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Ensemble-based virtual screening for cannabinoid-like potentiators of the human glycine receptor $\alpha 1$ for the treatment of pain

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Abstract

The human glycine receptors (hGlyRs) are chloride-selective ion channels that mediate inhibitory neurotransmission in the brain stem and spinal cord. They are also targets for compounds of potential use in analgesic therapies. Here we develop a strategy to discover analgesic drugs via structure-based virtual screening based on the recently published NMR structure of the hGlyR- $\alpha 1$ transmembrane domain (PDB ID: 2M6I) and the critical role of residue S296 in hGlyR- $\alpha 1$ potentiation by ⁹-tetrahydrocannabinol (THC). We screened 1549 FDA-approved drugs in the DrugBank database on an ensemble of 180 hGlyR- $\alpha 1$ structures generated from molecular dynamics simulations of the NMR structure of the hGlyR- $\alpha 1$ transmembrane domain in different lipid environments. Thirteen hit compounds from the screening were selected for functional validation in *Xenopus laevis* oocytes expressing hGlyR- $\alpha 1$. Only one compound showed no potentiation effects; seven potentiated hGlyR- $\alpha 1$ at a level greater than THC at 1 μ M. Our virtual screening protocol is generally applicable to drug targets with lipid-facing binding sites.

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ANCILLIARY INFORMATION

Supporting Information

Tables S1 and S2 provide the top 200 screened compounds in the presence and absence of lipids, respectively. Table S3 lists the patent publication numbers for compounds selected for *in vitro* functional validation and other leading compounds in the virtual screen. Figure S1 shows the RMSD convergence of MD simulations. Figures S2-S4 show the lowest energy docking poses for all experimentally tested drugs. Figure S5 shows no functional modulation of the first tier drugs on $\alpha 7$ nAChR. Figure S6 provides the ¹H NMR spectrum of pimozone from Sigma-Aldrich. This material is available free of charge via the Internet at <http://pubs.acs.org>.

PDB ID Codes: 2M6I for the NMR-determined structure of the open-channel hGlyR- $\alpha 1$ TMD.

Keywords

Glycine receptors; pain; analgesia; positive allosteric modulators; pLGICs; drug repurposing; ensemble-based virtual screening; structure-based virtual screening

INTRODUCTION

Glycine receptors (GlyRs) belong to the Cys-loop receptor superfamily that also includes nicotinic acetylcholine receptors (nAChRs), 5-hydroxytryptamine type 3 (5-HT₃) receptors, and type A γ -amino-butyric acid receptors (GABA_ARs). These receptors are pentameric ligand-gated ion channels (pLGICs). GlyRs are chloride-selective channels that mediate inhibitory neurotransmission in the central nervous system and regulate a variety of behaviors, including neuromotor activity, pain sensation, muscle relaxation, and anxiety.^{1,2,3} Thus, they are promising targets for therapeutic interventions.

Structurally, each GlyR subunit consists of an extracellular domain (ECD), a transmembrane domain (TMD) with four transmembrane helices (TM1 to TM4), and an intracellular domain (ICD). Agonist binding to the ECD transiently opens the channel allowing Cl⁻ ions to pass through the pore formed by the TM2 helices.^{4,5} High-resolution crystal structures for GlyRs are currently unavailable. Recently, we determined the structure for the human GlyR α 1-subunit (hGlyR- α 1) TMD using nuclear magnetic resonance (NMR) spectroscopy and electron micrographs.⁶ The hGlyR- α 1 TMD spontaneously forms channels permeable to chloride ions.⁶ The NMR structure of the hGlyR- α 1 TMD provides a valuable structural basis for screening and designing drugs that act on the hGlyR- α 1 TMD.

Most of the principal effects of Δ^9 -tetrahydrocannabinol (THC), the primary psychoactive component in cannabis, are mediated through the activation of the cannabinoid type 1 (CB1) receptor. THC has serious negative effects on human health, including motor impairment and psychosis, but it has been found to provide relief in alleviating chronic pain.⁷ Evidence indicates that some effects of THC are independent of CB1 receptors.⁸ In particular, the analgesic effects of THC are mediated through THC potentiation of hGlyR.^{9,10} Cannabinoid-induced analgesia is absent in mice lacking GlyRs, but not in those lacking CB1 and CB2 receptors.¹⁰ THC potentiates GlyRs with high affinity.⁹ Residue S296 in TM3 of hGlyR- α 1 and the equivalent residue S303 of hGlyR- α 3 have been identified as critical for THC potentiation of hGlyR- α 1 and hGlyR- α 3, respectively.¹⁰ The S296A or S303A mutation abolished THC potentiation of hGlyR- α 1 or hGlyR- α 3, respectively.¹⁰ THC binding to the S296 site of hGlyR- α 1 was determined using high resolution NMR.¹⁰ Thus, the THC binding site at residue S296 of hGlyR- α 1 offers an ideal structural template for virtual screening to identify novel positive modulators acting at the same binding site as THC but without exhibiting its negative effects.

