Journal of Biomolecular Structure and Dynamics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbsd20

Investigation of cannabidiol's potential targets in limbic seizures. *In-silico* approach

Olabimpe Olafuyi, Karina Kapusta, Alexander Reed, Wojciech Kolodziejczyk, Julia Saloni & Glake A. Hill

To cite this article: Olabimpe Olafuyi, Karina Kapusta, Alexander Reed, Wojciech Kolodziejczyk, Julia Saloni & Glake A. Hill (2022): Investigation of cannabidiol's potential targets in limbic seizures. *In-silico* approach, Journal of Biomolecular Structure and Dynamics, DOI: 10.1080/07391102.2022.2124454

To link to this article: https://doi.org/10.1080/07391102.2022.2124454

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
https://doi.org/10.1080/07391102.2022.2124454

Investigation of cannabidiol's potential targets in limbic seizures. *In-silico* approach

Olabimpe Olafuyi, Karina Kapusta, Alexander Reed, Wojciech Kolodziejczyk, Julia Saloni and Glake A. Hill

Interdisciplinary Center for Nanotoxicity, Department of Chemistry, Physics and Atmospheric Sciences, Jackson State University, Jackson, MS, USA

Communicated by Ramaswamy H. Sarma

ABSTRACT

Even though the vast armamentarium of FDA-approved antiepileptic drugs is currently available, over one-third of patients do not respond to medication, which arises a need for alternative medicine. In clinical and preclinical studies, various investigations have shown the advantage of specific plant-based cannabidiol (CBD) products in treating certain groups of people with limbic epilepsy who have failed to respond to conventional therapies. This work aims to investigate possible mechanisms by which CBD possesses its anticonvulsant properties. Molecular targets for CBD's treatment of limbic epilepsy, including hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1), gamma-aminobutyric acid aminotransferase (GABA-AT), and gamma-aminobutyric acid type A receptor ($GABA_A$), were used to evaluate its binding affinity. Interactions with the CB1 receptor were initially modeled as a benchmark, which further proved the efficiency of proposed here approach. Considering the successful benchmark, we further used the same concept for *in silico* investigation, targeting proteins of interest. As a result of molecular docking, molecular mechanics, and molecular dynamics simulations models of CBD-receptor complexes were proposed and evaluated. While CBD possessed decently high affinity and stability within the binding pockets of GABA-AT and some binding sites of $GABA_A$, the most effective binding was observed in the CBD complex with HCN1 receptor. 100 ns molecular dynamics simulation revealed that CBD binds the open pore of HCN1 receptor, forming a similar pattern of interactions as potent Lamotrigine. Therefore, we can propose that HCN1 can serve as a most potent target for cannabinoid antiepileptic treatment.

ARTICLE HISTORY
Received 21 March 2022
Accepted 8 September 2022

KEYWORDS
Limbic seizures; cannabinoids; CBD; molecular dynamics; *in silico*

1. Introduction

Epilepsy is one of the most prevalent neurological brain disorders in the world, targeting about 50 million people worldwide (World Health Organization. Epilepsy. Key Facts, 2019) and 3 million adults and 470,000 children in the United States in 2020 (Epilepsy Fast Facts | CDC, 2021). Epilepsy is characterized by abnormal nerve cell activity and recurrent seizures that negatively affect the quality of a patient's life. Most people diagnosed with epilepsy have focal (partial) epilepsy and temporal lobe epilepsy, or so-called limbic seizures (Blair, 2012). Limbic epilepsy is diagnosed when seizure foci arise in limbic brain areas, regions in the temporal and frontal lobes, which are involved with memory and emotion. It is a cause of serious disability, with significant mortality due to trauma and suicide. It also is associated with considerable cognitive and psychiatric comorbidity, which adds greatly to disability and impaired quality of life (Salpekar & Mula, 2019; Napolitano et al., 2021). Although there is a vast armamentarium of FDA-approved antiepileptic drugs (AEDs)

CONTACT Karina Kapusta karina.kapusta@icnanotox.org Interdisciplinary Center for Nanotoxicity, Department of Chemistry, Physics and Atmospheric Sciences, Jackson State University, Jackson, MS 39217, USA

Supplemental data for this article can be accessed online at http://dx.doi.org/10.1080/07391102.2022.2124454

currently available, over one third of patients with epilepsy develop drug resistant epilepsy and do not respond to medication (Kwan et al., 2011; Tang et al., 2017; Larivière et al., 2021). The lack of effectiveness and toxicity of current AEDs on the market has led to unconventional methods, traditional medicine, such as cannabis (Karaźniewicz-Łada et al., 2021).

