ARTICLE

Identification of Alternative Binding Sites for Inhibitors of HIV-1 Ribonuclease H Through Comparative Analysis of Virtual Enrichment Studies

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S *Supporting Information*

ABSTRACT: The ribonuclease H (RNase H) domain on the p66 monomer of HIV-1 reverse transcriptase enzyme has become a target for inhibition. The active site is one potential binding site, but other RNase H sites can accommodate inhibitors. Using a combination of experimental and computational studies, potential new binding sites and binding modes have been identified. Libraries of compounds were screened with an experimental assay to identify actives without knowledge of the binding site. The compounds were computationally docked at putative binding sites. Based on positive enrichment of natural-product actives relative to the database of compounds, we propose that many inhibitors bind to an alternative, potentially allosteric, site centered on Q507 of p66. For a series of hydrazone compounds, a small amount of positive enrichment was obtained when active compounds were bound by induced-fit docking at the interface between the DNA:RNA substrate and the RNase H domain near residue Q500.
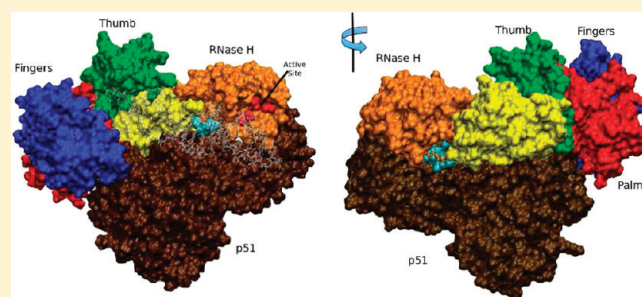
1. INTRODUCTION

Identifying the binding site for a group of active compounds that inhibit a target protein is often not a trivial task. There might be several potentially druggable sites on the target in addition to the substrate binding site that could accommodate an inhibitor. Sometimes, experimental evidence identifies actives and inactives without indicating where they bind. Structural information about the inhibition site might be incomplete. Computational methods can be used to identify possible druggable sites, but cannot definitively point to the preferred binding site.[1−9] Docking programs can be used to place actives into these various sites and provide estimates of the binding energy, but the errors in the estimated energies are often large enough to make unequivocal identification of the binding sites difficult without additional information. The true binding sites have physicochemical features that favor binding active compounds over inactives. The focus of the work reported in this article is the identification of putative binding sites for inhibitors of the ribonuclease H (RNase H) domain of HIV-1 reverse transcriptase (RT), a new target for anti-AIDS drug design.[10−13] One of our groups has developed a fluoresence assay that was used to identify active inhibitors of the RNase H activity of RT in high-throughput screens of ligand libraries.[14] Unfortunately, we do not have complementary X-ray

structures of complexes for any of the active compounds bound to RT. More generally, relatively little information is available about the structures of complexes of RNase H inhibitors bound to their receptors, and the little information that is available suggests the possibility of multiple binding sites.[15−17]

In this work, we attempt to leverage the experimental information obtained by identifying the active inhibitors of the RNase H function of RT in three ligand libraries by performing in silico docking studies against several possible targets. The idea is that the true binding sites will show stronger enrichment of the active inhibitors than nonspecific sites. Our previous experience with high-throughput in silico docking and enrichment[18] provides a framework against which to measure the quality of the in silico enrichment curves obtained in the present study. Furthermore, we recently obtained crystallographic data concerning ligand fragments that bind to HIV-1 RNase H that provide some corroboration of the results reported here. (A PDB file of these results is presented in the Supporting Information.)

Although several effective drug combinations for treating the human immunodeficiency virus (HIV) that causes acquired

immunodeficiency syndrome (AIDS) have been developed, they have not been effective in every patient ,and they have been prone to become ineffective due to mutations caused by misreads during the viral life cycle. The life cycle of HIV includes (i) entry/fusion of the virus into/to the host cell; (ii) release of key enzymes [reverse transcriptase (RT), protease (PR), and integrase] and the viral RNA; (iii) translation by RT of the viral RNA into double-stranded DNA; (iv) integration of the viral DNA into the host's genome, where it is transcribed to new viral RNA and a polypeptide that is an assemblage of the viral proteins; (v) processing of the polypeptide by PR into new viral proteins; and (vi) assembly of new viruses. Currently, the medications available and in development have disrupted the viral life cycle at most of these stages by inhibiting fusion, RT, PR, and integrase. (See ref 19 and references therein.) To deal with viral mutations, new drugs have been required to continue to be able to stop HIV replication. RT has been an attractive target because of its multiple and central roles in the life cycle of HIV. RT is a heterodimer with a p66 monomer and the smaller p51 monomer.[20] The p66 monomer has two active sites: the polymerase site, which builds the DNA onto the viral RNA, and the ribonuclease H (RNase H) site, which removes the viral RNA from the newly synthesized DNA:RNA duplex.[21] This free DNA strand is finally converted to the duplex strand of DNA for insertion into the host cell's nucleus. Currently, inhibitors of RT have existed that either bind directly to the polymerase site [the nucleoside RT inhibitors (NRTIs) and nucleotide RT inhibitors (NtRTIs)] or adjacent to it causing an allosteric change disabling polymerase activity [the non-nucleoside RT inhibitors (NNRTIs)]. Work has continued to develop medications that target RT at these two sites.[19,21]

Recently, work has been focusing on the other RT catalytic site that is located in the RNase H domain on p66. An inhibitor of viral RNase H would break the viral life cycle by stopping the removal of viral RNA from the DNA:RNA duplex and preventing RT from assembling the DNA duplex for insertion into the host's genome. Currently, inhibitors that bind to the metal-chelated active site of RNase H have been identified, along with compounds whose binding modes are unclear, but they have yet to be developed into viable medications.[10−13] Compounds chelating to the metals in the catalytic site of RNase H have included nucleotide derivatives,[22−24] diketo acids,[25−27] N-hydroxyimides,[28] hydroxylated tropolones,[29−32] 2-hydroxyisoquinoline-1,3(2H,4H)-dione derivatives,[33] pyrimidinol carboxylic acids,[34] and naphthyridinone-based compounds.[35] Compounds with unknown binding modes or with putative binding modes outside the catalytic site have included quinones from natural products,[36−39] hydrazone compounds,[15,40−42] vinylogous ureas,[16] thiocarbamates, and triazoles.[43]

As suspected for the quinones, hydrazones, vinylogous ureas, thiocarbamates, and triazoles, the active site might not be the only place on the RNase H domain to accommodate an inhibitor. With the exception of one hydrazone[15] and one of the naphthyridinone-based compounds,[35] the known X-ray crystallographic structures of RNase H inhibitors have them chelating to the active site.[32,34,35] However, some evidence suggests that the active site might be inaccessible to inhibitors when the DNA:RNA substrate is bound to RT. In the case of the hydroxylated tropolone β-thujaplicinol, it "appears unable to compete with the intact nucleic acid substrates".[31] The potential exists that inhibitors could bind to alternative sites. Such inhibitors might induce an allosteric change in RNase H disabling the active site similarly

to an NNRTI acting on the adjacent RT polymerase site. It might also be possible that an inhibitor will bind to the RNase H domain in a fashion that will prevent the substrate from having access to the active site. The advantage of allosteric inhibitors is that these binding sites are exclusive to the viral RNase H domain, whereas the active site itself is very similar to human RNase H1.[44] Inhibitors that bind to the active site of viral RNase H might also bind to human RNase H1, potentially leading to unwanted side effects. Through a synergistic combination of experimental evidence and computational modeling, we investigated the possibility that a collection of natural-product and druglike active inhibitors were preferentially bound to alternative sites on viral RNase H domain. The identification of possible binding sites where these active inhibitors bind could be utilized for further structure-based drug design.

