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Allosteric inhibitors of Coxsackie virus A24 RNA polymerase

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Abstract

Coxsackie virus A24 (CVA24), a causative agent of acute hemorrhagic conjunctivitis, is a prototype of enterovirus (EV) species C. The RNA polymerase (3D^{pol}) of CVA24 can uridylylate the viral peptide linked to the genome (VPg) from distantly related EV, and is thus, a good model for studying this reaction. Once UMP is bound, VPgU primes RNA elongation. Structural and mutation data have identified a conserved binding surface for VPg on the RNA polymerase (3D^{pol}), located about 20Å from the active site. Here, computational docking of over 60,000 small compounds was used to select those with the lowest (best) specific binding energies (BE) for this allosteric site. Compounds with varying structures and low BE were assayed for their effect on formation of VPgU by CVA24-3D^{pol}. Two compounds with the lowest specific BE for the site inhibited both uridylylation and formation of VPgpolyU at 10–20 μM. These small molecules can be used to probe the role of this allosteric site in polymerase function, and may be the basis for novel antiviral compounds.

Graphical abstract

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1. Introduction

1.1 Need for inhibitors of enteroviruses

Enteroviruses (EV) include poliovirus (PV), Coxsackie viruses (CVA and CVB), rhinoviruses (RV) and many other human pathogens¹. Although vaccination campaigns have essentially eliminated PV in most countries, they left behind many diverse EV that continue to cause outbreaks. In the US, the CDC estimates that there are over 20 million EV infections per year, each of which may result in the loss of 1–3 days of work or school time. More serious EV infections can lead to pneumonia², aseptic meningitis³ and PV-like paralysis^{4, 5}, especially in those with asthma and cystic fibrosis⁶ as well as in immunosuppressed individuals, neonates, and the aged. EV are easily spread through the oral/fecal route, and can survive for long periods of time in the intestines of asymptomatic individuals. Despite the millions of symptomatic infections with non-polio EV in the US every year, there are no clinically approved, wide spectrum therapies available.

Species C-EV, including CVA24 and its variants, are frequently isolated from outbreaks around the world⁷. The CVA24 variant strain chosen here as an RNA polymerase (3D^{pol}) prototype has been associated with epidemics of acute hemorrhagic conjunctivitis⁸. There is also a report of CVA24 associated acute flaccid paralysis, in a starving child in East Timor⁹. The CVA24 sequences for RNA polymerase (3D^{pol}) and VPg, are very similar to those of the more extensively studied Poliovirus (PV)¹⁰. However, vaccination against PV does not protect against CVA24, or other EV. The omnipresence of EV in the human microbiome, along with their sequence diversity, ability to generate recombinant viruses¹¹, and high mutagenesis rate, means that vaccination is not a promising strategy for dealing with these pathogens. Thus, there is a need for wide spectrum inhibitors of EV replication¹².

1.2 Targeting an allosteric site on the 3D^{pol}

One possible route to obtain EV inhibitors is to target the essential first step in replication of the viral RNA that is carried out by all EV-3D^{pol}, uridylylation of VPg to VPgUpU (Figure 1, left). VPg is also referred to as “3B”, as it is cleaved from the third viral protein (3ABCD, where 3D^{pol} is the RNA polymerase)¹³. VPg is then modified by transferring a UMP from UTP to a Tyrosine at position 3¹⁴. *In vitro*, this reaction requires only a divalent metal ion (Mg²⁺ or Mn²⁺), a suitable template (poly A or a small RNA segment from the viral genome called the Cre) and the 3D^{pol} and UTP¹⁵. Once uridylylated, VPgUpU primes the synthesis of the viral RNA and is found covalently linked to the 5' end¹⁶. Free VPgUpU and polyU-linked VPg can be isolated from cells infected with PV. The reaction is also essential for virus replication, as anti-VPg antibodies prevent virus replication^{17–20}. If after uridylylation, VPg cannot be cleaved from its precursor, a larger protein called “3BC”, the polymerase can make genomic RNA linked to 3BC. However, 3BC-RNA is only quasi-infectious, and no viral progeny is produced²¹.