Cannabis sativa has been used for centuries, presumably since 500 BC, as plant-based treatment for various diseases. It has recently become a subject of intensive research investigations devoted to various medicinal properties of this plant and its main constituent substances (ElSohly et al., 2017; Nuutinen, 2018; Sampson, 2021). There are 113 known phytocannabinoids in the cannabis plant, including the most known cannabidiol (CBD) and tetrahydrocannabinol (THC; Dhir, 2018). The human body contains a specialized system called the endocannabinoid system, which regulates various functions, including sleep, appetite, pain, and immune system response. The Cannabinoid Receptor 1 (CB1R) participates in the regulation of excitation/inhibition in the neurons, while Cannabinoid Receptor 2 (CB2R), having a lower concentration in the brain, is mainly expressed in the immune system (Klein et al., 2003). Based on *in vitro* and *in vivo* studies, , phytocannabinoids other than THC are thought to have a major anticonvulsant effect. In particular, CBD has received great attention in the scientific community and media (Mathern et al., 2015). Although CBD has proven to have anticonvulsant effects in clinical and preclinical studies, target proteins remain uncertain and the precise mechanisms by which CBD exerts its anticonvulsant properties in humans remain unknown. CBD has little to no binding affinity for CB1 receptor (Mathern et al., 2015). Targets of conventional AEDs that can play a role in limbic seizures provide a great starting point in the investigation of CBD treatment of epilepsy.

This work aims to investigate the possible targets for CBD against limbic seizures. Herein, we concentrate on Hyperpolarization-activated cyclic nucleotide-gated ion channels 1 (HCN1), γ-aminobutyric acid aminotransferase (GABA-AT), and γ-aminobutyric acid type A ($GABA_A$) receptors (Meldrum & Rogawski, 2007). HCN receptors are widely expressed in the hippocampus, cortex, and thalamus regions of the brain and mediated by cAMP, are responsible for conduction of the hyperpolarization-activated current (I_h). Subunits HCN1-4 can be combined into homomeric or heteromeric tetramers, being different in their functions and affinity to cAMP. The expression and functionality of HCN receptors were shown to play a crucial role in animal absence epilepsy (Kole et al., 2006) and later have been proposed to influence a human model of epilepsy. However, the role of HCN receptors in epilepsy is quite complex. In the case of limbic seizures, AEDs are proposed to target HCN1 (Meldrum & Rogawski, 2007). It was shown earlier (Albertson et al., 2011) that genetic deletion of HCN1 receptors may accelerate epileptogenesis, increase the severity of seizures, and provoke a high death rate among people with epilepsy. Among known blockers of HCN receptors there are also ZD7288, zatebradine, cilobradine, ivabradine, clonidine, and its derivative alinidine, bupivacaine, lidocaine, and mepivacaine (McClure et al., 2011). Drugs like lamotrigine and gabapentin also have action on HCN channels (Albertson et al., 2011). The GABA system plays a significant role in limbic seizures. When the concentration of the inhibitory neurotransmitter GABA diminishes below a threshold level, seizures occur. Raising the brain's GABA level can terminate seizures (Silverman, 2018). One of the principal methods to raise GABA levels in the human brain is to use small molecules that cross the blood-brain barrier and inhibit the activity of GABA-AT, a pyridoxal 5′-phosphate-dependent enzyme that degrades GABA (Denesyuk et al., 2002). GABA-AT is found in many organs, including the brain, liver, kidney and pancreas; it is present at higher specific activity in glial cells and presynaptic neurons (Jeremiah & Povey, 1981). Vigabatrin is an FDA-approved drug used to treat epilepsy by irreversibly binding to GABA-AT and inactivating the enzyme, leading to an increase in brain GABA levels. However, the drug is toxic, and large doses are needed for it to be effective. Other inhibitors of the enzyme include valproic acid, ethanolamine-O-sulfate (EOS), and phenelzine. AEDs, such as benzodiazepines, barbiturates, carbamazepine, and others, act directly on the $GABA_A$ receptor by enhancing its activity. When GABA is produced, it opens the transmembrane channels and allows chloride or other negatively charged ions to pass through the cell. The excitability of the cell is decreased while compounds that increase the cells activity enhance neuronal inhibition (Excitatory et al., 1997). $GABA_A$ receptors are pentameric transmembrane receptors with different isoforms of its subunits including α(1–6), β(1–4), γ(1–3), δ, ε, θ, π and ρ(1–3) (Wongsamitkul et al., 2017). Subunits' composition can vary and can be associated with specific functions (Galanopoulou, 2008). Drugs that target $GABA_A$ receptors have effects on the receptors activation, which silences the neurons. Benzodiazepines, GABAA agonistagonists, reduce seizures by increasing chloride influx, inhibiting$_A$ agonists, reduce seizures by increasing chloride influx which inhibits the neuron through hyperpolarization. Phenobarbital and diazepam are well-known $GABA_A$ antagonist that lessens current by diminishing the initial recurrence and the channels mean open time. Even though it was proven that CBD binds $GABA_A$ receptor (Bakas et al., 2017), it is still not clearly investigated which exact binding site is predominant and what is the mechanism of action.