It is well documented that membrane composition affects the activity of GlyRs and other pLGICs.^{11,12,13} Particularly, cholesterol has been shown to affect channel functions.^{14,15} In addition, the NMR structure of the hGlyR- α 1 TMD shows that residue S296 faces away from the intra-subunit helical bundle (**Figure 1**) and is exposed to lipids. The conformations of lipids around the site may affect the binding of THC and other modulators. Therefore, the

potential involvement of lipids and/or cholesterol in the binding of THC and other modulators should be considered in virtual screening.

Virtual screening allows for fast and economical evaluation of compounds in a large chemical library, but the accuracy of virtual screening strongly depends on conformations of the target receptor. Most virtual screening programs allow ligands to move flexibly around a rigid receptor. In reality, receptor motion often plays a critical role in the dynamic process of drug binding. A dynamic ensemble of receptor structures can reveal binding pockets that could not be found in a single static structure. More accurate results can be obtained by performing virtual screening on an ensemble of receptor structures generated through molecular dynamics (MD) simulations instead of a single structure.^{16,17}

In this study, we performed virtual screening with 1549 FDA-approved drugs in the DrugBank database at the S296 site in the hGlyR- α 1 TMD. The promiscuous nature of small molecules and the interconnectedness of cell signaling pathways offers the potential for drug repurposing, which enjoys the benefit of readily available pharmacokinetic and toxicological data obtained in previous clinical trials. Drug repurposing also significantly reduces the risks and timeline associated with clinical development. To increase the success of our virtual screening, we generated an ensemble of protein conformations through MD simulations with different lipid compositions and screened drugs in the presence and absence of lipids. The study served two purposes: 1) identify approved drugs that have potential analgesic effects by potentiating hGlyRs and 2) establish a protocol for screening lipid-exposed druggable sites. More than a dozen compounds identified from the virtual screening were validated experimentally for their abilities to potentiate glycine-activated currents in *Xenopus laevis* oocytes expressing hGlyR- α 1. The study has not only revealed drugs acting on the hGlyR- α 1 TMD with a mechanism similar to that of THC, but also paved a path for discovering new analgesic agents.

RESULTS

Effects of Lipid Composition on Conformations of hGlyR- α 1 TMD

We performed MD simulations of the NMR structure of the hGlyR- α 1 TMD in three systems with different lipid environments (**Figure 2a-c**): (1) pure 1-palmitoyl-2-oleoylphosphatidylcholine (POPC); (2) POPC/cholesterol with five cholesterol molecules initially within 4 Å of TM1 to TM3; and (3) POPC/cholesterol with eight cholesterol molecules initially within 4 Å of TM1 to TM3. Each system was subjected to three parallel 50-ns simulations. In all simulations, the RMSD of the hGlyR- α 1 TMD converged or stabilized after 10-20 ns (**Supporting Information Figure S1**). To examine whether the lipid composition affected the structure of the hGlyR- α 1 TMD, we calculated the pairwise RMSD values of the protein over the course of the MD simulations for each system. From each of the nine 50-ns trajectories, frames were extracted every 100 ps for analysis. 1500 snapshots from simulations in pure POPC and 3000 snapshots from simulations in POPC/cholesterol were collected. The presence of cholesterol did not greatly affect the tertiary structure of individual subunits. The mean pairwise RMSD for individual hGlyR- α 1 TMD monomers was 2.2 Å. In contrast, the mean pairwise RMSD calculated on pentamer structures was 3.5 Å, indicating more significant variations in quaternary structures. **Figure**