Programs are available that attempt to identify potentially druggable binding sites based on their geometry and physicochemical nature.[1−9] The physicochemical features that are considered in the interaction between the protein and a potential ligand include desolvation from hydrophobic and hydrophilic surfaces and hydrogen-bonding donor and acceptor regions. To probe for putative binding sites, we have chosen SiteMap, which has a reported accuracy of identifying over 80% of the known druggable sites.[8] However, as is the case for other druggability prediction programs,[45] SiteMap has identified multiple sites per target along with actual binding sites. For RT, there are multiple potential sites to which a druglike molecule could bind, even multiple sites located on the RNase H domain. The challenge is to identify which are the actual sites where RNase H inhibitors could preferentially bind.

Favorable simulated binding energies are not sufficient to determine whether an active inhibitor will preferentially bind to a particular site. Many concavities in a given target can provide favorable van der Waals, electrostatic, and/or hydrogen-bonding interactions to accumulate comparable nonspecific binding for active inhibitors and inactive compounds. True binding sites have physicochemical features that favor, on average, active compounds over other inactive weak binders. Our strategy was to use docking and in silico enrichment studies to seach for binding sites capable of making the distinction between actives and inactives based on the predicted binding energy scores for a large set of compounds. A binding site's discrimination between active and inactive compounds is manifested in a robust enrichment curve that describes how well actives are found at the top of the ranked database. During scanning of the ranked database from lowest energy (most favorable) to highest, the percentage of actives encountered is determined for the percentage of database that has been scanned. The percentage of encountered actives versus the percentage of the database can be plotted as a receiver operating characteristic (ROC) curve (also referred to as an enrichment curve).[46,47] The area under the ROC curve indicates how rapidly the active compounds were encountered when scanning a ranked database: an area of 1 indicates that all of the actives were found at the top of the database (perfect enrichment); an area around 0.5 would indicate that the actives were randomly distributed in the database (no enrichment). Enrichment can also be expressed as a percentage of the actives encountered after a small sample (for instance, the top 10%) of the ranked database has been searched.

Previous work using ROC or enrichment curves with a database of known inhibitors and druglike decoys has focused on benchmarking how well a docking algorithm and its scoring

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dx.doi.org/10.1021/ci200194w |J. Chem. Inf. Model. 2011, 51, 1986–1998

function can discriminate actives from inactives when binding to an experimentally determined site.[18,48−53] It has been argued that this ability should be a key feature for any docking procedure.[54] In this work, we employed Glide XP[55] for the in silico enrichment studies.[18,48−51] Recently, we benchmarked Glide XP against other docking algorithms in terms of its ability to predict enrichment. Glide XP performed well in database screening, finding, on average, 85% of the actives in the top 10% of the ranked database.[18] Based on its past performance, we believe that Glide XP is reasonably accurate in database screening and discriminating actives from inactives using estimated binding energies when docking to known sites. In this work, we did not know a priori where the actual binding site (or sites) might be. By docking the experimentally identified actives and inactives in putative sites (several chosen with SiteMap) with Glide XP, we sought to determine whether that site is the actual binding site based on how well actives ranked relative to inactives in the virtually screened database. If it was a high-affinity binding site, there ought to be substantial enrichment when the database of compounds was docked against that site. Based on our previous work,[18] we believed that an enrichment of around 8-fold would be strong evidence that the investigated site is the true binding site for those inhibitors. In contrast, if enrichment was absent, then this would be evidence against the site being a high-affinity site.

2. METHODS

2.1. Selection of Putative Binding Sites. The first step in our invesitgation was to identify all potential binding sites on the RNase H domain. We used SiteMap[7,8] to indicate where those sites might be. SiteMap placed a grid over the entire target protein. It assigned vertices that lie inside concavities, but not in the protein itself, as site points. Site points were clustered with other neighboring points to characterize a binding site. van der Waals and electrostatic probes were placed at the sites to map out the hydrophobic and hydrophilic surfaces, locations where metal chelation could occur, and regions that might accommodate hydrogen bonds with a drug candidate. The SiteScore was calculated to measure how druggable a site might be. SiteScore is an empirical function consisting of a weighted sum of exposure/enclosure, contact, hydrophobic/hydrophilic, and hydrogen-bonding terms. The weights were optimized on 538 proteins.[8] A SiteScore greater than 1.0 is correlated with a site that might be druggable, a score between 0.8 and 1.0 generally indicates that a region might be "difficult" with regard to finding a druglike compound that binds to it, and a score below 0.8 represents a site that is not druggable according to the knowledge-based metrics in SiteMap.[8] SiteMap returned the top binding sites ranked based on their SiteScores. We performed SiteMap calculations on RT with metal cofactors in the active site of RNase H, on RT with those metals removed, and on RT with the DNA:RNA substrate present.

2.2. In Silico Protein−Ligand Docking Simulation. Once binding sites were selected, we used the protein−ligand docking program Glide XP to predict how compounds might bind to RNase H.[55] During the docking procedure, all conformations (rotamers of substituents about a core structure) of the ligand were generated and superimposed (clustered) about some central chemical group of the ligand. Glide XP generated a grid over the binding site on which the ligands were placed. Based on the size of the ligand cluster, Glide XP eliminated all grid points
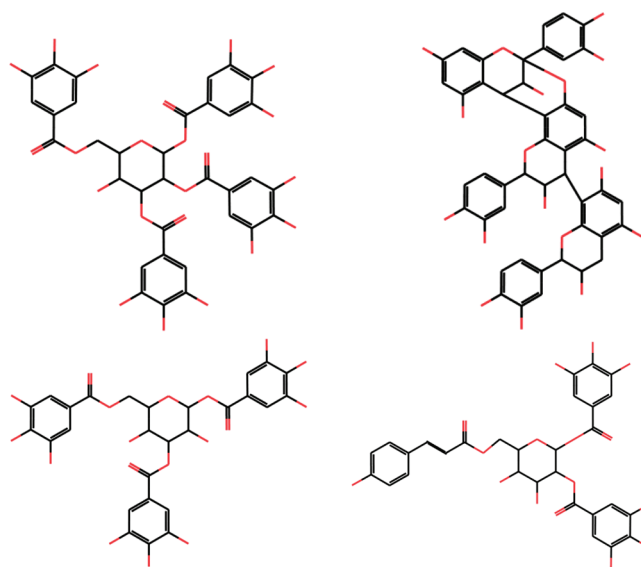

Figure 1. Representative AnalytiCon compounds, with carbon in black and oxygen in red. Clockwise from top left, the compounds are NP-003686, NP-004204, NP-011987, and NP-005114. The abundance of hydroxyl groups is the reason these compounds are very water-soluble and not prone to aggregation.

that were too close to or too far from the surface of the pocket. Ligands placed on grid points that were too close would overlap with receptor atoms; ligands placed on grid points that were too far would not make contact with the receptor surface. Also, Glide XP eliminated any grid points that directly overlapped the protein. Removal of grid points based on their distance from the receptor eliminated over 90% of the grid points. This was followed by a rough sampling of orientations about each remaining grid point, eliminating an additional 90% of the remaining orientations. Using the final, accepted set of grid points and orientations, an exhaustive search was then carried out for each ligand conformation in the receptor pocket. The best few hundred poses of the ligand in the receptor pocket from this exhaustive search were minimized using a precomputed grid of van der Waals and electrostatic interactions.[56,57] A Monte Carlo seach of torsional minima and orientations of substituents on the core was performed for a select few poses to further minimize and refine the pose of the ligand in the pocket. The minimized poses were scored with GlideScore XP, which is an expanded version of the empirical program ChemScore.[58] For the best poses, Glide XP docked explicit water molecules around the ligand and receptor pocket to assess desolvation penalties (such as removing waters from polar and nonpolar regions of the receptor to accommodate the ligand) and to calculate the solvation energies of exposed polar and charged groups on the ligand and receptor. Other contributions such as filling in a hydrophobic enclosure in the receptor with a ligand were also calculated. Salt bridges, π-cation interactions, and other medicinal chemistry motifs have been incorporated in GlideScore XP as described in ref 55.