We recently demonstrated that four diverse 3D^{pol} from EV-species A–C were able to uridylylate five VPgs whose sequences varied by up to 60% of their residues. Although all four EV-3D^{pol} tested showed preference for their cognate VPg, they could also uridylylate a PCP-consensus^{24–27} VPg designed to represent the physical chemical properties of 31

different EV-VPgs²⁸. Thus, the residues and mechanism required for uridylylation must be similar in most, if not all, EV, indicating that specific inhibitors of this reaction could be promising pan-EV therapeutics. The polymerases of CVA24 and PV, which are 97% identical, were both able to efficiently uridylylate all of the VPgs examined.

Others have sought inhibitors of targeted RNA elongation in EV-3D^{pol}, by measuring fill in of a variety of "self-priming" hairpin RNA's^{29, 30}. However, the best inhibitors found from high throughput screening had IC₅₀ values in the 50–80 μ M range²⁹. Compounds tested in another study bind within the template binding channel of EV-polymerase³⁰, a location also found for inhibitors of the Dengue virus (DENV) RNA polymerase, which does not use protein priming³¹. We found this site lacks specificity, as many of compounds from high throughput screening (HTS) libraries bind there. To obtain more specific inhibitors, we selected compounds based on their specific docking to the surface, about 20Å from the active site, where free VPg binds to the 3D^{pol} of species C and B EV (Figure 1, right). This surface was hypothesized initially to play a role in cleavage of VPg from the 3rd viral protein³², and later was suggested to be where VPg uridylylation occurs³³. The results shown here support yet another role for the surface region: as an allosteric effector of RNA synthesis (consistent with³⁴). Small molecules selected to bind to the surface site on PV-3D^{pol} with intermediate binding energies (BE) inhibited or slightly stimulated VPg uridylylation and synthesis of polyU-RNA. Two compounds with the best specific binding to the site inhibited VPg uridylylation by CVA24 3D^{pol} in a concentration range of 10–20 μ M. Further structure-based analysis of these active compounds and derivatives thereof can be used to probe the mechanism for uridylylation and priming by VPg.

2. Methods

2.1 Compounds and proteins

Small molecules were initially selected from the Maybridge Hitfinder XD (version 10) compound library, which consists of 14,400 compounds stored as 10mM solutions in DMSO (dimethyl-sulfoxide) at –20°C. Compounds were also purchased in lyophilized form from Chembridge/Thermo Fisher or Enamine. Aliquots of the dried compounds were weighed out on a balance with accuracy within 100 μ g, dissolved in DMSO to 10mM and stored at –20°C. Compounds and DMSO controls were diluted 1:50 or 1:100 (only E9) in polymerase buffer (PB: 50mM HEPES, pH 7.5, 8% glycerol) before further dilution in the assay.

CVA24 and the PCP consensus VPgs were synthesized and CVA24 3D^{pol} and Dengue virus (DENV) NS5 polymerase (used for control assays not shown) were purified as described elsewhere^{28, 35}. The CVA24 polymerase was concentrated to about 3 mg/ml and stored at –20°C in PB with additional glycerol added to a final concentration of 18%.

2.2 Docking

The PyRx program was used for all dockings³⁶. The target protein structure was the high resolution PV1-3D^{pol} structure, PDB accession code 2IM2. The sequence of the protein used for this crystal structure differs from that of the CVA24 3D^{pol} at 9 out of 461 residues total, only 3 of which are likely to have any effect on the polarity of the underlying position (Fig.

S1). All compounds were initially docked to a grid limited to the VPg binding site (white box, Figure 2). The sequence in this area is conserved in EV-3D^{pol} of species C and B²⁸. The “small” docking grid, which covered the conserved allosteric site, was centered on five residues (F377, R379, A380, D381 and E382) shown by mutagenesis to be important for uridylylation but not RNA elongation³². This location was determined based on docking³³ and mutagenesis studies³⁷, and comparison to the CVB3 co-crystal structure³⁸ (Figure 1, right). Ligands were downloaded as mol2 or structure data files (SDF) from the online database ZINC³⁹ and converted to pdbqt files with BABEL. The libraries were the Maybridge library (ca. 46,000 compounds, which also includes the Hitfinder compounds), and the Zbc Leads special subset (ca. 26,000 compounds) from ZINC³⁹ which includes some compounds from the Maybridge library as well as more diverse compounds available from other vendors. Finally, compounds structurally related to those with the best binding energies were selected from ZINC using the integrated search tools.

