

Affinity of rimantadine enantiomers against influenza A/M2 protein revisited

Antonios Drakopoulos[†], Christina Tzitzoglaki[†], Chulong Ma,[‡] Kathrin Freudenberger[‡], Anja Hoffmann[§],
Yanmei Hu,[‡] Günter Gauglitz[‡], Michaela Schmidtke[§], Jun Wang,^{‡,*} Antonios Kolocouris^{†,*}

[†] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Greece

[‡] Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721, USA

[‡] Institut für Physikalische und Theoretische Chemie, Eberhard-Karls-Universität Tübingen, Germany

[§] Jena University Hospital, Department of Virology and Antiviral Therapy, Hans Knoell Str. 2, D-07745 Jena, Germany

Keywords: rimantadine enantiomers, isothermal titration calorimetry, free energy perturbation, Bennett's acceptance ratio, electrophysiology, synthesis, antiviral assay, membrane protein, influenza M2 pore

1/13/2017

Running title: Binding of rimantadine enantiomers to influenza A/M2 protein

A.D. and C.T. contribute equally.

*Corresponding Author Addresses:

(A.K.) Panepistimioupolis – Zografou, Athens 15771, Greece; Tel: (+301) 210-7274834, Fax: (+301) 210 727 4747; E-Mail: ankol@pharm.uoa.gr

(J.W.) University of Arizona, Tucson, AZ 85721, USA; Tel: 520-626-1366, fax: 520-626-0749, E-Mail: junwang@pharmacy.arizona.edu

Table of Contents

Experimental Part	S3
Ligands	S3-S4
Peptide synthesis, ITC measurements	S5
FEP/MD Simulations	S6,S7
Table S1: Measures from the MD simulations of M2TM _{Udom/72} -ligand in DMPC bilayer	S8
Two-Electrode Voltage Clamp (TEVC) Assay, Antiviral assay: cells and viruses	S9
Abbreviations	S10
Supplementary references	S11-S13

Experimental Part

Ligands

1 was purchased from Merck and **2** from Alfa chemicals (> 99 % purity). Enantiomers of **2** were purchased from Enamine; **2-S** has 95% chemical purity and **2-R** has 90% chemical purity and the enantiomeric excess of each enantiomer sample is 99%; the enantiomeric excess (*ee*) of both **2-R** and **2-S** is 99% (Mosher's method⁴¹); the purity of compound **3** used was > 99%; the purity of compound **3** used was > 99%.

2-(Tricyclo[3.3.1.1^{3,7}]dec-1-yl)-2-propanol (AdMe₂C-OH) 7a. Methylmagnesium iodide was prepared from magnesium turnings (1.99 g, 83.1mmol) and methyl iodide (10.7 g, 75.6mmol) in 40 mL of dry diethyl ether. A solution of 1-adamantanecarbonyl chloride **6** (2.5 g, 12.6 mmol) in 60 mL of dry diethyl ether was added dropwise under Ar atmosphere and stirring. The reaction mixture was heated at gentle reflux for 4h under stirring and Ar atmosphere. The mixture was treated with an equal volume of saturated solution of ammonium chloride under ice-cooling. The organic layer was separated and the aqueous phase was extracted with diethyl ether 2 times. The combined organic phases were washed with water and brine, dried (Na₂SO₄) and evaporated under vacuum to yield a white colored solid residue of 2-(1-adamantyl)-propan-2-ol **7a**. Yield 2.09 g (85.5%); IR (Nujol): $\nu(\text{OH})$ 3400 (br s, O-H) cm⁻¹; ¹H-NMR (400MHz, CDCl₃) δ : 1.12 (s, 6H, 2xCH₃), 1.62-1.69 (m, 12H, 2,4,6,8,9,10-H, adamantane H), 1.99 (br s, 3H, 3,5,7-H, adamantane H); ¹³C-NMR (200 MHz, CDCl₃) δ : 24.34 (CH₃), 28.74 (3,5,7-C, adamantane C), 36.35 (2,8,9-C, adamantane C), 37.22 (4,6,10-C, adamantane C), 38.84 (1-C, adamantane C), 74.88 (C-OH).