2.3. Libraries of Compounds. One of the central goals of this project was to find either natural-product or druglike inhibitors of HIV RNase H. A collection of natural-product compounds was obtained from AnalytiCon Discovery (AnalytiCon Discovery GmbH, Potsdam, Germany). The AnalytiCon library consists of natural-product compounds that contain many hydroxyl and carboxyl groups on carbon backbones. Examples of AnalytiCon
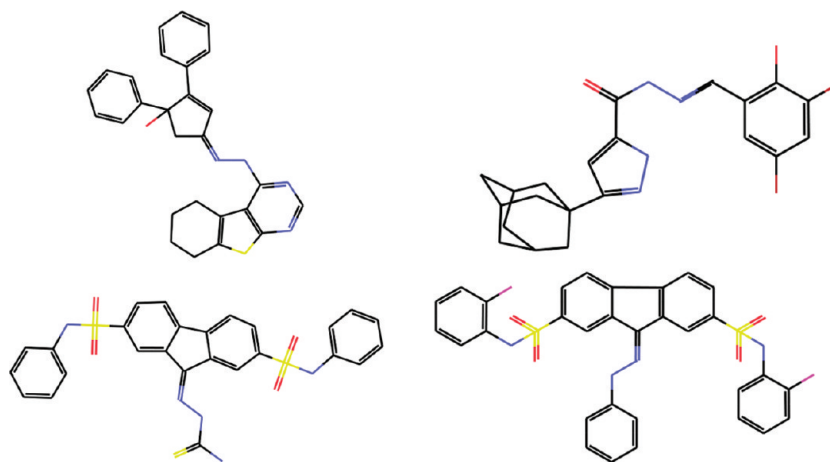
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dx.doi.org/10.1021/ci200194w |J. Chem. Inf. Model. 2011, 51, 1986−1998

Figure 2. Representative Life Chemicals hydrazone and hydrazine compounds, with carbon in black, oxygen in red, sulfur in yellow, iodine in magenta, and bromine in brown. Clockwise from top left, the compounds are F0745-0032, F1092-0760, F1345-0373, and F1345-0193.

compounds are shown in Figure 1. There are two sublibraries: MEGx compounds, which have >90% purity, and Natx compounds, which have >95% purity, as determined by liquid chromatography/mass spectroscopy and by NMR spectroscopy. There were 2319 compounds in this library with two-dimensional representations. Without information about the three-dimensional structure and chirality, we prepared each compound using LigPrep (Schrödinger, LLC) to generate three-dimensional representations and all possible enantiomers and protonation states for each compound. The library increased to 11247 structures. The structure of the complex with the lowest energy among each compound's three-dimensional structures was retained for comparison to the low-energy structures of the other compounds. For each potential binding site, high-throughput virtual screening with Glide XP required between two and three weeks with the library of 11247 structures divided among eight Opteron 2354 and 2384 processors.

Based on previous favorable results with hydrazones as inhibitors,[15,40−42] a library of hydrazone/hydrazine compounds was collected from Life Chemicals (Life Chemicals, Burlington, ON, Canada) for testing. Examples of the Life Chemicals compounds are shown in Figure 2. These compounds have >95% purity as measured by NMR spectroscopy. This library contains 5544 structures in two-dimensional representations. For docking with Glide XP, three-dimensional structures were created from the two-dimensional representations. For each potential binding site, high-throughput virtual screening with Glide XP required between one and two weeks with the library of 8199 structures divided among eight Opteron 2354 and 2384 processors. Less time was required for the hydrazone/hydrazine library than for the AnalytiCon library because the former comprised compounds that are less flexible than the latter's compounds.

The inhibitory activities of the 5444 hydrazone/hydrazine compounds and the 2319 natural-product compounds were determined using purified recombinant wild-type HIV-1 reverse transcriptase and a high-throughput fluorescent assay for the specific measurement of RNase H activity. According to this assay, an 18-nucleotide 3′-flourescein-labeled RNA is duplexed to a complementary 18-nucleotide 5′-Dabcyl-modified DNA strand. The duplex has very low fluorescent signal, but when RNase H hydrolyzes the RNA from the duplex, the labeled

fragment dissociates from the DNA, resulting in a 50-fold gain in fluorescent signal. The lack of a gain in fluorescent signal in the presence of a particular compound thus indicates that the compound has inhibited RNase H in an unspecified manner.[14] The possibility that aggregation of the compounds might occur has been raised in the literature.[59] The AnalytiCon compounds contain a large amount of hydroxyl functionality, rendering them very water-soluble. The hydrazone/hydrazine compounds are less soluble, but we believe that aggregation of the actives was not a concern because we retested the best inhibitors in the presence of low amounts of nonionic detergents and observed no impact on the inhibitory potency.

To maximize the identification of potential high-affinity binders, we chose a high cutoff of $\geq 80\%$ inhibition at the screening concentration of 10 μM. With this inhibition cutoff, the AnalytiCon 2319-compound natural-product library yielded 84 confirmed hits (3.6%). The Life Chemicals 5444-compound hydrazone/hydrazine library yielded 338 confirmed inhibitors (6.2%). Compounds with activities close to but below the 80% inhibition threshold degrade the enrichment signal because they can be scored as false positives whereas they can exhibit substantial inhibition. For the AnalytiCon library, this was not an issue because the distribution of activities was close to bimodal. However, for the hydrazone/hydrazine compounds, approximately 20% of the library exhibited moderate (between 40% and 80%) inhibition at a concentration of 10 μM. To minimize the false positive problem with scoring the hydrazone/hydrazine library because of its more continuous activity distribution, we retained the 50 compounds with the lowest half-maximal inhibitory concentrations (i.e., IC$_{50}$ scores) among the 338 actives for docking along with all of the inactives. The IC$_{50}$ scores ranged from 0.17 to 9.22 μM; the highest IC$_{50}$ value for the 50 compounds was 1.14 μM. (The weakly binding active inhibitors were not included in the list of docked compounds.) Had we also removed inactive compounds that showed moderate inhibition, the enrichment signal might have been stronger than what was observed. The library of known inactive AnalytiCon and hydrazone/hydrazine compounds are universal decoys according to the classification by Nicholls.[60] The decoys are from libraries of commercially available compounds that share common motifs with the known actives. It should be noted that the decoys employed in our study were experimentally determined
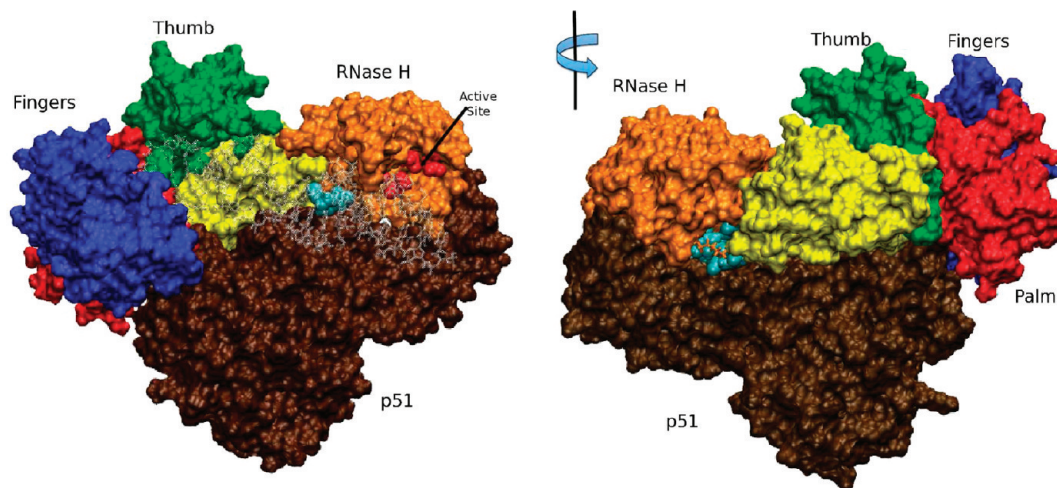
Figure 3. Images pointing out where the binding sites explored in this study are located on RT. On the left, the complex of RT and the DNA:RNA substrate is shown with p66 in blue ("fingers"), red ("palm"), green ("thumb"), yellow ("connection"), and orange for the RNase H domain with the active site residues in red spheres. The p51 domain is in brown, and the DNA:RNA substrate (stick representation) is in transparent gray. Site 500S is highlighted with the bound hydrazone inhibitor F1345-0193 shown in cyan spheres behind the transparent gray substrate and located at the intersection of the yellow, orange, and brown domains. On the right, the opposite side is shown after a 180° rotation of the molecule on the left, showing the AnalytiCon compound NP-003686 (in cyan) bound to site 507 (with residue 431 represented in sticks for a better perspective on NP-003686) located at the intersection of the orange and yellow domains of p66 and the p51 brown domain. These representations were generated using VMD.[70]