The aim of this investigation is to identify the molecular target/targets for the CBD treatment of limbic epilepsy. Scanning cannabinoids such as CBD, THC, and 2-AG against HCN1, GABA-AT, and $GABA_A$ receptors and comparing its affinities to known activators/inhibitors can help to score and evaluate the efficiency of cannabinoids.

2. Methods

2.1. Target protein and ligand preparation

All calculations were carried out using Schrodinger. Three-dimensional structure of CB1R, $GABA_A$, GABA(AT) and HCN1 receptor has been retrieved from RCSB PDB (RCSB PDB: Homepage, 2021). For CB1R, GABA(AT), and $GABA_A$ crystallographic structures with PDB IDs: 5XRA, 6B6G, and 6X3W. respectively, were used. For HCN1 receptor close-pore

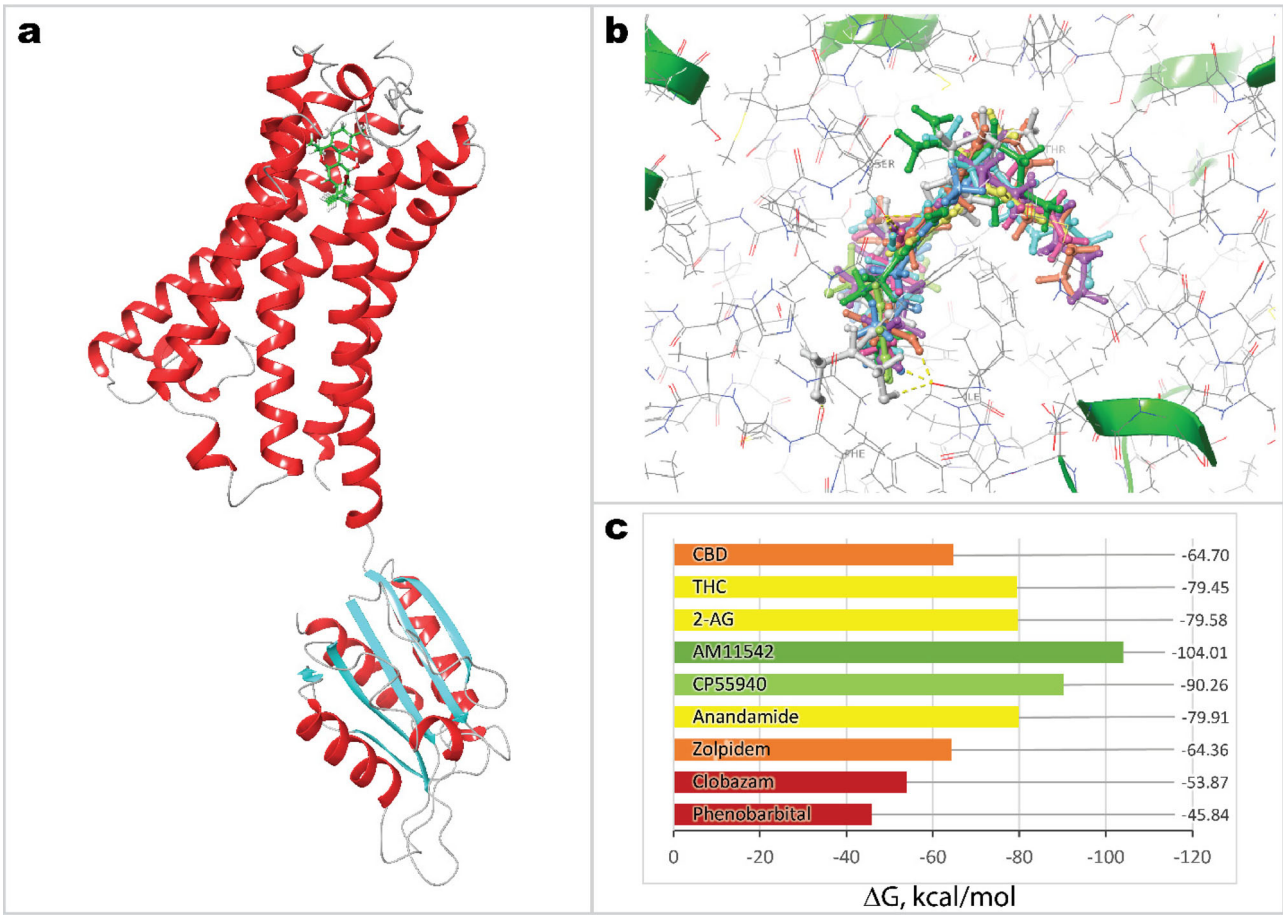

Figure 1. Results of benchmark MM-GBSA calculations: (a) protein structure with co-crystallized ligand (PDB ID: 5XRA); (b) superposition of all studied ligands bound to CB1 receptor (2-AG – gray; CBD – green; THC – magenta; AM11542 – cyan; Anandamide – orange; Clobazam – light green; CP55940 – purple; Phenobarbital – yellow; Zolpidem – blue); (c) – Free Gibbs energies of interaction between receptor and studied compounds.

protein with (PDB ID: 5U6O) and without (PDB ID: 5U6P) its C-terminus were used. However, molecular docking produced no poses for any of the compounds under investigation. Thus, the homology model of the open-pore HCN1 protein built by Tanguy et al. (2019) was used instead.

