Evaluation of adamantane derivatives as inhibitors of dengue virus mRNA cap methyltransferase by docking and molecular dynamics simulations

Victor Luzhkov, Etienne Decroly, Bruno Canard, Barbara Selisko, and Johan Åqvist

a Department of Kinetics of Chemical and Biological Processes, Institute of Problems of Chemical Physics, Russian Academy of Sciences, Chernogolovka, Moscow region, 142432, Russia

b Centre National de la Recherche Scientifique and Universités d’Aix-Marseille I et II, UMR 6098, Architecture et Fonction des Macromolécules Biologiques, AFMB-CNRS-ESIL, Case 925, 163 avenue de Luminy, 13288 Marseille Cedex 9, France

c Department of Cell and Molecular Biology, Uppsala University, BMC, Box 596, S-751 24 Uppsala, Sweden

* Corresponding author:

Victor Luzhkov, e-mail: vbl@icp.ac.ru,
Abstract:

Binding of the dengue virus S-adenosyl-L-methionine (AdoMet) - dependent mRNA cap methyltransferase (NS5MTaseDV) with adamantane derivatives was explored using molecular modelling methods and (nucleoside-2’O)-methyltransferase bioassay. The studied compounds include urea derivatives of adamantane and antiviral drugs amantadine and rimantadine. The urea derivatives of adamantanes had previously been identified as inhibitors of NS5MTaseDV. The docking simulations using GOLD, Glide, and Dock give consistent binding modes and binding affinities of adamantanes in the AdoMet-binding site of NS5MTaseDV and, in particular, yield similar positions for the previously found inhibitors. Combined, they perfectly correspond to the bioassay measurements of nucleoside-2’O-methyltransferase activity of NS5MTaseDV, which confirmed inhibitory properties of the active urea adamantane but did not show inhibitory activity for amantadine and rimantadine. We also employed microscopic molecular dynamics (MD) simulations and a linear interaction energy (LIE) method to verify the docking results. The MD/LIE binding free energies of selected protein–inhibitor complexes agree overall with the binding affinities from docking and demonstrate that amantadine and rimantadine only weakly bind at the explored site. The MD simulations also demonstrated flexible character of a protein loop that is located between the β2 and β3 strands and is part of the AdoMet-binding pocket of NS5MTaseDV.
1 Introduction

The addition of a cap structure at the 5’ end of eukaryotic mRNAs plays an essential role in the life cycle of mRNAs by protecting them from degradation by host endonucleases and by enhancing initiation of translation.[1] RNA capping in eukaryotic cells is a co-transcriptional event which involves conversion of the 5’-triphosphate end of mRNA to a 5’-diphosphate by an RNA triphosphatase, transfer of a guanosine moiety to the 5’-diphosphate by a guanylyltransferase and cap methylations catalyzed by a (guanine-N7)-methyltransferase (N7-MTase), leading to a cap-0 structure $^{7}$MeGpppN, and by a (nucleoside-2’O)-methyltransferase (2’O-MTase), leading to a cap-1 structure $^{7}$MeGpppN$_{2’OMe}$N.[2-4] Many viruses have been shown to encode their own capping enzymes. Viral mRNA cap MTases[5,6] are considered as promising targets for potential chemotherapeutic agents against human viruses and, in particular, flaviviruses[7-10] that are cause of several serious diseases in humans including encephalitis and haemorrhagic symptoms.[11,12]

Methyltransferases play an important role in cellular biochemistry since major cellular components, like DNA, RNA, proteins, lipids and polysaccharides, are subject to methylation by these highly specific enzymes. Most of them use S-adenosyl-L-methionine (AdoMet) as the donor of a methyl group and generate S-adenosyl-L-homocysteine (AdoHcy) as the reaction co-product. In flaviviruses, the N7-MTase and the 2’O-MTase activities are linked to the non-structural NS5 protein in which case N7-methylation precedes 2’O-methylation.[13] X-ray structures of the MTase domain of NS5 from several flaviviruses have been determined in complexes with AdoMet, AdoHcy and GTP analogues.[13-18] These MTases have an overall globular shape with a central subdomain adopting a typical fold of AdoMet-dependent MTases[19] characterized by a seven-stranded central β-sheet surrounded by six helices. Structure determination of the MTase complexes from several flaviviruses defined one binding site for AdoMet/AdoHcy in the central subdomain, and the second binding site for GTP analogues in the N-terminal subdomain. Because of the appropriate distance to the
AdoMet-binding site, the GTP-binding site was proposed to host the cap guanine during 2’O-methylation whereas an intervening positively charged zone indicates the RNA binding region, which continues beyond the GTP- and the AdoMet-binding sites.[14–16]