Overall, SDFs or mol2 files (converted to pdbqt files using the BABEL utility incorporated in the PyRx platform before docking) for about 60,000 compounds were docked to the site shown in Figure 2. Our previous work^{40–42} indicated that the BE from Autodock could be used to select compounds with a likely affinity for a given area of a protein. However, they cannot predict specificity for a given site. Compounds, which according to PyRx VINA, exhibited the lowest BE to the VPg binding region were thus re-docked to a “large grid” covering the entire polymerase. The test for specificity for the allosteric site was based on whether one or more of the eight best binding poses to the whole polymerase fell within the small grid area.

To screen for specific inhibitors, 300 of the best compounds (BE to the allosteric site ranging between –7 and –8.4) were re-docked to a large grid covering the whole polymerase molecule (middle, Fig. 2). Then separate dockings were done of compounds similar to those showing specificity, according to structure-based search. The most selective compounds were thus obtained after several cycles of dockings, using compounds similar to those with the best BE in the larger library, to the small and large grid.

2.3 Uridylylation assay

Compounds were assayed at concentrations between 1 and 25 μ M for their effects on synthesis of VPgpU and on VPgpolyU (see Figure S2 for more details). All compounds were initially tested at 25 μ M, and those with activity were further diluted to determine an approximate IC₅₀ (here, that giving 50% or less of the VPgpU or VPgpolyU produced with added DMSO alone). The reaction mixtures (10 μ l) contained 50 mM HEPES, pH 7.5, 8% glycerol, 0.5 μ g of the template RNA (polyA (Sigma)), 0.5 mM manganese (II) acetate, 1–2 μ g purified 3D^{pol}, 1 μ g synthetic VPg, and 10 μ M UTP (+ α -UT³²P from Amersham)¹⁵. As described previously¹⁵, Mn²⁺ gives more efficient uridylylation than Mg²⁺ in the in vitro assay when the RNA template is polyA. Except where noted otherwise, multiplex assays were performed in siliconized PCR plates or individual PCR strip tubes and incubated for the indicated times at 30°C in a thermocycler (Biorad). The reactions were terminated by rapid cooling to 0°C and addition of 2 μ l/sample 6 $\times$ Tris-Tricine-SDS loading buffer (12% SDS, 30% Glycerol, 0.05% Coomassie Blue G250 and 150 mM Tris/HCl pH 7.0). Samples

were heated at 40 °C for 15 minutes before loading onto a 16% denaturing (6M Urea) Tris-Tricine/SDS-PAGE minigels (15 slot), prepared in house as described elsewhere⁴³. The uridylylated VPg³²PU and VPg³²PpolyU products were quantified with a Phosphorimager (PMI; BioRad). Due to the limits on how many samples can be done in one experiment, gel results shown are representative of several assays.

3. Results

3.1 Selection of compounds based solely on docking scores to the allosteric site

Docking experiments revealed that less than 0.5% of the ligands from all the docking compound libraries had affinity for the surface site, as indicated by $BE < -6.0$. To determine experimentally whether these results correlated with an effect on the activity of the 3D^{pol}, 26 compounds of varying structures and BE between -8.1 and -7.4 to the allosteric site were selected from Maybridge Hitfinder diversity library. Details of the structure and physicochemical properties of these compounds are summarized in Table S1 (EV1–26). The compounds were assayed at 25 μ M concentration (the highest concentration we considered likely to indicate realistic inhibition of the polymerase) in the uridylylation assay after 60 minute incubation (Fig. S2, summarized in Fig. 3) and the amount of uridylylated VPg (VPgpU) and VPg linked polyU formed in the assay was determined by relative CPM incorporated in the SDS-PAGE bands. Of these, three compounds within the 10 best (lowest) BE scoring group inhibited the reaction. The effect was most pronounced in the elongation step, as indicated by reduced amounts of VPgpolyU (top bands on gel, Figure S2). Figure 3 shows the amount of UMP incorporated into VPgpolyU relative to the normalized BE of each compound. Even at 25 μ M, none of the compounds completely prevented uridylylation, and when further diluted, the compounds did not give significant inhibition.

Furthermore, several compounds with higher BE (*i.e.*, weaker predicted binding) activated the reaction slightly, especially in the absence of VPg (Figure 3 and data not shown). This suggested that the site could be an allosteric activation site for the polymerase, as had been previously suggested based on other evidence³⁴. Lyle et al.³² showed that mutations at this site could prevent uridylylation, while having little effect on elongation. Elongation in this case was the ability of the polymerase to fill in the strands of a ds-RNA template.