2d shows the pairwise RMSDs calculated from pentamers simulated in the absence and presence of cholesterol molecules. Snapshots were classified by the number of cholesterol molecules within 4 Å of TM1 to TM3. Note that during the simulation, the number of cholesterol close to the hGlyR- α 1 TMD varied due to the migration of cholesterol molecules. Structures with nine cholesterol molecules within 4 Å of TM1 to TM3 show similar mean pairwise RMSDs (2.5 Å) to structures from pure POPC simulations, but this is likely due to the much smaller number of structures in this cholesterol group (33 structures) than in the POPC group (1500 structures). More importantly, structures within the same group exhibit less structural variations than those belonging to groups with different numbers of cholesterols. These data demonstrate that MD simulations in the presence of different lipid compositions generate distinct ensembles of protein structures. The effect of cholesterol on the quaternary structure of hGlyR- α 1 TMD was also evidenced by the change in the pore radius (**Figure 2e**). Without cholesterol, the average minimum pore radius is 3.1 ± 0.3 Å, whereas the average minimum pore radius from simulations with cholesterol is 1.8 ± 0.4 Å. The penetration of cholesterol molecules into the intra-subunit space between TM3 and TM4 may have caused the conformational change of the channel and reduced the pore radius.

Virtual Screening on an Ensemble of hGlyR- α 1 TMD Structures

Representative protein conformations were generated through structural RMSD clustering. We examined the major structural clusters from each simulation using the Quality Threshold Clustering Algorithm,¹⁸ which provides clusters within a given size threshold. This algorithm optimizes the intra- and inter-cluster RMSD. Through this process, we found that most of the simulations contained four major structural clusters. For a given cluster, the pentamer with the minimum average RMSD between itself and all other structures was selected as a representative structure. Each representative pentameric structure was divided into five screening units (see the Experimental Section), each unit comprising two adjacent subunits in order to include potential intra- and inter-subunit binding sites in virtual screening. This yielded a total of 180 screening units for docking calculations. In order to consider direct lipid involvement in the S296 binding site, we screened all compounds against the same 180 structures in the presence and absence of the surrounding lipids (POPC or POPC/cholesterol) from the MD simulations. The lipid molecules may help to form the binding pocket, but they could also potentially block the binding site. Nevertheless, the inclusion of a variety of conformations of lipid molecules is biologically relevant. We reasoned that compounds scoring well in both the absence and presence of lipids might increase the success of *in vitro* functional validation.

Virtual screening in the presence of lipids showed that most of the hGlyR- α 1 TMD structures bound no more than 25 hit compounds, defined as those with predicted binding disassociation constants (K_d) < 1 μ M (**Figure 3a-c**). However, when lipids were excluded from the virtual screening, the number of hit compounds on individual structures over all three systems increased significantly ($p < 0.001$ in the two-sample Kolmogorov-Smirnov test) (**Figure 3d**). The data suggest a higher degree of selectivity when the screening is in the presence of lipids.

For a given hGlyR- α 1 TMD structure, we examined how lipid molecules affected drug docking to the S296 site. The presence of lipids effectively reduced the number of hit compounds. Moreover, the lowest predicted binding energies of each compound in the presence and absence of lipids were not necessarily obtained on the same hGlyR- α 1 TMD structures, demonstrating the necessity of screening with an ensemble of structures. For the same hGlyR- α 1 TMD structures, the presence of lipids can enhance or reduce the binding affinity of certain drugs. **Figure 4** shows examples of both cases. For a given structure, we observed that the predicted binding affinity of adapalene improved when screened with lipids (**Figure 4a-b**). We also observed the opposite situation with a different drug: lipids blocked telmisartan binding to residue S296. The predicted K_d of telmisartan on this hGlyR- α 1 TMD significantly decreased in the absence of lipids (**Figure 4c-d**). Additional images showing docked conformations of the screened compounds as well as THC in complex with the hGlyR- α 1 TMD are shown in **Supporting Information Figures S2-S4**.

Rank and Selection of Screened Compounds

The 1549 FDA-approved screened compounds were separately ranked, with and without lipids, based on their predicted binding energies -8.18 kcal/mol (equivalent to $K_d = 1$ μ M) summed over the 180 screening units. The top 200 compounds from the virtual screenings in the presence or absence of lipids are provided in **Supporting Information Tables S1-2**. Among the top 25 hits from the screenings with and without lipids, there were 14 overlapping compounds (**Table 1**). These 14 compounds were considered for further experimental testing.