New pharmaceutical agents have been screened and developed against the target AdoMet-dependent MTases.[20–26] In several cases structure-based drug design was employed. In particular, a low-micromolar inhibitor aurintricarboxylic acid (IC$_{50}$ = 2.3 µM) for the dengue virus NS5 MTase (NS5MTase$_{DV}$) has been identified by Milani et al. from docking of the virtual Library of Pharmacologically Active Compounds and inhibitory activity measurements.[25] Several inhibitors of NS5MTase$_{DV}$ with IC$_{50}$ < 10 µM has been found by Podvinec et al. [26] using virtual screening of large chemical libraries and bioassay tests. Recently, we explored inhibition of NS5MTase$_{DV}$ by several new classes of compounds selected from large chemical databases as analogues of AdoHcy using a combination of pharmacophore filtering and docking.[24] In particular, our work predicted new inhibitors, containing a polycyclic adamantane cage (Fig. 1). The most active of this series in a subsequent inhibition bioassay, using an 2’O-MTase test, was 2-((3-(4-methylphenyl) adamantane-1-yl)carbamoyl) amino)-3-phenylpropanoic acid (hereafter referred to as compound 1, Fig. 1) with an IC$_{50}$ value of 60.5±3.2 µM. According to the docking simulations the adamantane moiety with two side chains of proper chemical composition enables selective fit of ligand 1 into the AdoMet-binding site. One of the aims of this work was to explore by computational methods how the molecular structure of adamantane derivatives affects their binding to the AdoMet-binding site of NS5MTase$_{DV}$.

Antiviral agents containing an adamantane group have in principle been known for long time,[7,27,28] including the marketed drugs against influenza virus – amantadine and rimantadine. The treatment of influenza virus by these compounds is related to the inhibition of the pH modulator membrane channel M2.[7,28] Besides, amantadine was reported to inhibit
dengue virus replication. Following that, we included amantadine and rimantadine in our study.

In silico approaches for evaluating binding propensities of new ligands are nowadays widely applied in rational drug design. In the present work we computed binding modes and binding energies of adamantanes using docking and molecular dynamics (MD) free energy simulations. Both computational approaches are essentially based on the knowledge of the 3D structure of the biomolecular target. The docking techniques predict positions of the bound ligands and their binding affinities from sampling the conformational space of protein–ligand complexes. Docking uses analytical scoring functions for quantifying ligand interactions with the biomolecular target. As a further refinement step, microscopic MD simulations provide information on thermally equilibrated statistical ensembles of structural models, allowing the stability of the protein–ligand complexes to be assessed. In many cases rather accurate estimates of binding free energies can be obtained from using such methods. Computational tools can in this way yield useful data of protein–ligand interactions on the atomic level when no direct crystallographic data for the protein–ligand complexes exist, as is the case with complexes of adamantane inhibitors with NS5MTaseDV.

2 Results and Discussion

Ligands: In exploring of how molecular structure affects binding of chemical compounds built around the nonpolar adamantane core we considered the previously found adamantane NS5-MTaseDV inhibitors 1 and 2 as starting points (Fig. 1). We also added adamantane 3 and the antiviral drugs amantadine 4 and rimantadine 5 with only a short side chain at the polycyclic cage. Besides, the ligand set included hypothetical analogues 6–12 of 1 and 2,
where the length of the side chains linked to the adamantane cage was reduced in a step-wise manner as compared to the starting structures.

Insert Figure 1

**Docking calculations:** For docking we used three programs with differing algorithms because in a situation where the experimental structures of the protein–ligand complexes are not known, the use of differing approaches can provide cross-validation of the results and in this way enhance the reliability of predicted docked positions and their scores. The first selected program was GOLD,\(^{[38,39]}\) which had previously been used by us at the final stage of virtual screening of the NS5MTase\(_{DV}\) inhibitors\(^{[24]}\) and allowed prediction of the active compounds 1 and 2. The computational procedure of GOLD employs a genetic algorithm for the local minima search and specially parameterized scoring functions for quantifying essential contributions to the free energies of binding. Similarly to the previous simulations the current docking scheme used the GoldScore fitness function which includes protein–ligand hydrogen bond energy, protein–ligand van-der-Waals interaction energy, ligand internal van-der-Waals interaction and torsional energies. It should be noted in this respect that the second scoring function of choice in GOLD, i.e. ChemScore, is not as good as GoldScore in predicting binding positions for AdoHcy in the AdoMet-binding site of NS5MTase\(_{DV}\).\(^{[24]}\) The second docking approach was Glide,\(^{[40–42]}\) which uses a multi-stage algorithm for filtering the low-energy docked structures. Glide employs at different stages of the docking protocol the ChemScore and GlideScore scoring functions, and, additionally, the OPLS-AA force field for calculating ligand-binding positions and binding affinities. Such a hierarchical search is claimed to provide good accuracy for ligand binding modes.\(^{[42]}\) The GlideScore function, which is invoked for the final assessment of the binding affinities, includes van-der-Waals, Coulomb, and hydrogen bond contributions, penalties for buried polar groups and frozen rotatable groups as well as special lipophilic and polar site terms for protein–ligand interactions.\(^{[40]}\) In selected cases we employed third docking program
Dock uses a matching algorithm for superimposing ligands onto the binding pocket and energy grids from the Amber force field for calculations of the protein–ligand interactions. All three methods consider conformationally flexible ligands. The test calculations by GOLD, Glide and Dock correctly place AdoHcy in the complex with NS5MTaseDV (PDB code 1R6A) where the corresponding ligand RMSD’s from the experimental position are only 1.0, 1.0, and 0.9 Å, respectively.