2-(Tricyclo[3.3.1.1^{3,7}]dec-1-yl)-2-azido-propane (AdMe₂C-N₃) 9a. The oily 2-(1-adamantyl)-2-azido-propane **8c** was prepared by treatment of the tertiary alcohol **7a** with CH₂Cl₂/NaN₃/TFA. To a stirring mixture of sodium azide (503 mg, 7.74 mmol) and dry dichloromethane (15 mL), trifluoroacetic acid (2.94 g, 25.8mmol) was added at 0 °C. The resulting mixture was stirred for 10 min at 0 °C and a solution of 2-(1-adamantyl)-propan-2-ol **7a** (500 mg, 2.58mmol) in 15mL of dry dichloromethane was added dropwise under ice-cooling. The mixture was stirred vigorously at 0-5 °C for 4 h and additional 24 h at ambient temperature. The mixture was made alkaline by adding NH₃ 12 % (40 mL) and the organic phase was separated and washed with 30 mL of water two times. The aqueous phase was extracted two times with dichloromethane (30 mL) and the combined organic phases were washed with water, brine and dried (Na₂SO₄). Solvent was evaporated in vacuo to afford 2-(1-adamantyl)-propan-2-azide **9a**. Yield: 80%; IR (Nujol): $\nu(\text{N}_3)$ 2098 cm⁻¹

(s); $^1\text{H-NMR}$ (400MHz, CDCl_3) δ : 1.23 (s, 6H, $2\times\text{CH}_3$), 1.60-1.71 (m, 12H, 2,4,6,8,9,10-H, adamantane H), 2.0 (br s, 3H, 3,5,7-H, adamantane H); $^{13}\text{C-NMR}$ (200 MHz, CDCl_3) δ : 20.79 (CH_3), 28.66 (3,5,7-C, adamantane C), 36.56 (2,8,9-C, adamantane C), 37.07 (4,6,10-C, adamantane C), 39.10 (1-C, adamantane C), 67.57 (C-N).

2-(Tricyclo[3.3.1.1^{3,7}]dec-1-yl)-propan-2-amine (AdMe₂C-NH₂) 3. A solution of 2-azido-2(1-adamantyl)-propane **9a** (250 mg, 1.14 mmol) in 10 mL of dry diethyl ether was added dropwise to a solution of lithium aluminum hydride (173 mg, 4.56 mmol) in 10 mL of dry diethyl ether under ice-cooling. The mixture was heated at reflux for 5 h under stirring. Then the mixture was hydrolyzed with a dropwise addition of 2 mL water, 2 mL of sodium hydroxide 10% w/v solution and 6 mL water under stirring and ice-cooling. The mixture was filtered under vacuum and the residue was washed 2 times with diethyl ether. Another 30 mL of diethyl ether was added to the ethereal filtrate and the solution was extracted with 60 mL (2×30 mL) of hydrochloric acid 6% w/v. The aqueous phase was separated and made alkaline through-addition of an excess solid sodium carbonate under ice-cooling. The aqueous phase was extracted two times with 30 mL of dichloromethane. The combined organic extracts were dried (Na_2SO_4) and evaporated under vacuum, to yield a light yellow colored solid residue of 2-(1-adamantyl)-propan-2-amine **3**. Yield: 73%; IR (Film): $\nu(\text{NH}_2)$ 3373 cm^{-1} (s); $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 0.99 (s, 6H, $2\times\text{CH}_3$), 1.60-1.68 (m, 12H, 2,4,6,8,9,10-H, adamantane H), 1.99 (br s, 3H, 3,5,7-H, adamantane H); $^{13}\text{C-NMR}$ (200MHz, CDCl_3) δ : 25.30 (CH_3), 28.87 (3,5,7-C, adamantane C), 36.23 (2,8,9-C, adamantane C), 37.26 (4,6,10-C, adamantane C), 38.12 (1-C, adamantane C), 53.68 (C-N). Anal. Hydrochloride ($\text{C}_{13}\text{H}_{24}\text{NCl}$) (EtOH-Ether).