All target proteins were prepared using Protein Preparation Wizard (Madhavi Sastry et al., 2013). Present crystallographic waters were deleted, the bond orders were assigned, and hydrogens were added after the removal of the original hydrogens. Prime (Jacobson et al., 2004) module was used for filling in missing loops and side chains, and Epik (Shelley et al., 2007) was employed for the generation of protonation states. Optimization of hydrogen bond networks and proteins' restrained minimization were carried out using OPLS3e (Roos et al., 2019) force fields. All ligand 3 D structures were downloaded from PubChem. Ligands were prepared using LigPrep Module (Elokely & Doerksen, 2013).

For the benchmark model of the CB1 receptor, known binders 2-AG, THC, AM11542, CP55940, and Anandamide were used as a positive control, while Clobazam, Phenobarbital, and Zolpidem were used as decoys. The grid for molecular docking was centered on a cocrystallized ligand of the CB1 receptor, AM11542. For all other protein structures 2-AG, THC, and CBD were used as ligands of interest, while the remaining six ligands would vary from protein to protein to compare ligands of interest with known binders. For the HCN1 receptor Alinidine, Clonidine, Ivabradine, Lamotrigine, Lidocaine, and ZD7288 were chosen, for GABA(AT) these ligands were Diazepam, EOS, 4-Amino-5-oxopentanoic acid, Vigabatrin, Gabapentin, and Phenelzine, and for $GABA_A$ for comparison were used Diazepam, Clobazam, Zolpidem, Phenobarbital, Loreclezole, and Ganaxolone.