The previous docking simulations using GOLD had predicted that the best-fitting position of ligand 1 closely overlaps with the experimental location of AdoHcy in the 1L9K structure. To enhance the robustness of the computational predictions we employed here a differing 3D data for the protein, namely the 1R6A structure of the complex of NS5MTaseDV with AdoHcy and ribavirin 5′-triphosphate. The two structures have similar resolution of 2.4 – 2.6 Å, and the root mean square deviation (RMSD) between them is about 0.4 Å. The 1R6A structure only differs from 1L9K by rotated side chains of several amino acids, including E111 near the AdoHcy-binding site, by slightly differing binding conformation of AdoHcy, and by positions of several crystallographic waters.

Using the 1R6A target structure for docking does not change the predictions from the earlier work. The adamantane cage of 1 in the low-energy docked structures is positioned in front of the β–strands β1, β2 and β4, and overlaps with that of the ribose ring of AdoHcy. The protein loops between β2 and β3, and β3 and β4 delineate the location of the toluene moiety while α–helices αA and αX harbour the phenylalanine functional group of 1 (Fig. 2). Docking of 1 with GOLD and Glide provided very similar results for both stereoisomers of this compound (Fig. 3). A minor difference is that GOLD preserves all-trans conformation for the urea group in all structures, whereas Glide predicts all-trans conformation of urea for 1(R) and cis-trans conformation for several low energy complexes of 1(S). Docking of 1 with Dock predicts structures that are similar to those from the other two methods (Fig.3).Docked
positions of inhibitor 2 are very similar to those of ligand 1. But unlike 1, all-trans conformation of urea was observed in all low-energy binding modes of 2.

**Insert Figures 2, 3**

Calculations of docked complexes of adamantane 3 predict three binding modes at the AdoHcy-binding site. Glide predicts binding of 3 either to the position of adamantane cage of 1 (referred to as Position 1) or in the cavity next to the pocket for the adenine group of AdoHcy (referred to as Position 3), with the latter site being slightly favored. GOLD places adamantane into the deep pocket delineated by S56, D79, C82, G85, W87, and D146 (Fig. 4). This site, referred to as Position 2 in Fig. 4, essentially overlaps with the location of phenylalanine moiety of 1. The corresponding binding cavities are shaped by G81, T104, K105, E111, I147 in Position 1, and by F133, I147, G148, R160, R163 in Position 3 (Fig. 4). Docking of the positively charged adamantane derivatives 4 and 5 using GOLD yields binding modes close to that predicted by this method for adamantane, i.e. in Position 2 (Fig. 5). Dock also places 4 and 5 only in Position 2. Docking using Glide places 4 only in Position 1, whereas 5 binds in Position 1 or 2. In all cases amine groups of 4 and 5 were aligned towards side chains of D79, D146 and backbone carbonyl of G81.

**Insert Figures 4, 5**

We have also explored docking modes of truncated analogues of 1, i.e. ligands 6–12 (Fig. 1). Docking of ligand 6 that only lacks phenyl ring in the polar tail of 1 predicted binding positions very similar to that of 1. Ligand 6 occupies a smaller volume in the polar region of the binding site which can provide greater conformational flexibility for the corresponding side chain. However, the binding modes of the polar tail of 6 from Glide and GOLD are quite close to the experimental position of amino acid tail of AdoHcy. The urea derivatives 9 and 10 were placed from docking into the adamantane’s binding positions 1 and 2. The singly substituted adamantanes, i.e. ligands 7, 8, 11, 12, also demonstrated multiple
binding modes within the AdoHcy-binding region. For instance the adamantane cage of compound 7 with a negatively charged carboxyl substituent was placed into the binding site 1 by GOLD and into site 3 by Glide, whereas nonpolar ligand 8 was docked to site 3 by Glide and to sites 1–3 by GOLD. Overall, inspection of the docking data shows that the AdoHcy-binding site is exceedingly spacious for small adamantanes and, hence, sufficiently bulky substituents at the adamantane cage would be required to tightly fill the site.