Peptide synthesis

M2TM peptides corresponding to residues 22-46 of Udorn/72 wildtype sequence of M2 (C-terminally amidated M2TM_{Udorn/72}: SSDPLVVAASIIGILHLILWILDRL) were synthesized by standard Fmoc solid phase peptide synthesis using an aminomethyl polystyrene resin loaded with the amide linker and purified by reverse phase HPLC. A purification procedure previously described¹ and modified was used.² The final peptide purity was 98%.

ITC measurements

Binding affinities of aminoadamantane derivatives (see Scheme 1 in the main text) for M2TM_{Udorn/72} were determined by ITC experiments for M2TM-ligand systems in DPC micelles at pH 8. All measurements were performed in triplicate with a TAM 2277 (TA instrument) at pH 8 and 20 °C in a buffer of 50 mM NaH₂PO₄ and 100 mM NaCl. The peptide and the aminoadamantane derivative were dissolved in a freshly prepared DPC solution with a concentration of 13 mmol L⁻¹. Measurements were conducted using 2 mL of 125 μM peptide (corresponding to 31.25 μM M2TM tetramer). A concentration of 1.1 mM of the ligand was used for the titrant, of which 7.6 μL (equivalent to 8.4 nmol) were dispensed in the peptide/DPC solution with each injection. The time interval between two injections was set to at least 6 minutes. Synthetic M2TM (residues 22-46) was reconstituted at a 1:57 monomer/lipid ratio - which guarantees the quantitative formation of M2TM tetramers (see ref. 13, 14 of the draft) - in DPC micelles at pH 8 by dissolving and sonicating 225 nmol of M2TM with the 57 fold amount of DPC in the aforementioned buffer system. Solutions of ligands **1**, **2-R**, **2-S**, **3** in the buffer were titrated into the calorimetric cell at 20°C. The heat evolved was obtained from the integral of the calorimetric signal. The heat associated with the binding of the ligand to M2TM was obtained by subtracting the heat of dilution from the heat of reaction.^{3,4} Data evaluation was carried out with Digitam for Windows v4.1. Affinity constants were calculated by non-linear regression of the measured heat per injection using Origin 8.0⁵ and are included in Table 1. For the calculation, the concentration of the peptide was kept variable because the M2TM tetramer formation is not complete. Data of three independent measurements was used, whereby all measurements were performed with the same experimental conditions using one stock solution. Data evaluation was done by plotting the measured heat per amount of substance against the molar ratio of titrant to peptide tetramer. The resulting titration curve was fitted using a global fit including the data of three independent measurements.

FEP/MD Simulations

Relative binding free energies for aminoadamantane derivatives (Scheme 1) bound to M2TM_{Udom/72} were computed following the BAR approach⁶ and applying a thermodynamic cycle (Scheme 2). Alchemical free energy calculations were carried out for M2TM-ligand complexes under periodic boundary conditions with Desmond⁷⁻⁹ using the settings and the simulation protocol described above and also in ref. 10, 11. The structures for the simulations of the aminoadamantane compounds in solution were generated and minimized in Maestro¹² using the MMFF94 force field implemented with Macromodel 9.6.^{13,14} The M2TM_{Udom-1} complex structure (PDB ID 2KQT^{15,16}) served as a model structure for M2TM_{Udom} with bound ligands **3-5** (Scheme 1 in main text) in DMPC.