We noticed that cholesterol had profound effects on the screening results. Of the 14 top ranking compounds, six would not be ranked high if screened only with POPC but without cholesterol. Since cholesterol is a component of native cell membranes, the inclusion of cholesterol in the screening is biologically relevant. Some of these top 14 compounds have been previously suggested to interact with receptors other than hGlyRs, such as the drug pimozone with the dopamine D2 receptors in the central nervous system.¹⁹ None of these compounds, however, have been previously indicated to modulate hGlyRs. All 14 compounds were filtered for Pan Assay Interference Compounds (PAINS)²⁰ and none were found to contain sub-structural features that would label them as “frequent hitters” in high throughput screens. In addition, none of these compounds are structurally similar to THC, as measured by low Tanimoto coefficients.²¹ A simple 2D structural similarity search would not have yielded the same leading compounds.

Sulindac and mefloquine, two compounds that ranked below the top 25 but higher than THC, were also selected for *in vitro* functional analysis (**Table 2**). Sulindac is a nonsteroidal anti-inflammatory agent with analgesic and antipyretic effects, but its mechanism of action was unknown prior to this study.²² Mefloquine is an anti-malarial agent that binds to adenosine receptors in the central nervous system.²³ Both of these compounds have a hydroxyl group, which was shown to be critical for THC binding to hGlyR- α 1.¹⁰ However, neither of them are structurally similar to THC, as measured by low Tanimoto coefficients.²¹ PNU-120596 (**Table 2**), a known highly specific positive allosteric modulator for the α 7nAChR,²⁴ was chosen as a negative control in functional assays. This negative

control was also included in the virtual screening and it was predicted to bind the hGlyR- α 1 TMD with low affinity. For all 180 hGlyR- α 1 TMD structures screened in the presence of surrounding lipids, no single PNU-120596 docked with $K_d = 1 \mu\text{M}$. When lipids were excluded from the screening, there was only one hGlyR- α 1 TMD structure that PNU-120596 bound to with $K_d = 1 \mu\text{M}$.

Functional Validation

After a thorough search, we were able to purchase 11 of the 14 top ranked compounds shown in **Table 1** for functional validation. Antrafenine, tasosartan and nandrolone phenpropionate were not commercially available at the time of our experiments. These 11 top-ranked compounds and two additional compounds (sulindac and mefloquine) from **Table 2**, along with the positive control THC and negative control PNU-120596, were tested on oocytes expressing hGlyR- α 1. Representative functional traces in **Figure 5a** demonstrate that pimozone has a much greater potentiation effect on the channel current than THC. In contrast, conivaptan shows no potentiation for hGlyR- α 1. Among all thirteen tested compounds, seven (first tier) were found to potentiate the glycine receptor better than THC at $1 \mu\text{M}$ (**Figure 5b**), the concentration used as a threshold in the virtual screening. Another five (second tier) also significantly potentiated the glycine receptor, but less effectively than THC (**Figure 5c**). Conivaptan and the negative control, PNU-120596, did not show potentiation up to the maximum tested concentration, $10 \mu\text{M}$ (**Figure 5c**). The measured potentiation and EC_{50} values of all tested compounds are summarized in **Table 3**. The compounds from the first tier give a hit rate of 54%, but the combined results from all tested compounds gives an overall hit rate of 92%.

To explore whether these drugs also act as modulators on other members of the Cys-loop receptor superfamily, we additionally tested each of the seven first tier compounds in **Figure 5b** on oocytes expressing α 7nAChR and found that none of them showed significant modulation of acetylcholine-activated channel currents (**Supporting Figure S5**). This is consistent with the previous finding that THC has little effect on α 7nAChR function, though some endogenous cannabinoids have been found to inhibit α 7nAChR.²⁵

The measured potentiation of the top ranked compounds shows a moderate correlation (Pearson correlation coefficient, $r = -0.72$) with their total binding energies predicted by the virtual screening in the presence of lipids (**Figure 6a**). In general, compounds with a higher rank in the virtual screening in the presence of lipids provided a higher degree of potentiation of the hGlyR- α 1 current. However, the ranking generated from the virtual screening in the absence of lipids showed no correlation ($r = -0.052$) with the experimental potentiation results (**Figure 6b**). The experimental data further highlights the importance of including lipids in the virtual screening.