3.2. Compounds with low BE and specificity for the allosteric site are efficient inhibitors

Having shown that some compounds with lower BE could inhibit the reaction, we next determined whether specifically selecting compounds that were most likely to bind to the allosteric site, as opposed to other areas of the polymerase (see Fig 2 for docking grids), would be better inhibitors. Compounds with low BE to the allosteric site were tested for their ability to bind specifically to the allosteric site, when the docking grid covered the whole 3D^{pol} surface (middle picture, Fig. 2). Of approximately 300 molecules with BE below -7 to the allosteric site, nearly all preferentially bound near the template binding channel (green oval in Fig. 4). The screenshot in Fig. 4, right, shows that only a few compounds bound in the allosteric site when allowed to dock to the whole 3D^{pol}. The nine compounds that bound in the allosteric site (yellow oval) in at least one of their top best

eight poses were purchased in powder form and tested in the uridylylation assay. While a few of these compounds showed some inhibition when assayed at 25 μ M (data not shown), they exerted little effect upon further dilution.

One compound from the two libraries with the best and most specific binding to the allosteric site (5/8 top poses) was not immediately available. However, structure files for a set of 56 commercially accessible compounds, similar in structure to this “hit” from the ZBC Leads set, were obtained. These were docked to both grids, and several were found to have even better specific binding energy (BE/mw) scores than the unavailable substance. After docking and comparing the physical chemical properties, ten of these compounds with the highest specific BE were purchased and assayed (Fig. 5A; Table S1). Even these structurally related compounds gave varying results in the assays, but several inhibited the polymerase when assayed at 25 μ M final concentration (Figure 5A). The two compounds displaying the lowest BE/mw, specificity for the site, as well as reasonable solubility, are also the best at inhibiting uridylylation. A time course confirmed that two compounds with the lowest BE/mw and specificity for the allosteric site (Table S1) were the most efficient inhibitors: E37 at 10 μ M and E38 at 20 μ M (Figure 5B). None of the compounds tested in Figure 5B inhibited DENV NS5 polymerase (Fig. S5), which was used as a negative control.

EV37 and EV38 may be competitive inhibitors of VPg binding, as both are less effective at higher VPg concentrations (Figure S4). However, quantifying the assay results is complicated by the non-linear effects of adding VPg to the *in vitro* assay, an issue also described by others³⁴. Addition of less than 0.5 μ g/10 μ l assay gives almost no polymerase activity; whereas, higher amounts may inhibit product formation.

The structures of EV35, EV37, EV38 are shown in Figure 5B. Inconsistent results with EV35 were most likely due to its low aqueous solubility (log P = 4.54, Table S1 and a white precipitate formed in diluted aqueous samples). EV35 had a methyl group on the benzoxazol-N, as did all of compounds EV28–36. The common feature of EV28–EV38 is that they are all aromatic compounds with a sulfonamide linkage to a benzoxazol moiety. There is insufficient data at this point to conduct a proper structure-activity analysis, as the compounds also had very different solubility in water, which will affect their performance in the assay.

The best docking pose of the most efficient inhibitor, EV37, is shown both space filling and with interacting residues of the 3D^{pol} in Fig. 6. In the pose shown, the fluorobenzene moiety is in direct contact with E382 and aromatic residues from other areas of the polymerase, and the benzoxazole moiety interacts with Arg 379. However, other poses with similar energy indicate other possible interactions that also involve the Phe 377, and with the ligand in the opposite orientation in the site. When allowed to dock to the whole polymerase, EV37 also docked near the conserved D358. However, it did not enter the substrate binding channel or the active site. The importance of the individual elements of the structure must await testing of additional derivatives and, especially, experimental determination of the binding sites of the selected inhibitors on the polymerase.

4. Discussion

4.1 The best inhibitors dock most specifically to the VPg binding site

The RNA dependent RNA polymerases (RdRp) of the EV and other plus strand RNA viruses are complex molecules despite their small size (typically 60–100 kD, well below the that of the multi-subunit polymerases of the cells they infect). The RdRp are similar in their overall structures, especially in the active site for nucleotide transfer. However, they differ in regions that mediate interactions with other factors produced by the virus itself or the cells they infect^{13, 45}. The RdRp of Hepatitis C virus (HCV), for example, has been shown to have as many as four different allosteric sites for binding by small molecule inhibitors^{46–54}. Identifying the allosteric binding sites for these co-factors is essential for producing specific inhibitors of RdRp.