by us to be inactive compounds; that is, they showed less than 80% inhibition at 10 μM concentration. The close similarity of actives and inactives presents a challenge to computational enrichment studies.

To add to the diversity of the AnalytiCon and hydrazone/hydrazine libraries, we included a set of druglike[60] decoy compounds to each set, consisting of 2000 compounds with an average molecular weight of 380 Da.[18] These libraries of decoys provided another challenge in the goal of distinguishing how well the binding sites preferentially select active compounds from the collection of inactives. If the binding site could not distinguish actives from inactives, these decoy compounds would have had comparable binding energies and would have prevented any enrichment at that site.

2.4. Receiver Operating Characteristic Curves. High-throughput virtual screening was carried out with Glide XP using the library of compounds docking to sites indicated as druggable by SiteMap and to the active site with metal cofactors to the crystallographic coordinates for an HIV-1 RT structure deposited in the Protein Data Bank (PDB)[61] (PDB accession code 3IG1[32]). Based on a low-resolution structure of RT with the DNA:RNA substrate in complex with the hydrazone, THBNH, which indicated that inhibitors might bind between the RNase H domain and the substrate, we also bound compounds at a SiteMap-determined druggable site around residue Q500 on p66 using the RT/substrate structure based on the crystallographic coordinates deposited in the PDB[61] (accession code 1HYS[62]). For each library, using the lowest-binding-energy structure for each compound, we ranked, from lowest to highest binding energies, the compounds into a ranked database for each site. Using the list of active compounds determined by the fluorescent assay,[14] we calculated receiver operating characteristic (ROC) curves (enrichment curves) by determining the percentage of active compounds found within the top 10% of the ranked database. The area under the ROC curve was also calculated as another measure of enrichment.

2.5. Induced-Fit Protocol for Docking Ligands with Substrate. One potential binding site was located between the

RNase H domain and the DNA:RNA substrate. However, there was not enough space for compounds to bind between the two moieties in the model that we selected of RT with the DNA:RNA substrate (PDB accession code 1HYS). This was a model of the crystal structure of RT with DNA:RNA without any other ligand bound. We initially docked compounds at this site with reduced van der Waals radii (i.e., a softened receptor). The top 10 compounds (based on binding energies) were selected to be used as a wedge between the DNA:RNA substrate and RT. Using full van der Waals radii with a docked compound in place, a conjugate gradient minimization of each entire complex was performed with the IMPACT package.[63] The compounds were removed, and the library of compounds was redocked to the new conformations of RT and the DNA:RNA substrate.

3. RESULTS AND DISCUSSION

3.1. Prediction of Putative Binding Sites. The SiteMap calculation identified several potential druggable binding sites on HIV RT without the DNA:RNA substrate present. Two sites were located in the vicinity of the RNase H domain. One site was on the substrate binding side of RT centered between p66 residues W406 and Q500 (referred to as site 406, because residue 406 is at the center of the site) between the active site and the p51 domain. The other site was centered on residue Q507 (referred to as site 507 for the central residue) on p66 on the opposite side from the substrate binding side of the RT and the RNase H domain. The right side of Figure 3 shows the location of site 507 on RT. Both of these sites consist of residues that provide hydrogen-bond donors or acceptors from either side chains or backbone and also consist of neighboring patches of residues that can make hydrophobic interactions with a ligand. For site 507, the hydrogen-bonding residues, either by side chains or backbones, are T403, E404, K431, E430, Q507, and W535 on p66 and K331 and L425 on p51. The hydrophobic interactions at site 507 are between the ligands and residues W401 and W535 on p66 and L422 and L425 on p51 and between the ligands and the base
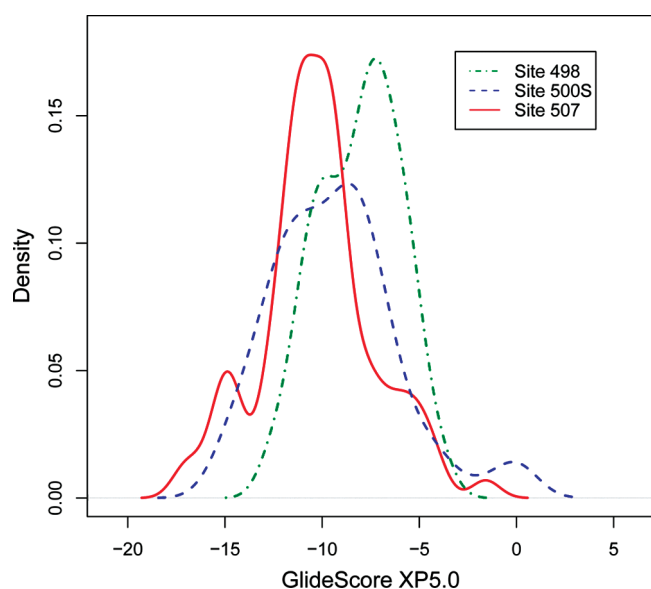
Figure 4. Distribution of the GlideScore XP5.0 binding energies for the 84 AnalytiCon active compounds plotted relative to each other for the binding sites at 498, 500S, and 507. The solid red curve is the distribution for site 507, the dot-dashed green curve is for site 498, and the dashed blue curve is for site 500S. Figure was generated using R.[71]

of the side chains of E404 and Q507 on p66 and K331 on p51. These two sites, 406 and 507, are separated by a thin layer of protein (only a few angstroms in thickness) at the interface of p66 and p51. During docking, because there was no significant separation between site 406 and site 507, the docking grid encompassed both sites. Consequently, attempts to bind to site 406 resulted in ligands placed preferentially in site 507. In other words, the best binding energies were found at site 507 instead of site 406. In light of this, our docking calculations focused on site 507 instead of site 406. SiteMap did not indicate that the active site around residue D498 (referred to as site 498) on p66 was a druggable site. (The SiteScore was estimated to be below 0.5, significantly lower than the threshold of 0.8.) This was due to the shallow-well geometry of the site. It might also have be due to the cofactor metals giving the site too much of a hydrophilic character.[8] Nevertheless, inhibitors have been identified that bind to the active-site cofactor metals.[32,34,35] We therefore also explored the active site (site 498) as a potential binding site.