DISCUSSION AND CONCLUSIONS

Using an ensemble of structures in the presence and absence of lipids for virtual screening is effective in identifying drugs that are positive allosteric modulators of hGlyRs. Ten of the fourteen top-ranked compounds from the virtual screening (**Table 1**), as well as sulindac and mefloquine, had patent applications related to the treatment of pain (**Supporting**

Information Table S3). Such a high hit rate for analgesia-related compounds to the hGlyR- $\alpha 1$ S296 site is unlikely a coincidence, even though prior to this study, these top-hit compounds were unknown to potentiate hGlyRs. Our results suggest that potentiation of hGlyR by these compounds may provide similar analgesic effects to the action on hGlyRs as exhibited by THC.¹⁰ These findings support the relevance of hGlyRs as a therapeutic target in pain pathways.

Virtual screening in the presence of lipids produced a subset of structurally diverse hit compounds distinct from those obtained in the absence of lipids. The presence of lipids affected the screening results at two stages: the generation of protein structures with the desired lipid composition over the course of MD simulations (**Figure 2a–c**) and virtual screening with lipids as a part of the binding pocket. The hGlyR- $\alpha 1$ TMD NMR structure provided a reliable starting point for generating an ensemble of protein conformations in both POPC and POPC/cholesterol environments. Using a large ensemble of conformations for virtual screening instead of a single conformer reproduces the dynamic nature of channel proteins and reduces bias in the screening process. The presence of a varied lipid composition during MD simulations increased the diversity of the ensemble (**Figure 2**). Limiting the number of protein structures used in the virtual screening certainly saves computation time. However, the “best” structure for one pre-selected compound is not necessarily the most suitable structure for other compounds in the screened library. In fact, among the 14 compounds shown in **Table 1**, only six of them share the “best” docking structure with THC. Therefore, the inclusion of an ensemble of protein conformations in the virtual screening is highly recommended if computational resources permit.

For an interior drug-binding site, it would be sufficient to directly use the ensemble of protein structures for screening without including lipids as part of the binding pocket. For the hGlyR- $\alpha 1$ S296 site or any similar lipid-facing binding sites, however, lipid molecules may help form the binding pocket and should be considered for their influence on drug binding. Molecular volumes for the top hits in **Tables 1-2** varied from below 300 to close to 500 Å³. Such flexibility in the molecular volume of the binding compound is likely a characteristic shared by binding sites at the protein-lipid interface in transmembrane proteins. Given the wide range of molecular volumes, it is not surprising that the presence of lipids during screening influenced the results. As shown in **Figure 4 and Supporting Figures S3-4**, lipids can have a profound influence on the predicted binding affinity by forming additional stabilizing contacts at the binding site. In this way, lipids help accommodate compounds of widely varied molecular volumes at lipid-exposed binding sites. The vast majority of the compounds identified from our screening in the presence of lipids overlap with the compounds identified from the screening without lipids, but the total number of hit compounds from screening in the presence of lipids is less. It appears that screening in the presence of lipids can effectively filter the results. Furthermore, the results in **Figure 6** clearly demonstrate the importance of including lipids in the virtual screening. Thus, for lipid-facing binding sites, including the surrounding lipid molecules in the virtual screening is highly recommended.

In conclusion, our virtual screening on an ensemble of hGlyR- $\alpha 1$ structures in different lipid environments has proved efficient to identify FDA-approved drugs that potentiate hGlyRs.

Among the identified drug candidates, several were known or suspected to have an analgesic effect. The potentiation of hGlyRs provides a plausible mechanism for the analgesic effects of these drugs. The screening results provide a valuable basis to further evaluate their analgesic properties. The protocol established in the current study can readily be transferred for discovering novel compounds of higher efficacy on hGlyRs by screening larger chemical libraries. Finally, this screening methodology can be applied to other membrane proteins with lipid-exposed binding sites.

EXPERIMENTAL SECTION

General Procedures

An overview of the step-by-step protocol used in this study is shown in **Figure 7**.

System Preparation

The NMR-determined structure of the open-channel hGlyR- α 1 TMD (PDB ID: 2M6I)⁶ was used for the initial coordinates of the protein in MD simulations. The protein was embedded into one of three different pre-equilibrated lipid bilayers: 1) pure POPC, 2) POPC/cholesterol in a 5/1 molar ratio with five cholesterol molecules initially within 4 Å of TM1 to TM3, and 3) identical to system 2, except with eight cholesterol molecules initially within 4 Å of TM1 to TM3. The 5/1 molar ratio of POPC/cholesterol was chosen because eukaryotic cell membranes typically are composed of approximately 20% cholesterol.²⁶ The GROMACS g_membed tool²⁷ was used to insert the protein into POPC or POPC/cholesterol lipid bilayers.^{28,29} Each system was solvated in TIP3P water, ionized with 25mM NaCl, and contained ~90K atoms.