The 3D^{pol} of EV, and indeed, the polymerases of all of the picornaviridae family (of which enterovirus is one genus⁵⁵), achieve specificity by using a protein to prime RNA synthesis. In EV, VPg remains bound to the 5' end of the viral RNA¹⁶, although it may be removed at some phase of replication⁵⁶. Here, we have shown that the previously identified binding site for VPg on the surface of the CVA24 3D^{pol} (Figure 1 & 2) is, indeed, an allosteric site, as compounds selected to bind there interfere with both uridylylation and subsequent elongation to VPgpolyU (Figures 3 & 5). However, to efficiently inhibit uridylylation, the compounds must also have specificity for the site. Compounds selected from the Maybridge Hitfinder based on binding energies alone (Fig. 3, S2, S3) were less efficient inhibitors than those selected to have at least one low energy pose within the site, when allowed to dock at any location throughout the entire 3D^{pol} structure. The most efficient inhibitors were also those with the most specific binding to the allosteric site (Figure 5B and 6).

Previous work showed that the CVA24 3D^{pol} can uridylylate VPg peptides from even very distantly related EV²⁸. As Figures 3 and 5 indicate, it is a very active protein. When supplied with VPg, UTP, a polyA template, and a buffer solution containing Mn²⁺, only a few of the compounds tested could interfere with its ability to uridylylate VPg and go on to produce VPgpolyU. Indeed, from all the compounds tested, only two were able to consistently block uridylylation of VPg in the 10–20 μ M range (Figs. 5, 6). Neither inhibited DENV polymerase that had been similarly purified (Figure S5).

4.2 The VPg binding site is an allosteric site on the polymerase

The two best inhibitors (Fig. 5B) are structurally related to each other, and to a compound from the original docking library that had the best overall BE to the allosteric site of any of the 60,000 compounds initially docked. They are both somewhat related to one of the Hitfinder compounds tested in the first assay series (Fig. 3, S2 & S3). Most of the Hitfinder compounds tested, that were originally selected solely by their affinity for the allosteric site when docking to the small grid (Fig. 2, left) preferentially docked to other areas of the 3D^{pol} than the allosteric site (Figure 4). In contrast, EV37 and EV38, selected from a group of structurally related compounds (Table S1), still bound preferentially to the allosteric site, even when given the large grid covering the whole polymerase (Fig. 2 middle). The effective concentration of EV37 is 10 μ M, better than that reported for an inhibitor of DENV

polymerase that binds within the template binding channel (as determined by ligand cross linking³¹) and for inhibitors of the PV-3D^{pol} selected from a 14,000 compound library in a random fashion²⁹. Our virtual screening results indicate that it is not difficult to find compounds that bind in the template binding channel⁴⁴ (green oval in Fig. 4) although remarkably few bind within the conserved metal binding, phosphotransfer site⁵⁷ at the heart of the polymerase (red box, Fig. 4). One interpretation for this outcome is that nucleotide-based compounds may be over-represented in HTS libraries, even those selected to contain a diversity of scaffolds such as the ones we selected for docking. Such compounds may bind nonspecifically to all sites that have evolved to bind nucleic acids, meaning the potential for undesired side effects might preclude therapeutic use.

Several of the small molecules from the Hitfinder series with lower docking scores (higher BE) activated 3D^{pol}, especially with respect to the synthesis of VPgpolyU (Fig. 3, S2, S3). Others have shown that RNA elongation by PV-3D^{pol}, using an artificial template, is accelerated up to eight fold by the presence of positively charged peptides, even those that bear little resemblance to VPg⁵⁸. However, these peptides cannot substitute for VPg in the production of full length viral RNA. The Hitfinder compounds that triggered a slight activation of the RNA polymerase in the assay are not clearly different in structure or physicochemical properties from the best inhibitors (Fig. S3). It is possible that these molecules may stabilize the 3D^{pol} during the reaction when added at relatively high concentrations (25 μ M). On the other hand, the lowest energy binding pose of the best inhibitor, EV37 (Fig. 6), sits within the allosteric site, interacting (Fig/ 6B) with two residues (R379 and E382). whose mutation inhibits uridylylation. In this pose, EV37 makes additional interactions with G425 and Y450, near residues in PV 3D^{pol} previously shown to be important for uridylylation⁵⁹.