HCN receptor consists of S5 helix, pore helix, selectivity filter and S5-S6 loop, and S6 helix. Inhibitors of hyperpolarization-activated cyclic nucleotide-gated channels were shownto bind the pore region between S5 and S6 helixes of a human HCN1 receptor. Grid for HCN1 was generated with a center in the middle of the protein's pore. For GABA(AT) grid was centered on a co-crystallized ligand. Meanwhile, since $GABA_A$ has several binding sites (each less or more favorable for particular drugs), one must consider investigating interactions with different subunits. 3 D structure of $GABA_A$ with PDB ID: 6X3W consists of two α1 (chains B and D), two β2 (chains A and C), and one γ2 (E) subunits. While classical benzodiazepines are known to bind at the α-γ interface near α1H101, α1Y159, α1T206, and α1Y209 residues (Sigel, 2002), recent studies (Kim et al., 2020) showed its possibility to bind in three other regions at α1(chain B)-β2(chain A), α1(chain D)-β2(chain C) and β2(chain C)-γ2(chain E) interfaces. Barbiturates bind α1(chain B)-β2(chain A) and β2(chain A)-γ2(chain E). All five possible binding sites were considered for further investigation.

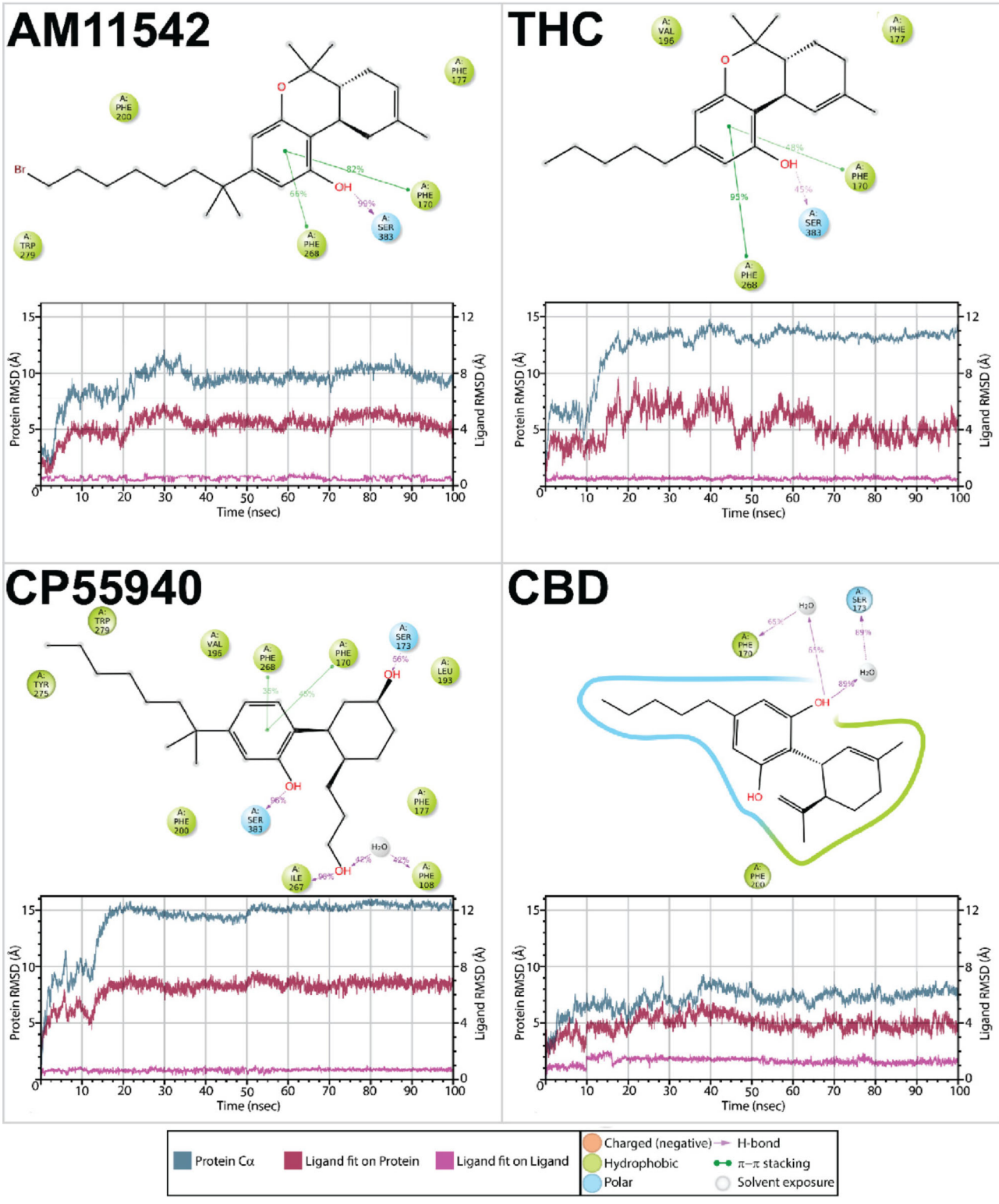

Figure 2. Results of 100 ns molecular dynamics simulation for selected complexes of studied compounds with CB1 receptor: 2 D diagrams with percent time of interactions and RMSD of a protein and a ligand.

2.2. Ligand docking, MM-GBSA calculation and MD simulation

The goal of molecular docking is to predict the predominant binding mode/modes of the ligand and a known three-dimensional structure of a protein. Molecular docking was carried out using the Glide module (Friesner et al., 2006). The protein structure was kept rigid, while the ligand was flexible inside the binding pocket. Planarity of conjugated π groups was enhanced, as well as Epik state penalties were. Extra-précised (XP) docking with flexible ligands' structure was used.

The best scoring poses produced by XP docking were further used to perform postprocessing with Molecular