Preliminary X-ray crystallographic results suggested that the compound trihydroxybenzoylnaphthyl hydrazone (THBNH) binds to RT with the DNA:RNA substrate in place. We explored whether the interface between the RNase H domain and the substrate could be defined as a druggable binding site with SiteMap. A large site centered around residue Q500 (referred to as site 500S for the central residue with the S to indicate the presence of the substrate) on p66 on the RNase H domain was predicted by SiteMap to be druggable, with a favorable SiteScore at 1.04. This site is not a conventional protein or nucleic acid target for binding, but a combination of the two, where the binding site consists of both protein and nucleic acid. Significantly, residue Q500 on p66 has been shown to bind to the nucleic acid template that is only one nucleotide base away from the scissile phosphate at the RNase H active site.[62] Based on the experimental and SiteMap results, we used this site at the interface of RNase H and substrate as a target for the libraries
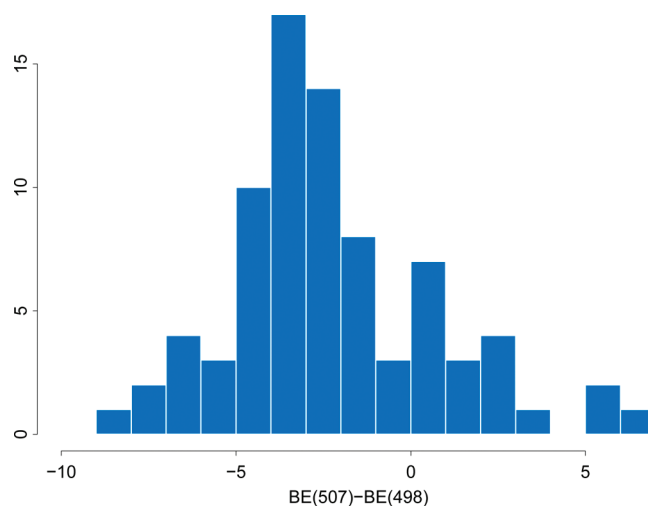

Figure 5. Histogram of the difference in GlideScore XP5.0 binding energies at site 507 [BE(507)] and at site 498 [BE(498)]. Bars with negative energy differences show actives that favor site 507; bars with positive energy differences show actives that favor site 498. Figure was generated using R.[71]

of compounds. The location of this site is shown on the left side of Figure 3.

3.2. AnalytiCon Library Results. The results from the Glide XP high-throughput virtual screening of the binding of the AnalytiCon and decoy compounds to sites 498, 500S, and 507 indicated that the active compounds prefer binding to site 507, as shown in the distribution of GlideScore XP energies in Figure 4. A histogram of the differences in binding energies for each active compound bound at sites 507 and 498 is shown in Figure 5. Although most of the differences were negative, indicating that more actives favored binding to site 507, several active compounds were found to prefer binding to the active site at 498. The top five compounds that preferentially bound to site 507 had an average binding energy of −14.04 kcal/mol. The largest component of this binding energy was found to be the hydrogen-bond interaction between the active inhibitor and the backbones of the residues in site 507, with an average energy of −6.93 kcal/mol and nine hydrogen bonds. The next largest component was a hydrophobic interaction that, on average, was −5.41 kcal/mol. This hydrophobic interaction was primarily between the actives and the following residues: L503 on p66, L425 on p51, and the base of the side chain of K424 on p51. The top five compounds binding to the active site, site 498, had an average binding energy of −11.83 kcal/mol. The largest component was the hydrogen-bond interaction at −5.50 kcal/mol, but a strong electrostatic interaction of −4.19 kcal/mol, on average, due to inhibitor carboxylate groups chelating to the cofactor metals located in site 498. The active site was not as hydrophobic as site 507: the hydrophobic score at site 498 was small at −2.18 kcal/mol. The question that we now address is whether the composition of either site selectively favors actives over inactives.

A method was recently proposed by Fukunishi and Nakamura to determine the correct binding site by docking a random library of compounds to all possible binding sites on a protein. They showed that the average docking score was best when the random collection of compounds was bound to the known site.[64] This approach is similar to our proposed method in that both attempted to identify the binding site by docking large
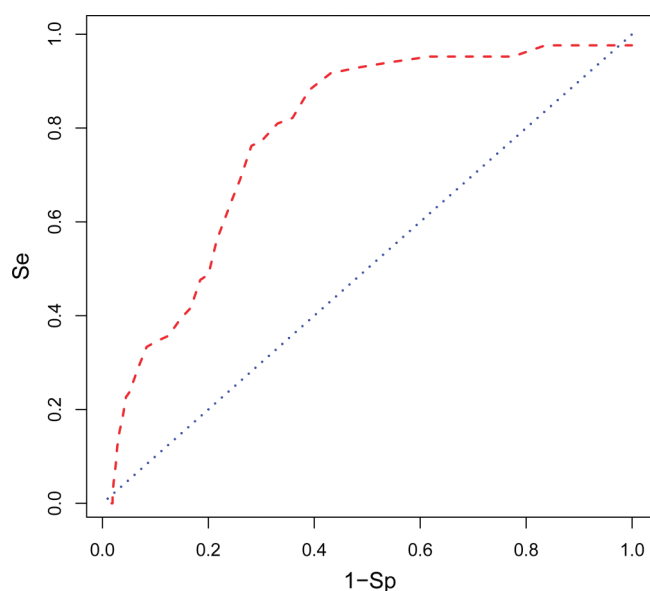
Figure 6. Receiver operating characteristic (ROC) curve in red showing the percentage of AnalytiCon active compounds found in the ranked database of all compounds bound to site 498. The selectivity (Se) of finding active compounds within some threshold is plotted against $1 - Sp$, where Sp is the specificity, which indicates the number of inactive compounds found within that threshold. The blue line indicates the ROC if the actives were randomly distributed in the database. The enrichment is 37% of the actives found in the top 10% of the screened database. The area under the ROC curve is 0.780. Figure was generated using R.[71]

libraries of compounds. However, they used libraries of compounds without prior identification of actives and inactives, whereas we experimentally identified the actives and inactives and used this additional information in our computational enrichment studies. As reported below, having access to the additional experimental information concerning which compounds in the library are active is helpful in distinguishing competing binding sites. Based on the formalism of Fukunishi and Nakamura,[64] we calculated the average energies of the AnalytiCon library of 2319 compounds and found that the averages were very similar at -6.0 ± 2.3, -5.9 ± 2.4, and -5.6 ± 2.1 kcal/mol for sites 507, 500S, and 498, respectively. The standard deviations indicate that the differences between these averages are not significant. As it is not possible to distinguish binding sites based on these averages, in this case, the construction of enrichment curves provides a better way to identify the most likely binding site for the AnalytiCon compounds.

Receiver operating characteristic (ROC) curves (or enrichment curves) detail how well these sites distinguish active from inactive AnalytiCon compounds. At site 498, 37% of the actives were found in the top 10% of the database, as shown in Figure 6, where the red curve is the ROC curve from the virtual screen and the blue curve is for comparison and shows what the ROC would look like if the actives were randomly distributed in the database (i.e., no preference for actives over inactives). This plot shows an almost 4-fold enrichment of actives in the top 10% of the database. The area under the ROC curve is 0.80, which indicates that the enrichment was better than random (for which the area under the ROC curve would be 0.5). Although this enrichment is significant, it is not as robust as the average 8-fold enrichment
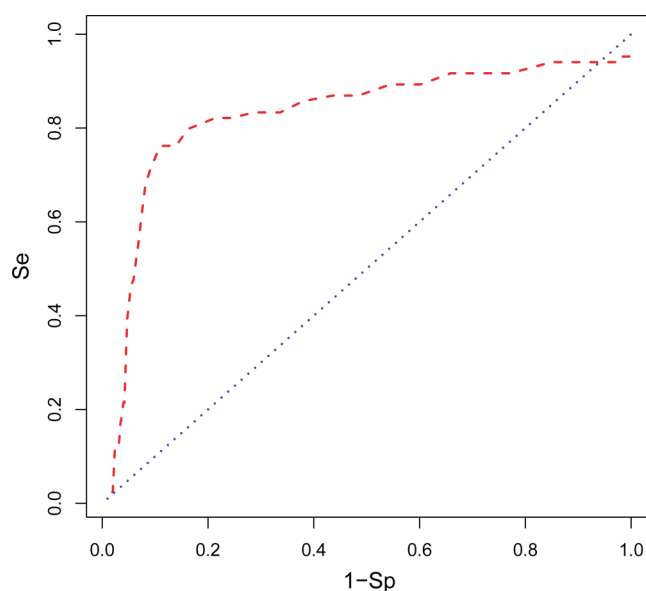

Figure 7. ROC curve in red showing the percentage of AnalytiCon active compounds found in the ranked database of all inactive compounds bound to site 507. The blue line indicates the ROC if the actives were randomly distributed in the database. Figure details can be found in the caption of Figure 6. The enrichment is 76% of the actives found in the top 10% of the screened database. The area under the ROC curve is 0.824. Figure was generated using R.[71]

reported in Zhou et al.,[18] which raises the question of whether this is the true binding site for the AnalytiCon inhibitors.