Insert Table 1

The docking scores from GOLD, Glide and Dock showed that the best binding ligands are inhibitors 1 and 2, and AdoHcy (Fig. 6, Table 1) The data of Fig. 6 illustrate a fair agreement between the rankings of the fitness scores from the GOLD and Glide methods. The corresponding Spearman rank correlation coefficient \( \rho \) is 0.75. As expected, the natural co-product AdoHcy received high docking scores, and is essentially predicted to bind better than all the adamantanes by GOLD and Dock, whereas in Glide it is outperformed by only 1(S). The docking scores agree overall with that from bioassay experiments AdoHcy binds better (IC\(_{50}\) of 0.3 \( \mu \)M\(^{[45]} \)) than racemic inhibitors 1 and 2. It can also be interesting to note that the conformationally flexible ligand 6 that lacks phenyl group in the polar tail gets lower scores than inhibitor 1, but still higher scores than inhibitor 2. The docking scores of 4 and 5 are less favourable than for 1, 2 and AdoHcy, yet these results do not exclude forming of energetically stable complexes at the AdoHcy-binding site.

Insert Figure 6

**Measurements of biological activity:** Since amantadine inhibits dengue virus replication\(^{[29]} \) we assessed the inhibition capacity of these antiviral drugs for the 2’O-MTase activity of NS5MTase\(_{DV} \). For this purpose, NS5MTase\(_{DV} \) was incubated with substrate \(^{7}\text{Me}\)GpppAC\(_5\) in presence of radiolabelled AdoMet and the reaction product (\(^{7}\text{Me}\)GpppA\(_2\)’OMeC\(_5\)) was quantified
by a filter binding assay (see Material and Methods). The control measurements in accordance to previous data\textsuperscript{[24]} showed that compound 1 inhibited the 2''O-MTase activity of NS5MTase\textsubscript{DV} by 75% at a concentration of 100 µM. However, inhibition of NS5MTase\textsubscript{DV} by rimantadine and amantadine was not observed at this concentration.

**Molecular dynamics simulations:** In order to get further insight into the binding patterns and inhibition capacity of adamantanes we evaluated structural stability and binding free energies of the complexes of NS5MTase\textsubscript{DV} with the tested compounds 1(R,S), 2(R,S), 4, 5, and AdoHcy using molecular dynamics simulations along with the linear interaction energy (LIE) method\textsuperscript{[46–48]} (see Material and Methods, Eq. 1). To increase sampling efficiency docked complexes from GOLD and Glide with differing binding positions were used as starting structures for MD (about 30 complexes were considered in total). Table 2 provides the simulation results for the complexes with the lowest values of Δ\textsubscript{G\text{bind}}. Negative values of the computed Δ\textsubscript{G\text{bind}} mean that the protein–ligand complex is thermodynamically more stable than the separated reactants.

**Insert Table 2**

First, we have examined molecular dynamics of the experimental complex of AdoHcy with NS5MTase\textsubscript{DV} (PDB code 1R6A). The molecule stays at the experimental binding position during 5ns of unrestrained MD simulations (Fig. 7) and its deviation from the starting position is less than for other ligands (Table 2). An interesting observation from the comparison of the MD-generated and starting structures concerns the ‘ribose-flanking’ loop K101–M116 (KGLTKGGPGHEEPIPM) between β2 and β3. This loop does not form direct H-bonds with AdoHcy and shields the binding site from water. In the crystallographic 1L9K\textsuperscript{[14]} structure the backbone atoms of G106 and E111 of the loop interact via a water molecule with the ribose moiety of the bound AdoHcy, however such a bridging water is not
present in 1R6A.\textsuperscript{[15]} The backbone atoms of the K101–M116 loop show only small deviation from the experimental position in the simulated NS5MTase\textsubscript{DV}–AdoHcy complex. However, the K101–M116 loop turns out to be quite flexible in MD simulations of the protein in the absence of AdoHcy. The latter is evident by visual inspection of the simulated structures (Fig. 7) and by RMSDs of 2.4 and 6.4 Å (after 5ns MD) for the whole protein and the loop, respectively (Table 2). Although the direct implications of such a feature for ligand binding are not fully clear the flexible character of the β2–β3 loop can in principle diminish steric limitations of inhibitor access to the AdoMet binding site.

Insert Figure 7,8

The examination of the molecular dynamics of the complexes of adamantanes showed that compound 1 in all cases stayed at the binding site during MD. However, the simulated positions of the 1(R) and 1(S) enantiomers in the low-energy trajectories diverged despite the fact that they were spatially close in the starting docked structures. Namely, 1(R) moved slightly in the direction of the RNA-binding site, whereas for 1(S) the adamantane cage shifted to enhance contacts with β4 while the β2–β3 and β3–β4 loops substantially deviated from the experimental structure (Fig. 8). The phenyl ring of the phenylalanine group of 1 showed the tendency to stay buried during the course of MD. Compounds 2(R) and 2(S) equally preserved their locations at the binding site during MD. Likewise compounds 4 and 5 preserved their location in the vicinity of S56, W87 and D146 during MD, i.e. in Position 2 (see Fig. 4). However, if MD of 4 and 5 started out from Position 1 the molecules migrated then during the MD simulations to Position 2.