MD simulations of the three systems were run to obtain ensemble conformations of the hGlyR- α 1 TMD equilibrated in lipids. All simulations were performed using GROMACS 4.6.1³⁰ with the AMBER03³¹ force field and additional parameters for POPC and cholesterol.³²⁻³⁴ The same simulation protocol was applied to each system. Energy minimization was performed for 10,000 steps with harmonic position restraints of 10,000 kJ/mol/nm² on the protein backbone atoms followed by 3 ns of equilibration, during which position restraints on the protein backbone were gradually reduced from 10,000 to zero kJ/mol/nm². NMR-derived distance restraints were included over the entire trajectory with a force constant of 1000 kJ/mol/nm². For each system, three 50-ns replicate simulations were performed at a constant pressure and temperature (NPT) of 1 atm and 310 K. After 10-20 ns, the backbone RMSD of the hGlyR- α 1 TMD in each simulation has converged to ~6 Å (**Supporting Figure S1**). All bonds were constrained using the LINCS algorithm.³⁵ The integration time step used for all simulations was 2 fs. Particle mesh Ewald was used for long-range electrostatic interactions. A 12-Å cutoff was used for nonbonded interactions. Full electrostatic and nonbonded interactions were evaluated every five steps. Systems were simulated with periodic boundary conditions in three dimensions.

Virtual Screening

To identify representative protein conformations in each system for virtual screening, we performed structural RMSD clustering using the Quality Threshold Clustering Algorithm¹⁸

implemented in VMD³⁶ with a threshold distance of 1.3 Å, which was chosen by testing a range of distances. This threshold distance divided the structures into similarly sized clusters with a smaller intra-cluster RMSD but larger inter-cluster RMSD. RMSD clustering was performed over the backbone atoms in TM1 to TM3 (residues 210 to 300) for each replicate simulation. TM4 was not included as it was furthest from the pore and most susceptible to random fluctuations. A representative structure was selected from each of the four major clusters. Each representative structure is the pentamer that has the minimum average RMSD between itself and all other structures in a given cluster. These representative pentameric structures were each divided into five sets of screening units, two adjacent subunits (**Figure 7**), in order to include potential intra- and inter-subunit binding sites in virtual screening. Four major clusters were identified for each simulation. This yielded a total of 180 structures (4 representative structures $\times$ 3 systems $\times$ 3 replicates $\times$ 5 subunit pairs) for docking calculations.

Virtual screening was performed on the DrugBank database of 1549 FDA-approved compounds. This database is already a small subset of compounds and thus no further filtering was done prior to screening. To account for the potential effects of lipids in drug binding, all drug compounds were screened on all 180 hGlyR- α 1 TMD structures twice: in the presence and absence of all lipid molecules within 7 Å of residue S296. The 7-Å threshold was selected to include only those lipid molecules that enclose the binding pocket. Docking calculations were performed using AutoDock Vina,³⁷ where the protein and lipids remained rigid while drug compounds were flexible. The search space was set to a 20 Å square box centered on residue S296, encompassing both potential intra- and inter-subunit binding sites. Hits were defined as any docked conformation of a compound with binding energy corresponding to a disassociation constant $K_d \leq 1 \mu\text{M}$ (binding energy $G = RT\ln K_d$, where $T = 298 \text{ K}$ and R is the ideal gas constant) on the 180 screening units. The screened compounds were ranked based on the total predicted binding energy of their hits among 180 screening units in the presence or absence of lipids. Using the total predicted binding energy in the ranking allowed us to account for both the number of hits and the binding energy of the screened compounds. Because of the small search space for molecular docking, high-scoring compounds were, by design, likely to be close to S296.