N- and C-termini of the M2TM model systems were capped by acetyl and methylamino groups after applying the protein preparation module of Maestro. The structures of the protein and **1** were saved separately and were used for the subsequent docking calculations. The ligands in their ammonium forms were built by means of Maestro 8.5 and were then minimized by means of Macromodel 9.6 and the MMFFs force field using the conjugate gradient (CG) method and a distance-dependent dielectric constant of 4.0 until a convergence value of 0.0001 kJ Å⁻¹ mol⁻¹ was reached. Docking poses of aminoadamantane derivatives in the M2TM bound state were generated by docking the prepared compound structures into the pore binding site of the M2TM. As a template structure was used the M2TM-**1** complex structure (PDB ID 2KQT^{15,16}). Docking was performed with GOLD 5.2^{17,18} using the ASP scoring function^{19,20}, after deletion of **1**, and considering six water molecules located within the M2TM pore-binding site between the ligand and His37. The option “toggle” was used to let the algorithm decide whether taking into account a water molecule or neglecting it based on an empirical desolvation penalty. The region of interest used by GOLD was defined to contain the atoms that were within ~15 Å of the ligand binding site in the receptor structure. The “allow early termination” command was deactivated. For all the other parameters, GOLD default values were used. Ligands were submitted to 30 genetic algorithm runs. Ten docking poses were produced for each ligand and were visually inspected using the UCSF Chimera package.²¹ The pose with the best score was used in FEP/MD simulations.

The M2TM complexes were embedded in a DMPC lipid bilayer extending 10 Å beyond the solutes. Complex and ligand systems were solvated using the TIP3P²² water model. Na⁺ and Cl⁻ ions were placed in the water phase to neutralize the systems and to reach the experimental salt concentration of 0.150 M NaCl. Membrane creation and system solvation were conducted with the “System Builder” utility of Desmond.⁷⁻⁹

The OPLS 2005 force field²³⁻²⁶ was used to model all protein and ligand interactions, and the TIP3P model²² was used for water. The particle mesh Ewald method (PME)^{27,28} was employed to calculate long-

range electrostatic interactions with a grid spacing of 0.8 Å. Van der Waals and short range electrostatic interactions were smoothly truncated at 9.0 Å. The Nosé-Hoover thermostat²⁹ was utilized to maintain a constant temperature in all simulations, and the Martyna-Tobias-Klein method³⁰ was used to control the pressure. The equations of motion were integrated using the multistep RESPA integrator²⁹ with an inner time step of 2 fs for bonded interactions and non-bonded interactions within a cutoff of 9 Å. An outer time step of 6.0 fs was used for non-bonded interactions beyond the cut-off. Periodic boundary conditions were applied.

Each system was equilibrated in MD simulations with a modification of the default protocol provided in Desmond, which consists of a series of restrained minimizations and molecular dynamics simulations designed to relax the system, while not deviating substantially from the initial coordinates. First, two rounds of steepest descent minimization were performed with a maximum of 2000 steps with harmonic restraints of 50 kcal mol⁻¹ Å⁻² applied on all solute atoms, followed by 10,000 steps of minimization without restraints. A series of four MD simulations was performed. The first simulation was run for 12 ps at a temperature of 10 K in the NVT (constant number of particles, volume, and temperature) ensemble with solute heavy atoms restrained with a force constant of 50 kcal mol⁻¹ Å⁻², followed by an identical simulation in the NPT ensemble. The temperature was then raised during a 25 ps simulation to 310 K in the NPT ensemble with the force constant retained. The temperature of 310 K was used in the MD simulations in order to ensure that the membrane state is above the melting temperature state of 297 K for DMPC lipids.³¹ Then an unrestrained NPT production simulation at 310³¹ followed saving snapshots in intervals of 4 ps.

Production simulations at each λ value were run for 4 ns or 6 ns for compounds without and with a cyclic group, respectively. A λ schedule comprising 12 windows was used (see Table S1 in sup. ref. 10). The complex systems were stable in the alchemical free energy simulations as indicated by an RMSD of the protein heavy atoms ≤ 1.5 Å such that the sampled structures could be used for computing relative binding free energies. Free energy differences ΔG were calculated by the BAR method⁶ and checked for convergence by computing ΔG based on increasing time intervals of the alchemical free energy simulations (see Table S1 in sup. ref. 10). Errors in the computed relative binding free energies were estimated using block bootstrapping³² as described in sup. ref. 10 .