The enrichment of actives for binding with Glide XP to site 507 was considerably more robust than that for site 498: the level of enrichment was roughly 8-fold, with 76% of the actives found in the top 10% of the database, as shown in Figure 7. This level of enrichment is consistent with the average level reported in Zhou et al.[18] We believe that this significant level of enrichment is achieved when the actives bind to a true active site. The 8-fold enrichment of the actives meaningfully reflects that site 507 is the main binding site for the AnalytiCon inhibitors. The shape of the ROC curve in Figure 7 displayed a very strong, steep response in selecting the actives from the inactives. (A steep response is absent at site 498, as seen in Figure 6.) Both the selectivity and specificity were robust in distinguishing true positive actives in general from true negative inactives. The area under the ROC curve for site 507 is 0.87, which is greater than that for site 498. The very favorable binding energies of actives and more robust distinction of actives from the rest of the database support the conclusion that more of the AnalytiCon inhibitors preferentially bound to site 507 than to site 498. The physicochemical composition of site 507 thus better differentiates between the AnalytiCon actives and inactives than site 498, supporting the suggestion that site 507 is the more likely binding site of the experimentally determined actives. As shown in Figure 3, this site is on the opposite side from the substrate binding region and the active site of RNase H (site 498).

There is recent experimental support for our enrichment studies suggesting that site 507 is a true alternative binding site for inhibitors of RNase H. Fragment-based drug design targeting RT is being carried out in the Arnold laboratory at Rutgers University. In this work, crystals of RT are soaked in a cocktail of several compounds. Through X-ray crystallography, compounds
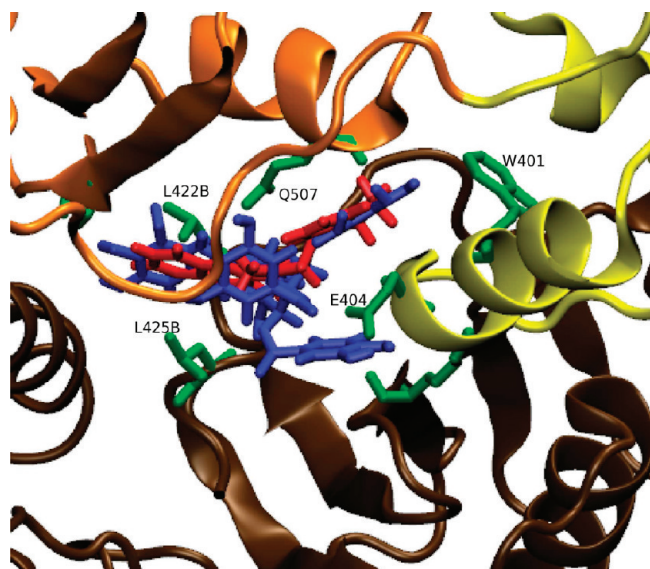
Figure 8. Binding orientations of AnalytiCon compound NP-003686 (in blue) and compound EN37 (in red) shown superimposed in site 507. The RNase H domain of p66 is in orange, the connection region of p66 is in yellow, and p51 is in brown. In green are residues that interact hydrophobically with the compounds. Residue labels ending in B indicate that the residue belongs to p51. This representation was generated using VMD.[70]

Figure 9. ROC curve in red showing the percentage of AnalytiCon active compounds found in the ranked database of all compounds bound to site 500S with the DNA:RNA substrate present. The blue line indicates the ROC if the actives were randomly distributed in the database. Figure details can be found in the caption of Figure 6. The enrichment is 58% of the actives found in the top 10% of the screened database. The area under the ROC curve is 0.815. Figure was generated using R.[71]

that bind to RT have been identified. One of these compounds is a 2-(((2-(3,4-dihydroquinolin-1(2H)-yl)-2-oxoethyl)(methyl)-amino)methyl)quinazolin-4(3H)-one (referred to as EN37) that binds at site 507 (the compound is centered on Q507). (A PDB file is available as Supporting Information.) Using Glide XP, we were able to redock EN37 to site 507 of RT to within 1.8 Å rmsd of the X-ray orientation. The profile of the binding energy between this quinazolinone and site 507 has contributions involving hydrogen-bond interactions and hydrophobic interactions similar to those of the AnalytiCon compounds that are the focus of the current study. An AnalytiCon active compound (NP-003686) is shown along with compound EN37 bound in site 507 in Figure 8. The two compounds overlap when bound in this site.

The RNase H domain on p66 makes contact with p51 by a thin interface between the two. It is possible that the RNase H domain could pivot about this interface between p66 and p51. The AnalytiCon active inhibitors are large; when bound in site 507, they might nudge the RNase H domain to a position in which the active site might no longer be able to catalyze the removal of RNA from the DNA:RNA duplex. (A few of the inactives had binding energies comparable to those of the actives; it is possible that these inactives might actually bind but might not be able to change the RNase H domain allosterically to provide inhibition.) We believe that this site might be a suitable target for new inhibitors. The advantage of binding to site 507 is that it is unique to RT. The RNase H active site is similar to human RNase H1;[44] therefore, inhibitors that bind to site 498 could interfere with human ribonuclease H and produce unwanted side effects.

An unconventional binding mode for RNase H inhibitors has been proposed based on limited crystallographic data. In this mode, the inhibitors would bind between the RNase H domain and the DNA:RNA duplex, preventing the substrate's access to the active site. SiteMap calculations supported the possibility that
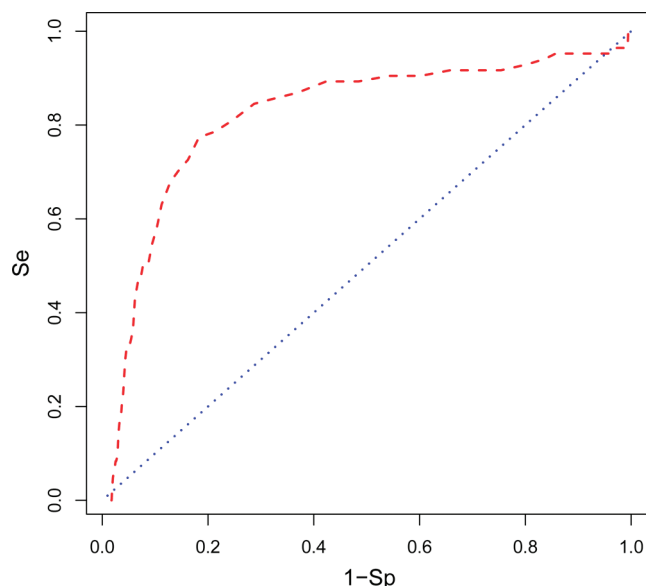
this region, site 500S, is druggable. Therefore, we also docked the AnalytiCon library to site 500S. We found a 6-fold enrichment of the AnalytiCon active compounds when binding to site 500S (see Figure 9). The selectivity and specificity in the ROC curve for site 500S was not as robust as that for site 507. Although there was a steep rise in the ROC curve for site 500S, it was not sustained, signifying that more false positives were encountered among the true positives of the active inhibitors. Nevertheless, there was some signal at site 500S. Does site 500S compete with site 507 for the same inhibitors, as is potentially indicated in Figure 4? A comparison of binding energies of the actives at site 507 with those at site 500S indicated that the compounds tend to prefer one site over the other based on the relative binding energies. The difference in energies for an inhibitor binding at each site was found to be significant, between 2 and 5 kcal/mol.