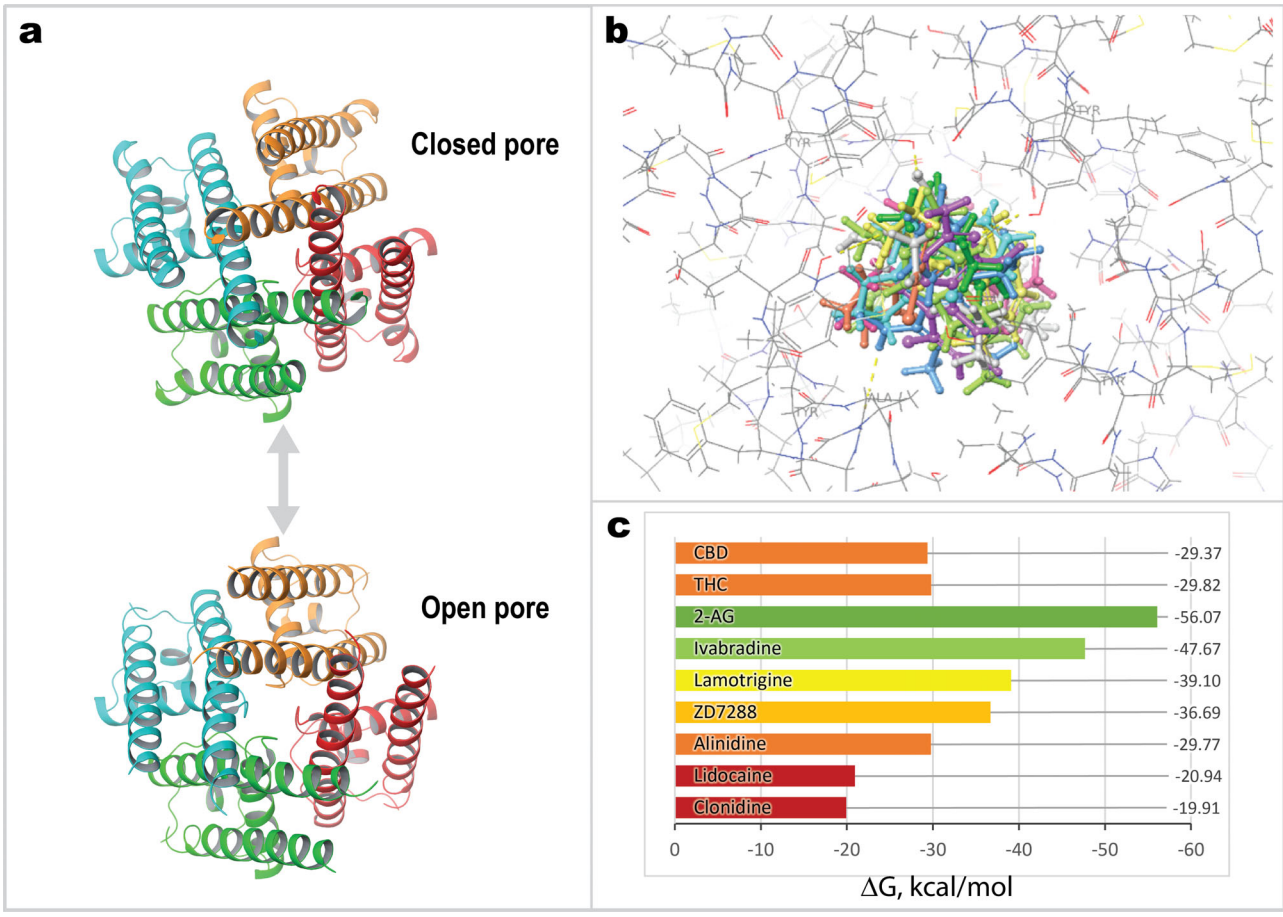

Figure 3. HCN1 receptor and results of MM-GBSA calculations: (a) side view of HCN1 protein; bottom view of HCN1 protein; (b) superposition of all studied ligands bound to the receptor as a result of MM-GBSA calculation (2-AG – grey; CBD – green; THC – magenta; Alinidine – cyan; Clonidine – orange; Ivabradine – light green; Lamotrigine – purple; Lidocaine - yellow; ZD7288 - blue); (c) Free Gibbs energies of interaction between HCN1 receptor and studied compounds.

Mechanics/Generalized Born Surface Area (MM-GBSA) calculation. MM-GBSA calculations were performed to estimate the free binding energy between the ligand and protein. Estimated free binding energies were calculated based on the equation below:

$$\Delta G = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{protein}})$$

All MM-GBSA calculations were carried out using the Prime module (Greenidge et al., 2013) with VSGB solvation model and OPLS3e force field. Residues located closer than 12 A from a ligand structure were set to be flexible, while the rest of the protein structure remained rigid, which may enhance the accuracy of default calculations. The MM-GBSA output files have been used as an input for MD simulations, implemented in the Desmond module (Guo et al., 2010).