Oocyte Expression and Electrophysiological Recordings

RNA encoding hGlyR- α 1 or α 7nAChR (25-50 ng) was injected into *Xenopus laevis* oocytes (stages 5-6) and channel activity was measured using two-electrode voltage clamp experiments as described previously.³⁸ Oocytes were maintained at 17 °C in a modified Barth's solution.³⁹ Functional measurements were performed 1-4 days after injection with an OC-725C Amplifier (Warner Instruments) in a 20- μ l oocyte recording chamber (Automate Scientific). Oocytes were clamped at a holding potential of -60 mV. The recording solutions contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 and 5 mM HEPES, pH 7.0. Channel modulation was measured by the simultaneous co-application of the desired compounds with an effective glycine concentration equivalent to ~5% of the maximal current (EC_{50}). Because α 7nAChR has fast channel desensitization, an effective acetylcholine concentration equivalent to 50% of the maximal current (EC_{50}) was used to ensure the accuracy of modulation assessments for the selected drugs. THC was obtained

from the National Institute on Drug Abuse. PNU-120596 was purchased from R&D Systems (MN). Conivaptan was purchased from Toronto Research Chemicals, cinacalcet and regorafenib from MedChem Express, and the rest from Sigma-Aldrich. The vendors have verified the compounds' purity ~98% by HPLC or TLC experiments. **Supporting Information Figure S6** shows the ^1H NMR spectrum of pimozone from Sigma-Aldrich. All compounds were first dissolved in DMSO and then diluted into buffer, with the final concentration of DMSO ~0.05%. Data were collected and processed using Clampex 10 software (Molecular Devices).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

GlyR	glycine receptor
THC	9 -tetrahydrocannabinol
NMR	nuclear magnetic resonance
nAChR	nicotinic acetylcholine receptor
5-HT₃R	5-hydroxytryptamine type 3 receptor
GABA_AR	type A γ -amino-butyric acid receptor
pLGIC	pentameric ligand-gated ion channel
ECD	extracellular domain
TMD	transmembrane domain
ICD	intracellular domain
TM	transmembrane helix
CBR	cannabinoid receptor
MD	molecular dynamics
POPC	1-Palmitoyl-2-oleoylphosphatidylcholine
RMSD	root-mean-square deviation
CNS	central nervous system
PAINS	Pan Assay Interference Compounds

HPLC	high-performance liquid chromatography
TLC	thin-layer chromatography
DMSO	dimethyl sulfoxide

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Figure 1.

Side view of the hGlyR- α 1 TMD. Residue S296 is highlighted in green in one of the subunits. A model of the ECD is positioned over the TMD for context.

Figure 2.

Lipid effects on the hGlyR- α 1 TMD structure during MD simulations. Top views of three hGlyR- α 1 TMD systems with adjacent lipid molecules for MD simulations. **(a)** System 1 with POPC; **(b)** system 2 with POPC/cholesterol; **(c)** system 3, identical to system 2 except having three more cholesterol molecule within 4 Å of TM1 to TM3 in the initial simulation set up. Residue S296 is highlighted in green. POPC and cholesterol molecules are shown as black and yellow sticks, respectively. **(d)** Mean pairwise RMSD (see color bar on right) of the hGlyR- α 1 TMD structures grouped by the number of cholesterol molecules within 4 Å of TM1-TM3. The number of structures in each group is noted along the diagonal. **(e)** The presence of cholesterol in systems 2 (green) and 3 (red) in MD simulations changed the pore profile of the hGlyR- α 1 TMD compared to system 1 (blue). The pore radii were calculated using the HOLE program.⁴⁰ The presented mean (solid line) and standard deviation (dashed line) of the pore radii were sampled from 500 structures from three replicate simulations for each system.

Figure 3.

Distributions of the number of hit compounds ($K_d \leq 1 \mu\text{M}$) from the virtual screening on hGlyR- $\alpha 1$ TMD structures from (a) system 1, simulated and screened with POPC; (b) system 2, simulated and screened with POPC/cholesterol; (c) system 3, identical to system 2 except having three more cholesterol molecule within 4 Å of TM1 to TM3 in the initial simulation set up; and (d) screened all structures from three systems, but with neither POPC nor cholesterol in the virtual screening.

Figure 4.

Representative examples of lipid effects on virtual screening. The presence of lipids improved adapalene (magenta) binding to the S296 site in the same structure and reduced the predicted K_d to (a) 179 nM from (b) 1.4 μ M in the absence of lipids. Lipids weaken telmisartan (orange) binding to the S296 site and increased the predicted K_d to (c) 1.4 μ M from (d) 7.2 nM in the absence of lipids. S296 is shown in green, POPC in black, and cholesterol in yellow.

Figure 5.