The calculations of the binding free energies $\Delta G_{\text{bind}}$ using the MD/LIE method predicted the best binding affinity in the considered series for 1(R). $\Delta G_{\text{bind}}$ for 2(S) is about 6 kcal/mol more positive than for 1(R) and is similar to that of AdoHcy. The binding of 1(S), 2(R) and 5 is about 1–3 kcal/mol weaker than for AdoHcy, whereas binding of 4 is
energetically unfavourable. Table 2 presents the data from one 4-ns of MD trajectory for each compound. The results for the trajectories that provide the lowest values of $\Delta G_{bind}$ out of several 4-ns of MD runs are displayed in this case. Besides the energy criterium, some MD trajectories were rejected if the overall positions of the ligands (and the counter ions $\text{SO}_4^{2-}$) in the protein cavity were not conserved. The omitted data in principle provide additional estimates of the robustness and statistical uncertainties of our calculations. For instance, the next low in rank values of $\Delta G_{bind}$ are $-8.4 \pm 7.6$ for 1(R), $-2.1 \pm 5.1$ for 1(S), $-0.7 \pm 1.9$ for 2(R), and $-4.6 \pm 0.9$ kcal/mol for 2(S).

In summary the MD simulations indicated that the AdoMet-binding site does not favour strong binding of amantadine and rimantadine. This corresponds to the results obtained in the inhibition assays. In general, exploring molecular dynamics of the microscopic models with explicit water molecules and the related binding free energies (obtained here using the MD/LIE technique) is deemed to enhance our understanding of the ligand-binding patterns compared to using only static docking methods with simplified description of the surrounding media (e.g.[34]). It should be noted nevertheless that despite such an improvement, the MD/LIE method still yields only estimates of the relative binding energies. The relatively large error bars of the computed binding energies for 1 are typical for complex multi-ion systems and are dominated by electrostatic interactions, including interactions with the adjacent $\text{SO}_4^{2-}$ ions. Hence, large fluctuations of the counterion positions increase statistical uncertainty for the energies of the charged ligands. Note also, that the computed $\Delta G_{bind}$ were not calibrated in this work against any absolute values of binding free energies.

The studied molecules carry either negative, positive or zero total charges. In this respect, due to the presence of several ionisable amino acids the binding site can apparently accept ligands with differing net charges and the binding patterns of the corresponding ligands differ. The highest binding affinities are predicted for the complexes of the negatively charged compound 1, neutral compound 2, and zwitterionic AdoHcy. In the complex with the
anion 1 the greatest single stabilizing energy contribution is from the electrostatic interaction of the ligand’s negatively charged carboxyl group with the positively charged R84. The complexes with cations 4 and 5 are less stable than those of 1 and AdoHcy. For 5 the greatest stabilizing contribution in the low-energy Position 2 arises from the electrostatic interactions of the amine group with D79 and D146. These amino acids surround the amine group of the bound AdoHcy or AdoMet as well, however carbocation moiety in AdoMet points in different direction and interacts with D146 and atom O of cap analogue $^{N7Me}GpppG$. [18]

3 Conclusions

In this work we studied interactions of derivatives of adamantane with dengue virus NS5 MTase using molecular modelling and biossays. We focused on the previously explored inhibitors 1, 2 and two antiviral drugs, amantadine 4 and rimantadine 5. Experimental data for the complexes of NS5MTase$_{DV}$ with these ligands is not available. Docking calculations using GOLD, Glide, and Dock predicted very similar binding modes of inhibitor 1 at the AdoHcy-binding site of the protein. These results are quite consistent despite differing approaches for searching configurational space and scoring protein–ligand interactions. It can also be noted that three binding positions at this site were found for bare adamantane and antiviral drugs 4 and 5. In total, the docking data provide further evidence that inhibitor 1 binds to the AdoMet/AdoHcy-binding site and mimics the position of bound AdoHcy. The docking scores predict in agreement with the bioassay data that 1(R,S) and 2(R,S) in general bind weaker than AdoHcy, whereas truncated analogues of the inhibitor 1 will be even weaker binders.

The bioassay measurements confirmed the inhibitory effect of compound 1, however no inhibition was observed for amantadine and rimantadine. To shed additional light on the measured inhibitory activities and docking predictions we performed microscopic MD simulations for the complexes of NS5MTase$_{DV}$ with these compounds in water. In accordance with the bioassay results the calculated MD/LIE free energies predict that amantadine and
rimantadine bind considerably weaker than 1, 2, and AdoHcy. Besides, the MD simulations revealed differences in the binding affinities of stereoisomers of 1 and 2. In view of complexity of the model system the computed binding free energies should be regarded with some caution. For instance, the present results probably overestimate the binding affinity of \(1(R)\) relative to that of AdoHcy. However, the data, that the AdoHcy-binding pocket of NS5MTase\(_{DV}\) allows effective binding of compounds 1 and 2, and only weak binding of amantadine 4 and rimantadine 5, can be considered as a robust result of the MD simulations. Altogether, the docking scores and the \(\Delta G_{\text{bind}}\) values from MD/LIE produced similar trends with the exception of 1(S) (Tables 1 and 2). An approach combining the two methods apparently gives sufficiently reliable estimates of the ligand binding propensities.