Other evidence supporting the various roles played by VPg in the activation of 3D^{pol} includes the non-linear increase of uridylylated products with a rise in VPg concentration (Fig. S4). Although the current experimental data show that adding twice as much VPg to the reaction increases the amount of VPgU produced (compare the first with the second lane at each time point in Fig. 5B), the effect is not proportional to VPg concentration and little activity is seen at lower VPg levels (Fig. S4). These results suggest that the polymerase does not bind very tightly to VPg, which may account for the free peptide's presence in the cytoplasm of infected cells.

4.3 Using small compounds to study the role of VPg in EV-replication

A binding site for VPg on the “back of the palm” in EV-3D^{pol} is widely accepted. However, the role of this site in uridylylation and priming is not understood. The inhibitors shown here may directly compete for VPg binding to the site, and thus, prevent uridylylation directly. Such competition is difficult to show conclusively, due to the non-linear effects of VPg concentration in the assay. Alternatively, by binding, they may further the conformational changes in 3D^{pol} needed for elongation, as has been shown for the allosteric, benzimidazole inhibitors of HCV RdRP, which bind within the thumb subdomain⁶⁰. In the absence of VPg, enterovirus 3D^{pol} can fill in the ends of double stranded RNA segments³⁰. Such fill-in activity is not believed to play a role in viral replication during infection. Many of the

inhibitors we identified show a more pronounced effect on the elongation phase than they do on VPgpU formation (Fig. S2, Fig. 5).

More advanced structural studies of the compounds' binding sites should aid in separating these effects. It would also be useful to test these compounds for their effects on the uridylylation mechanisms in other viruses, such as Norwalk and Caliciviruses, that use a larger VPg protein to prime synthesis of their RNAs⁶¹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

3D^{pol}	enterovirus RNA polymerase, cleaved from the 3rd viral protein; 3ABCD, where 3B is VPg
BE	binding energy
CVA	Coxsackie virus A
DENV	Dengue virus (a Flavivirus that does not use protein priming)
EV	enterovirus
HCV	hepatitis C virus
PB	polymerase buffer
PV	poliovirus
RdRp	RNA dependent RNA polymerase
SDF	structure data file
VPg	viral protein linked to the genome
VPgpU	uridylylated VPg
polyU	polyuridine

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Figure 1. Left: Enterovirus replication begins with uridylylation of the 22 amino acid peptide VPg at Tyr3

The NMR structures of PV-VPg (PDB accession code 2BBL²²) and PVV-PgpU²³ are shown to illustrate the stabilization of VPg after uridylylation. VPgpU then goes on to prime RNA synthesis. The peptide backbone is gray, side chains of N- and C-terminal & 3 Pro residues are sticks; the Tyr3 (green, with O red) or TYU3 (the covalently linked UMP is colored according to atom type, C is black, P is yellow and N blue) are shown as colored spheres **Right: Free VPg binds to a site on 3D^{pol} about 20 Å from the RNA synthesis site** (Y327-D330). The co-crystal structure of CVB3 VPg with its 3D^{pol} (PDB accession code 3CDW) is shown to highlight the surface site. The residues in the binding area are conserved in all EV species C and B. The 3D^{pol} is shown in ribbon format, with selected residues in CPK. VPg is shown as pale blue, space filling spheres.

Figure 2. Screen shots from PyRx showing the small and large grids used for selecting compounds with selectivity for the allosteric site

Left: Screen shot, showing the PV-3D^{pol} crystal structure (PDB accession code 2IM2) in stick format. The docking grid around the allosteric site is shown as a white box, drawn to include the area around F377, R379, A380, D381 and E382. The middle box shows the expanded grid, centered on the active site of the 3D^{pol} (red rectangle). The 300 compounds with the lowest BE were allowed to dock within this larger grid and the docking poses were screened to select those that still preferred to dock to the allosteric site (yellow oval), which were selected for further analysis. Compounds structurally similar to the most specific compounds were then docked to both grids, and individual molecules selected for experimental testing.

B.

