active site metal ions and coordinating waters are shown in ball and stick mode. In the same figures, an asterisk (*) is used to point out the position of the primer 3' O atom, which undergoes nucleophilic attack on P_{α} during reaction.

Figure 6.

Symmetrical primer/template substrate used in the RNA synthesis assays to test incorporation of CTP analogues is shown above the gel. G indicates incorporation of the radiolabeled nucleotide opposite template position 14, which is illustrated by the migration pattern of product 14 that is indicated to the left of the gel. Product 17 illustrates the migration pattern of RNA primers terminated with CTP and various CTP analogues. Different migration patterns of CTP analogues reflect their structural differences. Nucleotide incorporations by NoV RdRp were monitored in the presence of [α -³²P]GTP, symmetrical RNA primer/template, 2.5 mM MgCl₂, and various combinations of 1 μM NTP and 100 μM of CTP analogues. The presence of ATP, CTP, and UTP allows full-length product formation up to position 20. The presence of ATP and UTP provides control from misincorporation. Lane m illustrates the migration pattern of the radiolabeled 13 nucleotide-long primer.

Figure 7.

Distances between the O atom belonging to the 5-nitro group of NCT and (A) the NCT P_{α} atom, or (B) the primer 3'O atom, plotted as a function of simulation time. In part (C) we plot the time dependence of the distance between the 7-amide group's NH_2 moiety on 2Me7Am-ATP and its P_{α} atom.

Table 1.

Calculated Relative Binding Free Energies of NTP and dNTP Analogues to NoV RdRp and Some Experimental EC_{50} Values and Nucleotide Incorporation Efficiencies

compound	G_{FEP} (kcal/mol)	experimental EC_{50} , μM	relative efficiency of nucleotide incorporation	previous experimental EC_{50} , μM^{22}
2'-Me-CTP	-0.95 ± 0.57	3.3 ± 1.2	60 ± 30	1.3
ACT	-0.63 ± 2.03	49.8 ± 1.3	220 ± 120	
2'-F-2'-C-Me-CTP	-0.21 ± 0.35			3.2
NCT	0.82 ± 1.84			
2Me7Am-ATP	-0.40 ± 1.08	6.2 ± 1.2		
2',3'-dideoxy-ATP	0.86 ± 0.94	>100		
Ara-CTP	0.00 ± 0.66		200 ± 100	

Table 2. Nucleoside Precursors of CTP Derivatives Identified in Computational Screen for Binding to NoV RdRp

structure	name	G_{FEP} (kcal/mol) ^a (Experiment) ^b	structure	name	G_{FEP} (kcal/mol) ^a (Experiment) ^b
N4 derivatives					
	aa101	-4.17 ± 1.61 (2100 $\pm$ 1100)		N4-Rgr35	-1.66 ± 0.10
C5 derivatives					
	C5-Rgr34	-3.11 ± 0.02		aa102	-1.71 ± 0.44 (4600 $\pm$ 2700)

structure	name	G_{FEP} (kcal/mol) ^a (Experiment) ^b	structure	name	G_{FEP} (kcal/mol) ^a (Experiment) ^b
	C5-Rgr23	-2.57 ± 0.35		C5-Rgr46	-1.87 ± 0.68
C4' derivatives					
	C4'-Rgr26	-2.94 ± 0.91		C4'-Rgr1	-1.67 ± 0.10

^a Simulations were performed without C2'-methyl groups, which were added during experiments to ensure chain termination.

^b Relative efficiency of nucleotide incorporation.

Table 3.

Minimum Distances between Residue, or Atom, Pairs Belonging to the NTP-Bound NoV RdRp Structure, Averaged over the Last 200 ns of MD Simulations Modeled with Each of Six NTP Molecules in the Active Site^a

distance (Å)	compound					
	CTP	ACT	2'-Me-CTP	NCT	ATP	2Me7Am-ATP
Asp242/metal A	1.8 ± 0.3 (2.1)	1.8 ± 0.2 (7.9)	1.8 ± 0.2	1.8 ± 0.3 (2.2)	1.9 ± 0.1	1.9 ± 0.1
Asp343/metal A	1.8 ± 0.3 (2.1)	1.8 ± 0.2 (2.2)	1.8 ± 0.2	1.8 ± 0.3 (2.2)	1.8 ± 0.0	1.8 ± 0.0
Asp344/metal A	1.8 ± 0.3 (2.1)	1.8 ± 0.2 (4.4)	1.8 ± 0.2	1.8 ± 0.3 (2.5)	1.8 ± 0.0	1.8 ± 0.0
α nonbridging O/metal A	4.0 ± 0.6 (2.2)	3.1 ± 0.5 (2.2)	3.2 ± 0.5	3.3 ± 0.5 (2.2)	1.9 ± 0.1	1.8 ± 0.0
Asp242/metal B	1.8 ± 0.3 (2.1)	1.9 ± 0.2 (8.3)	1.9 ± 0.2	1.9 ± 0.3 (2.1)	1.8 ± 0.0	1.8 ± 0.0
Asp343/metal B	1.8 ± 0.3 (2.1)	1.8 ± 0.2 (2.2)	1.9 ± 0.2	1.8 ± 0.3 (2.1)	1.9 ± 0.0	1.8 ± 0.0
Arg182 NH1/ α,β -bridging O	2.1 ± 0.6 (3.0)	3.0 ± 0.5 (3.7)	2.9 ± 0.5	2.3 ± 0.7 (3.1)	5.0 ± 0.3	3.2 ± 0.3
Arg182 NH2/ α,β -bridging O	2.7 ± 0.5 (3.7)	4.1 ± 0.7 (5.2)	4.7 ± 0.7	3.7 ± 1.0 (5.4)	4.1 ± 0.3	3.9 ± 0.6
Lys374 <i>e</i> amino/ γ phosphate	19 ± 3.6 (3.6)	3.6 ± 4.7 (K374 absent)	16 ± 3.7	8.5 ± 7.9 (3.6)	17 ± 3.8	4.4 ± 0.4
Lys174 <i>e</i> amino/ γ phosphate	2.1 ± 0.5 (3.5)	17 ± 3.0 (3.7)	1.7 ± 0.2	1.8 ± 0.7 (3.5)	9.5 ± 2.2	3.9 ± 0.8
Lys180 <i>e</i> amino/ γ phosphate	9.5 ± 1.8 (5.9)	2.5 ± 1.5 (6.2)	5.5 ± 1.8	5.2 ± 1.9 (5.7)	2.1 ± 0.8	9.2 ± 1.7

^aStandard deviations are reported as errors. For the CTP-, ACT-, and NCT-bound complexes, the measurements from the crystal structures 3BSO, 3H5X, and 3BSN, respectively, are shown in parentheses for comparison.