Molecular dynamics considers a ligand-protein complex as a dynamic system, which can move freely inside of the explicit solvent cell. This type of simulations provides more accurate data and can effectively deal with the influence of conformational changes on ligand's binding. In our study, receptor-ligand complexes were subjected to 100 ns MD simulation with a time step of 25 ps. An orthorhombic box filled with water molecules was used to build a system. Neutralization was performed by adding sodium or chlorine ions (depending on a total charge of a complex). OPLS3e force field and VSGB solvation model were used. Temperature and pressure were set to 300 K and 1.01325 bar, respectively. Obtained trajectories were analyzed using Simulation Interaction Diagram.

3. Results and discussion

3.1. Benchmark

Interactions with the CB1 receptor were modeled as a benchmark. To validate the methodic used here four decoys (CBD, clobazam, phenobarbital and zolpidem) and five known binders (2-AG, THC, AM11542, CP55940 and Anandamide) were docked into the active site of CB1 receptor (Figure 1a,b). Binding poses produced by docking were used for MM-GBSA calculation. 2 D diagrams of interactions between CB1 receptor and studied compounds can be found in Figure S1 (Supporting Information). All ligands showed the presence of hydrophobic interactions, being tightly sitting inside of the hydrophobic pocket. Compounds with benzene ring in its structure (except for Clobazam) all formed a $\pi-\pi$ stacking with PHE 170 and PHE 268. The same pattern of hydrogen bonding was detected for CBD, THC, AM11542 and CP55940 acting as hydrogen bond donors for SER383. 2-AG formed hydrogen bonds with ILE 267 and PHE108 and Anandamide