An interesting observation refers to the K101–M116 loop between the \(\beta2\) and \(\beta3\) strands that turns out to be quite labile according to the MD simulation results. This loop is part of the AdoHcy-binding site in NS5MTase\(_{DV}\) and is open to the surrounding water. Besides, in the 1L9K pdb file this loop provides bridging stabilizing interactions with AdoHcy via the intervening water molecule. In the MD simulations the position of the K101–M116 loop closely followed the experimental structure for the NS5MTase\(_{DV}\)–AdoHcy complex but showed large fluctuations for the empty binding site and for the NS5MTase\(_{DV}\) complexes with adamantane derivatives. Difficulties with handling flexible biomolecular targets in the docking simulations are well recognised in modern structure-based drug design (e.g.\([34,36]\)). The related flexibility of the AdoHcy-binding site in NS5MTase\(_{DV}\) could possibly exert smaller steric limitations on inhibitors in the binding site and may also arguably imply a broader range of potentially active inhibitor scaffolds compared with those derived earlier from the rigid protein structure.\([24]\]

4 Materials and computational methods
**Molecular modelling:** The structure of the protein–ligand complexes and the binding propensities were computed using docking programs GOLD 3.2, Glide 4.5, Glide 4.5, and Dock6.4. The all-atom description was employed for all docking programs. The 3D structure of the protein was taken from the PDB entry 1R6A for the ternary complex of NS5MTaseDV, AdoHcy, and ribavirin 5'-triphosphate at a resolution of 2.6 Å. Molecular models were displayed and manipulated using Swiss-PDBViewer 3.7, UCSF Chimera, and DSViewerPro 5.0 from Accelrys (www.accelrys.com).

Search for the binding positions in calculations with GOLD was performed in a 15.0Å-radius sphere about the O atom of Gly81 using GoldScore. Two crystallographic waters HOH10 and HOH11, which are in contact with AdoHcy in 1R6A, were retained in the binding site and their occupancies were varied by the program GOLD during simulations. The settings for genetic algorithm (GA) in the search of the energy minima included 350000 iterations per docked solution. The best solutions were selected from 15 GA runs for each ligand. Other program settings correspond to the default values. During docking with Glide the search space for ligand positions was restricted by a 36Å cubic box with the center at the same position as in the simulations using GOLD. Extra-precision (XP) enhanced settings were employed for the computational procedure of Glide. Partial charges for the ligand atoms were generated from the OPLS-AA force field by the Maestro 8.0 program suite (see Supplement). Unlike the case with GOLD, the crystallographic waters from 1R6A were not included in docking with Glide and Dock. A rectangular box of 33.5×44.0×33.3 Å around the AdoHcy binding pocket was used in docking with Dock6.4. Ligand matching was limited to 20000 orientations, the energy cutoff in the conformer search was 20 kcal/mol. Partial charges from the Amber force field were used for the protein atoms, whereas Gasteiger charges were used for ligands (see Supplement). In all docking methods ligands were conformationally flexible during docking.
Molecular dynamics simulations were performed for several low-energy docked structures for each compound. The protein–ligand complexes were solvated by explicit water molecules (including crystallographic waters) and the whole system was modellled as a periodic rectangular cell of 62.1 Å × 68.3 Å × 77.7 Å. A periodic cell of the same size was also employed in simulations of the free ligands in water. MD simulations did not involve restraints except for positional restraints with a small force constant of 1.0 kcal mol\(^{-1}\) Å\(^{-2}\) applied to selected counterions to prevent possible large spurious positional changes. The ligand partial charges were taken from the docking calculations with Glide. All ionizable amino acids were modelled in their charged forms except for histidines that were taken uncharged. The model systems were made electrically neutral by compensating the excess charge with the crystallographic SO\(_4^{2-}\), and additional Cl\(^-\) and Na\(^+\) counter ions (if required). In particular, the simulated NS5MTase\(_{DV}\) possesses positive charge of +9 which was compensated by including in the model four SO\(_4^{2-}\) ions 903–905 and 907 from the 1R6A structure and an additional Cl\(^-\) ion. Sulfates 903–905 are located near the presumable RNA-binding site, whereas Cl\(^-\) was placed near the positively charged side chain of R119. For the protein–ligand complexes where the ligand had the net charge of +1 an extra Cl\(^-\) ion was added to the system (with the starting position near K99 and R157) whereas for the ligands with the net charge of −1 only four SO\(_4^{2-}\) ions were included in the model.