For structural analyses, snapshots of the different systems were created with VMD³³ or Maestro.¹² Trajectories were analyzed with Maestro, Gromacs,^{34,35} and VMD.³³ For the calculation of hydrogen bonds, a cut-off angle of 30° deviation from 180° between the donor-hydrogen-acceptor atoms and a cut-off distance of 3.5 Å between the donor and acceptor atoms were applied.

Table S1. Structural and dynamic measures from FEP/MD trajectories of M2TM_{Udom/72}-ligand complexes in DMPC bilayer.

Ligand ¹	RMSD(Ca) ²	Angle C-N vector ³	Angle C-C vector ⁴	V27-Ad ⁵	A30-Ad ⁵	G34-Ad ⁶	G34 Ca-lig.CH ₃ ⁷	G34 CH ₃ -lig.CH ₃ ⁸	H-bonds with water ⁹	RMSF ligand ¹⁰
1	1.5 ± 0.1	11.2 ± 5.9	-	4.2 ± 0.3	1.1 ± 0.3	7.0 ± 1.5	-	-	2.7 ± 0.5	0.1
2-R	1.5 ± 0.2	44.7 ± 7.6	13.7 ± 6.1	3.9 ± 0.3	1.4 ± 0.3	5.7 ± 0.3	2.9 ± 0.4	3.9 ± 0.3	2.6 ± 0.6	0.3
2-S	1.1 ± 0.2	55.7 ± 6.1	13.9 ± 7.0	4.2 ± 0.3	1.0 ± 0.3	4.8 ± 0.3	3.2 ± 0.3	3.5 ± 0.3	2.8 ± 0.4	0.3
3	1.4 ± 0.2	51.5 ± 4.9	5.3 ± 6.6	4.4 ± 0.3	0.8 ± 0.3	5.0 ± 0.4	2.2 ± 0.3	3.8 ± 0.3	2.9 ± 0.3	0.2

¹ See Scheme 1; values taken from ref. 27 of the draft; measures for **1** were added for comparison reasons.

² Maximum root-mean-square deviation (RMSD) for Ca atoms of M2TM relative to the initial structure (PDB entry: 2KQT) after root-mean-square fitting of Ca atoms of M2TM; in Å.

³ Angle between the vector along the bond from the carbon atom of the adamantane core to the ligand nitrogen atom and the normal of the membrane; in degree.

⁴ Angle between the vector along the bond from the carbon atom of the adamantane core to the carbon bridge of rimantadine analogue; in degree.

⁵ Mean distance between center of mass of A30 and centers of mass of adamantane calculated using Gromacs tools; in Å.

⁶ Mean distance between center of mass of G34 and centers of mass of adamantane calculated using Gromacs tools; in Å.

⁷ Mean distance between center of mass of rimantadine methyl and G34 Ca calculated using Gromacs tools; in Å.

⁸ Mean distance between center of mass of rimantadine methyl and A30 methyl calculated using Gromacs tools; in Å.

⁹ Mean number of H-bonds between ligand's ammonium group and waters.

¹⁰ Root-mean-square fluctuation of a ligand after fitting of the ligand to the average structure considering all ligand atoms; in Å.

Two-Electrode Voltage Clamp (TEVC) Assay

The inhibitors were tested via a TEVC assay using *X. laevis* frog oocytes microinjected with RNA expressing the M2 protein as in a previous report.³⁶ The blocking effect of the aminoadamantane derivatives against M2 was investigated with electrophysiology experiments using M2_{Udom/72}. Because WSN/33-M2-N31S which will be used to compare antiviral potencies using a whole cell assay, M2_{WSN-N31S} was generated and studied in parallel. The potency of the inhibitors was expressed as the inhibition percentage of the A/M2 current observed after 2 min and 5 min of incubation with 100 μ M of compound.