3.3. Hydrazone/Hydrazine Library Results. No enrichment was observed for the Life Chemical hydrazone and hydrazine compounds at sites 498 and 507 with Glide XP high-throughput virtual screening. The level of enrichment of the 50 strong-binding actives was essentially no better than that corresponding to a random mixture of the actives in the database. The enrichment was absent; only 8% of the actives were found in the top 10% of the ranked database. The areas under the ROC curves ranged between 0.505 and 0.555, showing that the actives were essentially distributed randomly in the database. These null results, from a combination of experimental and virtual screenings, do not support the selective binding of active hydrazone and hydrazine compounds to sites 498 and 507.

Recently, preliminary X-ray-crystallographic evidence was obtained suggesting that the hydrazone compound THBNH might bind between the RNase H domain and the duplex DNA:RNA substrate. We decided to explore the enrichment targeting
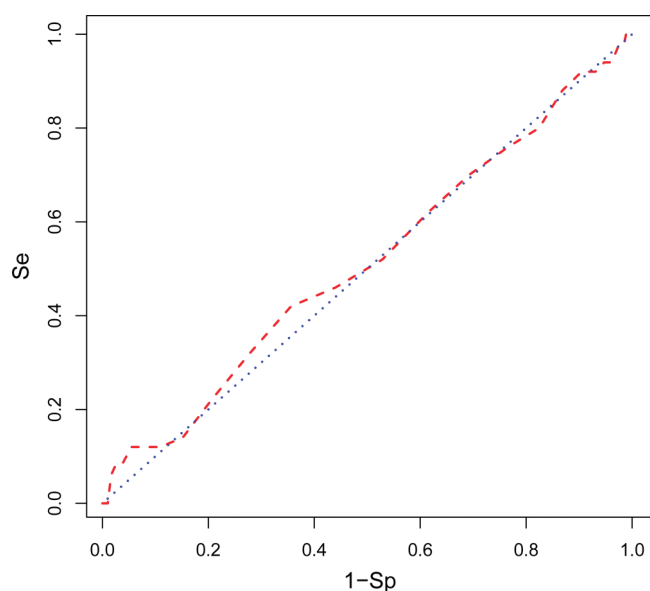
Figure 10. ROC curve in red showing the percentage of hydrazone/hydrazine active compounds found in the ranked database of all compounds bound to site 500S (between RNase H and the DNA:RNA substrate). The blue line indicates the ROC if the actives were randomly distributed in the database. Figure details can be found in the caption of Figure 6. The enrichment is 15% of the actives found in the top 10% of the screened database. The area under the ROC curve is 0.512. Figure was generated using R.[71]
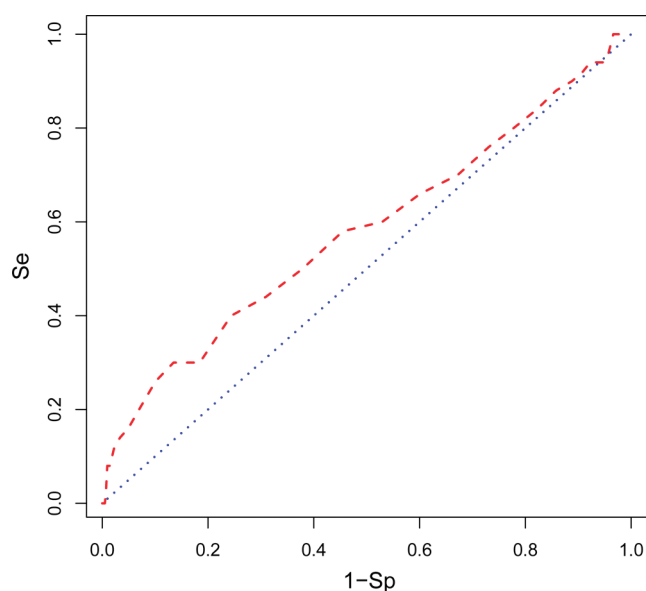

Figure 12. ROC curve in red showing the percentage of hydrazone/hydrazine active compounds found in the ranked database of all compounds bound to site 500S of the minimized RT-DNA:RNA complex. The blue line indicates the ROC if the actives were randomly distributed in the database. Figure details can be found in the caption of Figure 6. The enrichment is 24% of the actives found in the top 10% of the screened database. The area under the ROC curve is 0.574. Figure was generated using R.[71]
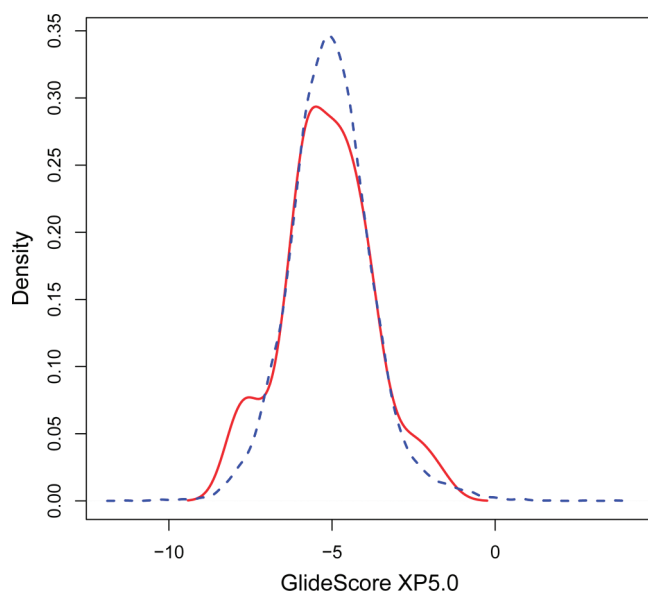

Figure 11. Distribution of GlideScore XP5.0 binding energies of the hydrazone/hydrazine active compounds (red solid curve) relative to the distribution of database energies (blue dotted curve) when docking to site 500S. Notice the shoulder peak comprises actives with binding scores between −9 and −6 kcal/mol. Figure was generated using R.[71]

this unconventional binding mode (a binding pocket that is a combination of protein and nucleic acid) for the library of the hydrazone and hydrazine compounds. Using the structure of 1HYS[62] as our model of RT with the DNA:RNA substrate, we targeted the binding of the compounds to the site around residue 500 on p66 (site 500S) based on the crystal structure and

SiteMap predictions (see Figure 3). The initial ROC curve in Figure 10 does not show very much enrichment (only 15% of the actives were in the top 10% of the ranked database), but the density plot in Figure 11 shows some signal of potentially useful enrichment with a shoulder peak in the actives' curve between −9 and −6 kcal/mol. We suspect that the binding region at site 500S was not open enough in the 1HYS model for all compounds to fit properly. It seems possible that the DNA:RNA substrate could be displaced partially from the RNase H domain by compounds binding in the vicinity of site 500S.