The MD simulations were performed with the program suite Q\(^{[47]}\) using the constant volume ensemble, the OPLS-AA\(^{[51,52]}\) force field and the TIP3P water model. Direct nonbonded cutoffs in the periodic box calculations were 10.0 Å for the protein-protein, protein-solvent and solvent-solvent interactions, and 31.0 Å for the ligand interactions with the surroundings. Beyond these cutoffs and up to an interaction distance of 31.0 Å the local reaction field expression\(^{[53]}\) with the first four terms in a multipole expansion was used to treat long-range electrostatic effects. The simulated box was stepwise heated to a temperature of T=300K during 100 ps MD and equilibrated for a total simulation time of 1000 ps. System
equilibration was followed by a productive MD phase of 4000 ps with a temperature coupling constant of $\tau_T = 0.1$ ps and a time step of 2 fs.

The data from the MD trajectories was collected each 10 fs for calculating the ligand binding free energies using the linear interaction energy (LIE) method.$^{[46-48]}$ In this method the ligand binding free energies, $\Delta G_{\text{bind}}$, are estimated from the scaled differences of the MD average intermolecular electrostatic and van der Waals (vdw) energies of the ligand bound to the protein and moving freely in aqueous solution

\[
\Delta G_{\text{bind}} = \Delta (E_{\text{ele}}) + \Delta (E_{\text{vdw}})
\]

The values of $\beta$ used in this work were taken from calibration on the solvation free energies of several hundred organic molecules,$^{[46]}$ while a standard value of 0.18 was used for $\alpha$ and $\gamma$ was set to zero.$^{[54]}$ The difference from the conventional LIE scheme is that in the present work the intermolecular energies for the free ligand in water include interactions not only with water but with the counter ions (if applicable) as well.

**Measurements of inhibitory activity:** Amantadine (A1260) and rimantadine (390593) were purchased from Sigma-Aldrich. The inhibitory activity of these molecules was tested on small RNA substrates $7^\text{Me}$GpppAC$_5$. The compounds were used at 125 µM final concentration. The MTase activity assays were performed in 30-µl samples containing 40 mM Tris, pH 7.5, 5 mM DTT, 5 µM AdoMet (0.3 to 2 µCi $[^3]$H)AdoMet, Amersham Biosciences), 1 µM NS5MTase$_{DV}$ and 2 µM of RNA substrate $7^\text{Me}$GpppAC$_5$ as described by Luzhkov et al.$^{[24]}$ Recombinant NS5MTase$_{DV}$ and the small capped RNA substrate ($7^\text{Me}$GpppAC$_5$) were synthesized as described previously.$^{[55]}$ In the inhibition assay, the inhibitor candidates were added to a premix containing buffer and enzyme and the reactions were then started with a premix of AdoMet and RNA substrate.
Acknowledgements

Support from the EU VIZIER program (CT 2004-511960), from the Uppmax computer center and from the computer center of ITP RAS is gratefully acknowledged. The authors also are thankful to Dr. J. Bujons and Dr. M. Almlöf for helpful discussions.

Keywords: Flavivirus methyltransferase inhibition, Drug design, Docking, Molecular dynamics, Adamantane derivatives
REFERENCES


Table 1. Docking scores for the complexes of NS5MTase<sub>DV</sub> with AdoHcy and ligands 1–5

<table>
<thead>
<tr>
<th>Ligand</th>
<th>GOLD</th>
<th>Glide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dock&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdoHcy</td>
<td>85.91</td>
<td>−8.3</td>
<td>−54.6</td>
</tr>
<tr>
<td>1(&lt;i&gt;R&lt;/i&gt;)</td>
<td>81.92</td>
<td>−7.4</td>
<td>−49.7</td>
</tr>
<tr>
<td>1(&lt;i&gt;S&lt;/i&gt;)</td>
<td>75.41</td>
<td>−8.7</td>
<td>−53.9</td>
</tr>
<tr>
<td>2(&lt;i&gt;R&lt;/i&gt;)</td>
<td>64.89</td>
<td>−5.9</td>
<td>−39.6</td>
</tr>
<tr>
<td>2(&lt;i&gt;S&lt;/i&gt;)</td>
<td>65.10</td>
<td>−7.5</td>
<td>−42.4</td>
</tr>
<tr>
<td>4</td>
<td>41.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>−21.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>48.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−3.3&lt;sup&gt;b&lt;/sup&gt;, −2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>−29.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> – scores in kcal/mol, <sup>b</sup> – ligand in position 2 (see Fig.4), <sup>c</sup> – ligand in position 1.
Table 2. MD/LIE calculations\(^a\) of the binding free energies for the complexes of NS5MTase\(_{DV}\) with AdoHcy and ligands 1–5