Anti-viral assay: cells and viruses

Madin-Darby canine kidney (MDCK) cells (Cat.no. RIE 328, Friedrich-Loeffler Institute, Riems, Germany) were propagated as monolayer in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and 2 mM L-glutamine. Amantadine-sensitive Udom/72, and WSN/33-M2-N31S³⁷ were used in this study. For the generation of WSN/33-M2-N31S³⁷ the plasmid pHW187-M2-N31 was altered by site-directed mutagenesis PCR and afterwards used as part of a plasmid set for virus recovery.³⁸ Both WSN/33-variants were propagated on MDCK cells in serum-free EMEM supplemented with 2 mM L-glutamine, 2 μ g/mL trypsin, and 0.1% sodium bicarbonate (test medium). Virus containing supernatant was harvested after about 48 h of incubation at 37 °C when cytopathic effect became microscopically visible. Aliquots were stored at -80 °C until use. The M2 gene identity was verified by sequencing.

CPE inhibition studies were performed on two-day-old confluent monolayers of MDCK cells grown in 96-well plates as published.³⁹ In CPE inhibition assay, 50 μ L of at least six serial half-log dilutions of compound in test medium and a constant multiplicity of infection of test virus (0.03 for WSN/33-M2-N31S) in a volume of 50 μ L of the test medium were added to cells. Then, plates were incubated at 37 °C with 5% CO₂ for 48 h. Crystal violet staining or neutral red staining and optical density determination were performed as described before.^{39,40} After log transformation of compound concentrations, linear regression was used to determine the 50% inhibitory concentration (IC₅₀). At least three independent assays were conducted to calculate the mean IC₅₀ and their standard deviations.

Abbreviations

M2TM, residues 22-46 of M2 protein comprising the transmembrane domain; CPE assay, cytopathic effect assay; DMPC, 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine; DPC, Dodecylphosphocholine; FEP, Free Energy Perturbation; MD, Molecular Dynamics; PME method, Particle Mesh Ewald method; ITC, Isothermal Titration Calorimetry; TEVC assay, two-electrode voltage clamp assay; BAR, Bennett acceptance ratio; PDB, Protein data bank; RMSD, root-mean-square deviation; RMSF, Root-mean-square fluctuation; HPLC, High Performance Liquid Chromatography; MDCK cells, Madin-Darby canine kidney cells; IC₅₀, 50% inhibitory concentration.

Supplementary References

1. Hansen, R. K.; Broadhurst, R. W.; Skelton, P. C.; Arkin, I. T. Hydrogen/deuterium exchange of hydrophobic peptides in model membranes by electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectr.* **2002**, *13*, 1376-1387.
2. Kolocouris, A.; Zikos, C.; Broadhurst, R. W. ¹⁹F NMR detection of the complex between amantadine and the receptor portion of the influenza A M2 ion channel in DPC micelles. *Bioorg. Med. Chem. Letters* **2007**, *17*, 3947-3952.
3. Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. N. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **1989**, *179*, 131-137.
4. Doyle, M. L. Characterization of binding interactions by isothermal titration calorimetry. *Current opinion in biotechnology* **1997**, *8*, 31-35.
5. *Origin 8.1G SRI*, v8.1.13.88; OriginLab Corporation, Northampton, MA, USA: **2009**.
6. Bennett, C.H. Efficient Estimation of Free Energy Differences from Monte Carlo Data. *J. Comp. Phys.* **1976**, *22*, 245-268.
7. *Desmond Molecular Dynamics System*, version 3.0; D. E. Shaw Research: New York, NY, **2012**.
8. Bowers, K. J.; Chow, E.; Xu, H.; Dror, R. O.; Eastwood, M. P.; Gregersen, B. A.; Klepeis, J. L.; Kolosvary, I.; Moraes, M. A.; Sacerdoti, F. D.; Salmon, J. K.; Shan, Y.; Shaw, D. E. Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters. In Proceedings of the ACM/IEEE Conference on Supercomputing (SC06), Tampa, Florida, 2006; Tampa, Florida, 2006.
9. *Maestro-Desmond Interoperability Tools*, version 3.1; Schrodinger: New York, NY, **2012**.
10. Gkeka, P.; Eleftheratos, S.; Kolocouris, A.; Cournia, Z. Free energy calculations reveal the origin of binding preference for aminoadamantane blockers of influenza A/M2TM pore. *J. Chem. Theory Comput.* **2013**, *9*, 1272-1281.
11. Ioannidis, H.; Drakopoulos, A.; Tzitzoglaki, C.; Homeyer, N.; Kolarov, F.; Gkeka, P.; Freudenberger, K.; Liolios, C.; Gauglitz, G.; Cournia, Z.; Gohlke, H.; Kolocouris, A. Alchemical Free Energy Calculations and Isothermal Titration Calorimetry Measurements of Aminoadamantanes Bound to the Closed State of Influenza A/M2TM. *J. Chem. Info. Model.* **2016**, **2016**, *56*, 862-876.
12. *Maestro*, version 8.5; Schrodinger, Inc.: New York, NY, **2008**.
13. Halgren, T. A. Merck molecular force field. V. Extension of MMFF94 Using Experimental Data, Additional Computational Data, and Empirical Rules. *J. Comput. Chem.* **1996**, *17*, 616-641.
14. Halgren, T. A. MMFF VII. Characterization of MMFF94, MMFF94s, and Other Widely Available Force fields for Conformational Energies and for Intermolecular-Interaction Energies and Geometries. *J. Comp. Chem.* **1999**, *20*, 730-748.