The induced-fit docking protocol in this study started with 10 active hydrazone or hydrazine compounds that successfully docked, using reduced van der Waals radii of the receptor, at site 500S (i.e., found in the shoulder peak of Figure 11). Each of these 10 compounds was used as a wedge to open up the space between the RNase H domain and the DNA:RNA duplex. A conjugate gradient minimization was performed with the IMPACT package[63] on each complex of the bound inhibitor compound, RT, and the substrate using full van der Waals radii. These minimizations created 10 new configurations of RT with the DNA:RNA substrate. The entire hydrazone/hydrazine compound library was then docked to site 500S on each of these 10 new configurations. As seen in the ROC curve in Figure 12, the enrichment grew from 15% to 24%, better than 2-fold enrichment at 10% of the ranked database, in the best of these 10 cases. The area under the ROC curve is 0.574, which is still a weak signal but an improvement relative to what was observed at the other sites for the hydrazone/hydrazine compounds. Although the presence of a weak signal at site 500S is an improvement over no signal, we are not as confident in these results based on our previous work with enrichment curves, which suggests that a true enrichment should be on the order of 8-fold[18] as observed for the

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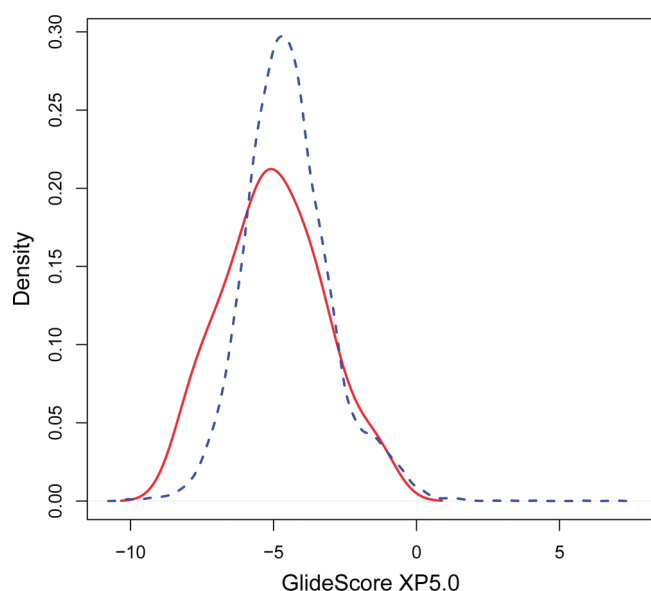dx.doi.org/10.1021/ci200194w |*J. Chem. Inf. Model.* 2011, 51, 1986–1998

Figure 13. Distribution of GlideScore XP5.0 binding energies of the hydrazone/hydrazine active compounds (red solid curve) relative to the distribution of database energies (blue dotted curve) when docking to site 500S of the minimized RT-DNA:RNA complex. Notice the shoulder peak comprises actives with binding scores between −9.5 and −6 kcal/mol. Figure was generated using R.[71]

AnalytiCon actives at site 507. To determine whether this weak signal could be a weak indicator that site 500S is a binding site for the hydrazone/hydrazine active inhibitors, we compared the density plots of the energies before and after our induced-fit procedure. The shoulder in the density plot of energies for the actives grew in magnitude without being shifted to lower energies by the inactive compounds in the database, as shown in Figure 13, which is clearly an improvement over Figure 11. On average, the inactive compounds shifted to less favorable energies, whereas several of the actives shifted to more favorable energies. Another, more computationally costly, approach would be to use a dummy atom positioned between the substrate and the RNase H domain and to adjust the radius of the dummy atom to incrementally move the substrate away from RT. With appropriate increments in the radius of the dummy atom, one could obtain a range of displacements that would accommodate more ligands. The high-throughput screening calculation would grow depending on the number of increments in the dummy atom radius used. Nevertheless, the approach using a bound inhibitor as described in this article is a simpler and more direct way to obtain a ligand-sized separation between substrate and RT. Our approach avoided having to repeat high-throughput virtual screening of the library on several dozen potential targets that were created with different increments.

The average GlideScore XP energy for the top five actives was −6.98 kcal/mol. The major component of that energy was found to be the hydrophobic interaction at −5.51 kcal/mol on average. The compounds were positioned to form a hydrophobic interaction with RT and with base pairs in the groove of the DNA: RNA substrate. The compounds were positioned between RT and the substrate between residues Y405 and W406 on p66 and between residues Q500 and Y501 on p66. Along with the evidence of THBNH bound to the complex of RT and substrate, there is evidence that mutations of Y501 result in resistance to

inhibition by the hydrazone BBNH [N-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone].[41] A mutation at Y501 would clearly have an effect on the binding of the library of hydrazone and hydrazine compounds at the target site 500S described by the ROC curve in Figure 12. This provides further support for the enrichment results described in this section, which indicate the possibility that site 500S with the bound substrate is a conceivable binding mode for compounds in the hydrazone/hydrazine library.

Our recent NMR studies of the interaction of the acylhydrazone inhibitor BHMP07 with an isolated HIV-1 RT RNase H domain fragment[17] provide compelling support for the site 500S binding. In these ^{1}H ^{15}N heteronuclear single quantum coherence (HSQC) experiments, the inhibitor was bound to the isolated domain without substrate present. Several residues were perturbed, based on changes in chemical shifts, in the presence of the acylhydrazone inhibitor. These residues included D499 and A502, which are adjacent to Q500.[17] These residues are part of the primer grip of the RNase H domain and play a role in aligning the DNA:RNA substrate with the active site.[62] An inhibitor such as the hydrazones binding at Q500 would clearly disrupt the primer grip's role in the activity of RNase H.

4. CONCLUSIONS

In this work, we have shown how combining experimental information concerning which compounds are active or inactive in large libraries with virtual screening through enrichment studies can provide valuable indications about the location of putative binding sites. Experimental high-throughput screening is a valuable tool for drug discovery by identifying active compounds that inhibit an enzyme of interest, even when the binding target is not known. The collection of active inhibitors can be aligned for three-dimensional quantitative structure—activity relationships (QSARs) to identify new compounds that might have similar binding activities.[65−68] Going further, knowing the location of the receptor pocket to which inhibitors bind can provide crucial information that can be used to guide structure-based design of new inhibitors. As in the current situation with RNase H, experimental information about where the actives bound to a target enzyme might not be known or might be ambiguous, such as the case with certain NMR spectroscopy data or low-resolution X-ray crystallography. Other sites in addition to the active site might be important. Programs such as SiteMap[7,8] attempt to identify binding sites based on geometry and physicochemical properties, but they also identify multiple sites at a time that might or might not be the actual binding sites.[1−9,45,69] Binding sites exhibit properties that differentiate between active inhibitors and inactive compounds. In this work, we have combined experimental identification of actives and in silico high-throughput virtual screening into enrichment (ROC) curves to provide powerful clues to identifying true binding sites of the actives. For the AnalytiCon library of compounds, we identified a pocket on the other side from the active site centered on residue Q507 on p66 with an 8-fold enrichment, on par with our previous work that benchmarked enrichment calculations with known systems.[18] We believe that inhibitors binding at site 507 could cause a conformational change at the interface between the RNase H domain and the p51 domain. This could conceivably change the orientation of the active site and affect its availability to act on the DNA:RNA substrate. For the hydrazone/hydrazine library, we identified a

new binding mode in which inhibitors bind between RT and the DNA:RNA substrate at residue Q500 (site 500S) with the only positive signal in the ROC curve for any site on RNase H. With this information, it should be possible to develop structure-based design strategies that target these allosteric sites. These allosteric sites have an advantage as drug design targets in that they are specific to HIV-1 RT, so that, ideally, compounds that target these sites will not interfere with human RNase H1.

■ ASSOCIATED CONTENT

ⓈSupporting Information. PDB file containing the coordinates of the complex of fragment EN37 bound at site 507 as shown in Figure 8 and an annotation of the residues which form the binding pocket for site 507 and site 500S. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

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