<table>
<thead>
<tr>
<th>Bound ligand</th>
<th>(\langle V_{\text{vdw}}^{\text{free}} \rangle_{\text{bound}})</th>
<th>(\langle V_{\text{el}}^{\text{free}} \rangle_{\text{bound}})</th>
<th>(\Delta G_{\text{bind}})</th>
<th>RMSD(^b), Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdoHcy</td>
<td>−52.3±0.6</td>
<td>−145.3±0.8</td>
<td>−4.4±0.7</td>
<td>2.4</td>
</tr>
<tr>
<td>1(R)</td>
<td>−56.2±3.1</td>
<td>−227.4±3.4</td>
<td>−11.4±2.4</td>
<td>3.9</td>
</tr>
<tr>
<td>1(S)</td>
<td>−57.1±0.4</td>
<td>−209.5±5.9</td>
<td>−3.5±3.4</td>
<td>7.1</td>
</tr>
<tr>
<td>2(R)</td>
<td>−62.8±0.6</td>
<td>−37.4±0.5</td>
<td>−2.8±0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>2(S)</td>
<td>−66.1±1.6</td>
<td>−41.4±0.1</td>
<td>−5.1±0.4</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>−19.7±0.2</td>
<td>−144.7±3.7</td>
<td>+1.1±2.7</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>−23.0±0.2</td>
<td>−147.7±9.9</td>
<td>−1.7±5.7</td>
<td>4.2</td>
</tr>
</tbody>
</table>

\(^a\) \(\Delta G_{\text{bind}}\) were calculated using Eq. 1 with \(\alpha=0.18\), \(\gamma=0\). The \(\beta\) coefficients in Eq. 1 are 0.45, 0.43, 0.52, 0.52, and 0.47, for ligands 1, 2, 4, 5, and AdoHcy, respectively. \(\langle V_{\text{vdw}}^{\text{free}} \rangle_{\text{free}}\) and \(\langle V_{\text{el}}^{\text{free}} \rangle_{\text{free}}\) of the ligands in water are for 1: −30.4±0.1 and −212.4±0.7, 2: −41.8±0.1 and −39.7±0.1, 4: −9.3±0.1 and −150.4±1.4, 5: −12.5±0.1 and −148.0±1.1, AdoHcy: −20.9±0.1 and −147.8±0.4 kcal/mol, respectively. Statistical uncertainties for the energy terms in the bound and free states were calculated by taking half of the difference between the average of the first and the second half of the productive phase of the 4-ns MD trajectories.

\(^b\) The root mean square deviation (RMSD) of the atom coordinates is provided for the docked and final (after 5 ns) MD structures. RMSDs were calculated for the CA atoms using iterative fit in Swiss-PDBViewer.\(^{[49]}\) The RMSDs for the protein and the ‘ribose-flanking’ loop were calculated for residues 23–260 and 101–116, respectively. Only heavy atoms were included in the calculations of RMSD for the corresponding ligand coordinates. Energies in kcal/mol.
Figure Captions.

Figure 1. Chemical structures of the studied compounds.

Figure 2. Overlayed positions of AdoHcy (experimental) and of inhibitor 1(S) docked by GOLD in the active site of NS5MTaseDV (1R6A pdb). The AdoHcy and GTP are displayed in sticks, 1(S) is displayed in ball-and-sticks.

Figure 3. Overlayed docked positions of inhibitor 1 in the AdoHcy-binding site of NS5MTaseDV. The structures with the best rank of the (R) – and (S) – stereoisomers from GOLD are displayed in ball-and-sticks (CPK) and in sticks (cyan), from Glide – in sticks (yellow and green), and 1(S) from Dock6.4 – in sticks (blue), respectively.

Figure 4. Overlayed docked positions of inhibitor 1(S) and adamantane 3 in the AdoHcy-binding site.

Figure 5. Overlayed docked positions of amantadine 4 and rimantadine 5 in the AdoHcy-binding site. Results from Gold are displayed for 4 in magenta and for 5 in yellow, from Glide for 4 are in cyan and for 5 in red and green, from Dock6.4 for 5 in blue, respectively.

Figure 6. Scatter of the highest-rank docking scores from Gold and Glide for the studied compounds. Note, that in GOLD binding affinity improves in an ascending order of the scores, whereas in Glide – in descending order.

Figure 7. Overlayed experimental (yellow) and MD equilibrated structures for the complexes of NS5MTaseDV with AdoHcy (magenta – simulation of the protein without AdoHcy in the binding site, cyan – in the presence of AdoHcy). Note significant displacement of the β2–β3 loop observed in MD simulation of the protein with an empty, solvent-filled binding site.

Figure 8. Overlayed experimental structure of NS5MTaseDV (yellow) and AdoHcy displayed in sticks (CPK), and the structures for the low-energy protein–ligand complexes with 1(R) and 1(S) after 5-ns MD displayed in green and blue, respectively.