15. Hu, J.; Fu, R.; Cross, T. A. The chemical and Dynamical Influence of the Anti-Viral Drug Amantadine on the M2 Proton Channel Transmembrane Domain. *Biophys. J.* **2007**, *93*, 276-283.
16. Cady, S. D.; Schmidt-Rohr, K.; Wang, J.; Soto, C. S.; Degrado, W. F.; Hong, M., Structure of the Amantadine Binding Site of Influenza M2 Proton Channels in Lipid Bilayers. *Nature* **2010**, *463*, 689-92.
17. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and Validation of a Genetic Algorithm for Flexible Docking. *J. Mol. Biol.* **1997**, *267*, 727-748.
18. Verdonk, M. L.; Chessari, G.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Nissink, J. W.; Taylor, R. D.; Taylor, R. Modeling Water Molecules in Protein-Ligand Docking Using GOLD. *J. Med. Chem.* **2005**, *48*, 6504-6515.
19. Korb, O.; Stützle, T.; Exner, T. E. Empirical Scoring Functions for Advanced Protein-Ligand Docking with PLANTS. *J. Chem. Inf. Model.* **2009**, *49*, 84-96.
20. Mooij, W. T.; Verdonk, M. L. General and Targeted Statistical Potentials for Protein-Ligand Interactions. *Proteins: Struct., Funct., Bioinf.* **2005**, *61*, 272-287.
21. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera--A Visualization System for Exploratory Research and Analysis. *J. Comput. Chem.* **2004**, *25*, 1605-1612.
22. Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* **1983**, *79*, 926-935.
23. Jorgensen, W. L.; Maxwell, D. S.; Tirado-Rives, J. Development and Testing of the OPLS All-Atom Force Field on Conformational Energetics and Properties of Organic Liquids. *J. Am. Chem. Soc.* **1996**, *118*, 11225-11236.
24. Rizzo, R. C.; Jorgensen, W. L. OPLS All-Atom Model for Amines: Resolution of the Amine Hydration Problem. *J. Am. Chem. Soc.* **1999**, *121*, 4827-4836.
25. Kaminski, G.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. L. Evaluation and Reparametrization of the OPLS-AA Force Field for Proteins via Comparison with Accurate Quantum Chemical Calculations on Peptides. *J. Phys. Chem. B* **2001**, *105*, 6474-6487.
26. Shivakumar, D.; Williams, J.; Wu, Y.; Damm, W.; Shelley, J.; Sherman, W. Prediction of Absolute Solvation Free Energies using Molecular Dynamics Free Energy Perturbation and the OPLS Force Field. *J. Chem. Theory Comput.* **2010**, *6*, 1509-1519.
27. Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald: An N log(N) Method for Ewald Sums in Large Systems. *J. Chem. Phys.* **1993**, *98*, 10089-10092.
28. Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. A Smooth Particle Mesh Ewald Method. *J. Chem. Phys.* **1995**, *103*, 8577-8593.
29. Martyna, G. J. T., D. J.; Klein, M. L. Constant-Pressure Molecular-Dynamics Algorithms. *J. Chem. Phys.* **1994**, *101*, 4177-4189.