Functional validation of virtual screening. **(a)** Representative current traces of *Xenopus laevis* oocytes expressing hGlyR- α 1 activated by glycine at 5% maximal effective concentration (EC_5) and modulated by 10 μ M pimozone, THC, or conivaptan. Black bars over the traces depict application of the indicated agonist or modulator. Scale bars indicate 30 seconds and 0.1 μ A current. Potentiation of hGlyR- α 1 by the indicated concentrations of **(b)** first tier and **(c)** second tier modulators normalized to the glycine-elicited current without

modulators. Data are fit to Hill equations with the parameters shown in **Table 3**. Error bars designate the standard error of the mean ($n = 4$).

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Figure 6.

Correlation between virtual screening predictions and *in vitro* functional validation.

Potentiation measured at 1 μ M for each of the top ranked tested compounds vs. the total docking energy obtained in (a) the presence of lipids or (b) the absence of lipids. Error bars show the standard error of the measured potentiation, $n = 4$ oocytes. The difference in the total binding energy between (a) and (b) reflects a higher degree of binding selectivity when the virtual screening was done in the presence of lipids.

Figure 7.
A flowchart highlighting our screening protocol.

Table 1

Top ranked compounds from the virtual screening.

Compound	Structure	Compound	Structure
Cholecalciferol 1 (6) ^a 423 Å ³ ^b 0.275 ^c		Risperidone 12 (22) 372 Å ³ 0.186
Adapalene 2 (13) 387 Å ³ 0.173		Conivaptan 14 (3) 446 Å ³ 0.072
Cinacalcet 3 (24) 325 Å ³ 0.158		Antrafenine 16 (1) 483 Å ³ 0.089
Pimozide 4 (7) 419 Å ³ 0.120		Telmisartan 21 (11) 470 Å ³ 0.121
Nandrolone phenpropionate 6 (17) 401 Å ³ 0.207		Tasosartan 22 (25) 360 Å ³ 0.113
Fluspirilene 7 (5) 439 Å ³ 0.113		Regorafenib 24 (21) 369 Å ³ 0.055
Dutasteride 9 (9) 445 Å ³ 0.152		Astemizole 25 (19) 428 Å ³ 0.134

^a Ranks from virtual screening with lipids (without lipids).^b Molecular volume.^c Tanimoto coefficient as compared to THC.

Table 2Additional compounds selected for *in vitro* functional validation.

Compound	Structure
THC 452 (134) ^a 325 Å ³ ^b 1.0 ^c
Sulindac 78 (114) 304 Å ³ 0.140
Mefloquine 172 (124) 297 Å ³ 0.182
PNU-120596 255 Å ³

^a Ranks from virtual screening with lipids (without lipids).^b Molecular volume.^c Tanimoto coefficient as compared to THC.

Table 3Potentiation at 1 μ M, EC₅₀ and maximum potentiation values for the tested compounds.

Compound	Potentiation at 1 μ M (%)	EC ₅₀	Maximum Potentiation (%)
Pimozide	219 $\pm$ 14	1.7 $\pm$ 0.8	510 $\pm$ 70
Cholecalciferol	216 $\pm$ 26	0.4 $\pm$ 0.1	220 $\pm$ 14
Cinacalcet	182 $\pm$ 3	0.32 $\pm$ 0.07	210 $\pm$ 6
Dutasteride	182 $\pm$ 11	0.33 $\pm$ 0.09	200 $\pm$ 8
Sulindac	181 $\pm$ 7	0.38 $\pm$ 0.05	190 $\pm$ 4
Risperidone	180 $\pm$ 6	0.32 $\pm$ 0.06	190 $\pm$ 5
Adapalene	172 $\pm$ 7	1.3 $\pm$ 0.8	260 $\pm$ 27
THC	162 $\pm$ 12	1.3 $\pm$ 0.6	260 $\pm$ 30
Fluspirilene	153 $\pm$ 2	1.2 $\pm$ 0.7	210 $\pm$ 15
Mefloquine	126 $\pm$ 4	2.5 $\pm$ 0.9	220 $\pm$ 19
Astemizole	122 $\pm$ 2	4.2 $\pm$ 1.8	280 $\pm$ 39
Telmisartan	120 $\pm$ 5	3.2 $\pm$ 1.5	210 $\pm$ 18
Regorafenib	116 $\pm$ 5	1.8 $\pm$ 0.2	200 $\pm$ 5
Conivaptan	106 $\pm$ 3	—	107 $\pm$ 5
PNU-120596	102 $\pm$ 4	—	103 $\pm$ 2