Figure 3. Correlating the effect of 26 Maybridge Hitfinder compounds (EV1–EV26) with BE to the allosteric site -7.4 . A) Assay results

The first bar (blue) shows the amount of VPgpolyU (top band on gels, see Figure 1S for details) formed in the standard uridylation assay (60 minutes) in the presence of each compound at 25 μ M. Band size is given relative to the average of four controls (labeled as ©, which were either diluted DMSO or PB alone added in place of a compound). The average CPM for the four controls is 352460 ± 76533 . The compounds either inhibited (*), activated slightly (@) or had no effect on the assay. The second bar (orange, brought onto the same axis as the relative CPM by adding 8.5 to each number) shows the BE to the

allosteric site for each compound, which ranges from -8.1 to -7.4 . **B. Scatter plot of the BE vs the relative incorporation into VPgpolyU** in the presence of each compound shows that the two values are only approximately related. However, compounds with higher BE (weaker predicted binding) were more likely to stimulate formation of VPgpolyU. The central bar in B shows the average, with the lighter bars marking the standard deviation.

Figure 4. Most of the compounds originally selected based on BE to the allosteric site bind within the template binding channel, if allowed to dock to the whole polymerase

To test their specificity for the allosteric site, 300 compounds with $BE < -7.0$ when docked to the small grid of Fig. 2 left were re-docked to a grid encompassing the whole polymerase molecule (Fig. 2 right). **Left:** For orientation, the backbone of the 3D^{Pol} is shown, with the side chains of residues forming the active site (red square) and the allosteric site (yellow oval) shown as colored sticks. The area where most of the compounds preferred to dock, which includes residues within the template binding channel⁴⁴, is indicated by a green oval.

Right: Screen shot from the PyRx analysis window, showing the best (lowest BE to the whole 3D^{Pol}) poses of about 100 compounds that were originally selected to have $BE < -7$ to the allosteric site. The compounds are shown as stick representations, with C=white, N=blue, O=red and halogens as green. The protein carbon molecules are de-emphasized as they are grey against a grey background, with only nitrogens (blue), oxygen (red) and sulfur (yellow) atoms emphasized. The poses of nine compounds (from all 300) that had at least one of their eight best poses within the allosteric site are indicated by the yellow circle. With one exception, the pose shown in the allosteric site is not the lowest energy one for these few compounds. Another view of the allosteric site in the whole polymerase is shown in Fig. 6C.

Figure 5. Compounds with the best binding energies and specificity for the allosteric site are efficient inhibitors of CVA24 3D-pol

A) Assay of 10 structurally related compounds, EV28–38, tested at 25 μ M in the assay for 60 minutes. The bands were quantified relative to the average of the four controls (2 buffer only, 2 DMSO diluted 1:40 added). **B)** Uridylylation time course in the presence of 3 compounds with related structure (EV35, EV37, EV38, pictured at right) at 20, 10 and 20 μ M, respectively) showing inhibition of uridylylation and the formation of VPgppolyU by the CVA24 3D^{pol}. The first sample in each series is a control assay mix, with 2 $\times$ the [VPg] and only DMSO added. Quantitation (middle) indicated that the bands in the presence of the inhibitors were 11–30% of those in the DMSO sample. None of these compounds inhibited DENV polymerase (Fig.S5).

Figure 6. Possible interactions of EV37 with 3D^{pol} residues within and around the allosteric site
A) Docking pose 1 of EV37 is shown as a space filling model in the allosteric site. The overview (**C**) is rotated clockwise to show the docked ligand from above, and highlight the allosteric site residues. Carbon atoms are green in the ligand and pink in the highlighted protein residues, all other atoms are colored red for O, blue for N, S is yellow, H is gray and the fluoride atom is a lighter shade of green, projecting to the front of the figure. Side chains of interest on the 3D^{pol} are shown as space filling here and as sticks in B and C. These include the allosteric site used for grid: F377,R379-E382, the conserved D358/K359 and the active site Y326-D329 (red box in C). Atoms are colored according to atom type except the carbon atoms are pink for the protein in A and B, and the ligand carbon is green in A and orange in B. **B) Residues of the 3D^{pol} that interact with the ligand** are labeled in dark black, with interaction spheres indicated. Other selected residues, labeled in italic, do not interact. **C) For orientation, the same pose is shown with the whole 3D^{pol}**, with the same side chains marked by thicker lines. The carbons of the ligand are purple, other atoms are colored as above. The yellow oval in B and C indicates the allosteric site, the active site is marked by the red box in C.