30. Humphreys, D. D.; Friesner, R. A.; Berne, B. J. A Multiple-Time-Step Molecular-Dynamics Algorithm for Macromolecules. *J. Phys. Chem.* **1994**, *98*, 6885-6892.
31. Koynova, R.; Caffrey, M. Phases and Phase Transitions of the Phosphatidylcholines. *Biochim. Biophys. Acta* **1998**, *1376*, 91-145.
32. Boyce, S. E.; Mobley, D. L.; Rocklin, G. J.; Graves, A. P.; Dill, K. A.; Shoichet, B. K. Predicting Ligand Binding Affinity with Alchemical Free Energy Methods in a Polar Model Binding Site. *J. Mol. Biol.* **2009**, *394*, 747-763.
33. Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual Molecular Dynamics. *J. Mol. Graph.* **1996**, *14*, 33-38.
34. Berendsen, H. J. C.; van der Spoel, D.; van Drunen, R. GROMACS: A Message-Passing Parallel Molecular Dynamics Implementation. *Comput. Phys. Commun.* **1995**, *91*, 43-56.
35. Hess, B.; Kutzner, C.; van der Spoel, D.; Lindahl, E. GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. *J. Chem. Theory Comput.* **2008**, *4*, 435-447.
36. Wang, J.; Wu, Y.; Ma, C.; Fiorin, G.; Wang, J.; Pinto, L. H.; Lamb, R. A.; Klein, M. L.; Degrado, W. F. Structure and inhibition of the drug-resistant S31N mutant of the M2 ion channel of influenza A virus. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 1315-1320.
37. Schade, D.; Kotthaus, J.; Riebling, L.; Kotthaus, J.; Müller-Fielitz, H.; Raasch, W.; Hoffmann, A.; Schmidtke, M.; Clement, B.; Zanamivir Amidoxime- and N-Hydroxyguanidine-Based Prodrug Approaches to Tackle Poor Oral Bioavailability. *J. Pharm. Sci.* **2015**, *104*, 3208-3219.
38. Hoffmann, E.; Neumann, G.; Kawaoka, Y.; Hobom, G.; Webster, R. G. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 6108-6113.
39. Schmidtke, M.; Schnittler, U.; Jahn, B.; Dahse, H.-M.; Stelzner, A. A rapid assay for evaluation of antiviral activity against coxsackie virus B3, influenza virus A, and herpes simplex virus type 1. *J. Virol. Methods* **2001**, *95*, 133-143.
40. Torres, E.; Duque, M. D.; Vanderlinden, E.; Ma, C.; Pinto, L. H.; Camps, P.; Froeyen, M.; Vázquez, S.; Naesens, L. Role of the viral hemagglutinin in the anti-influenza virus activity of newly synthesized polycyclic amine compounds. *Antiviral Res.* **2013**, *99*, 281-291.
41. Dale JA, Mosher HS. Nuclear magnetic resonance enantiomer reagents. Configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, O-methylmandelate, and a methoxy-a-trifluoromethylphenylacetate (MTPA) esters. *J. Am. Chem. Soc.* **1973**, *95*, 512-519; Ohtani I, Kusumi T, Kashman Y, Kakisawa H. High-field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. *J. Am. Chem. Soc.* **1991**, *113*, 4092-4096.