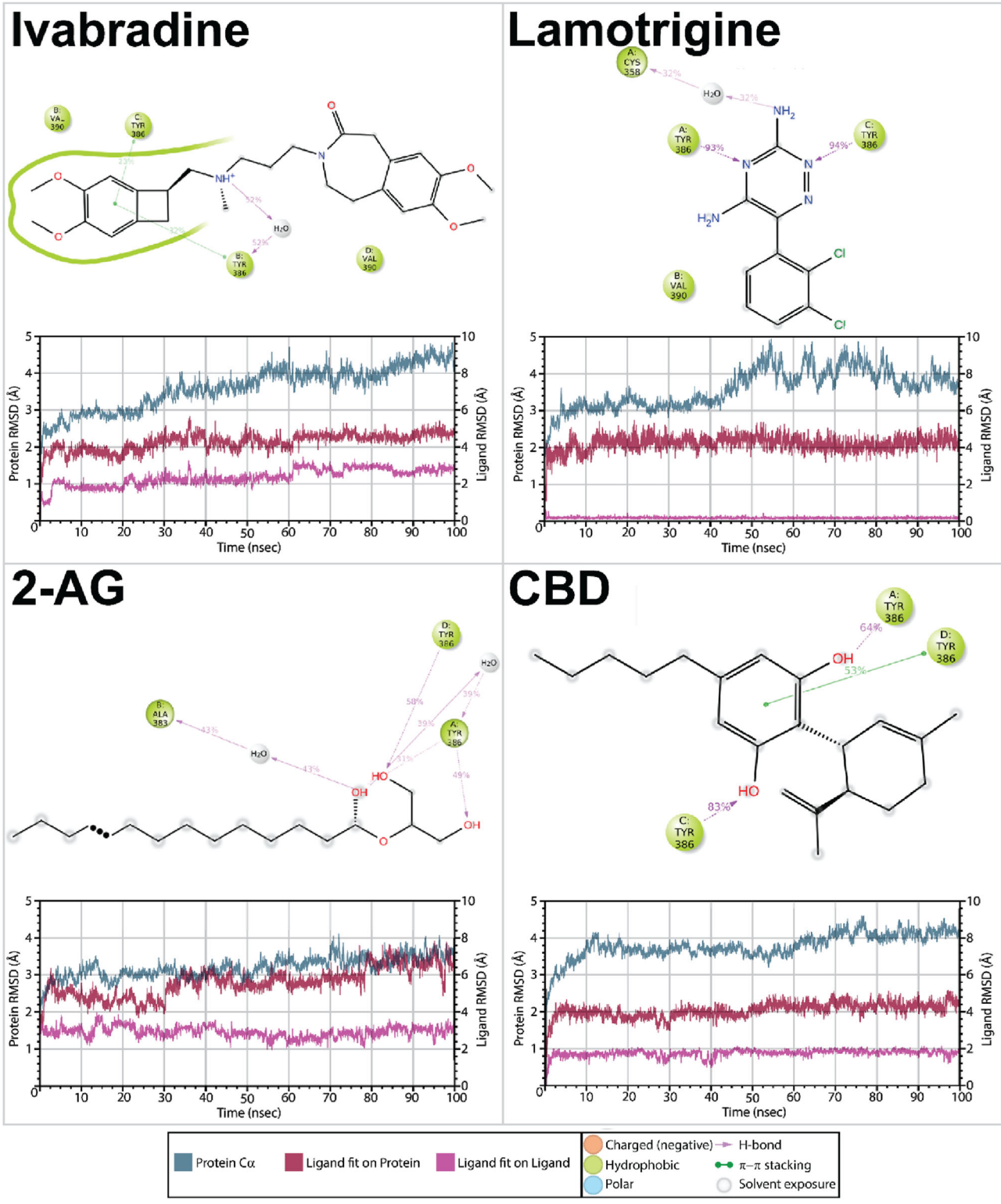

Figure 4. Results of 100 ns molecular dynamics simulation for selected complexes of studied compounds with HCN1.

solely with ILE 267. Interestingly, with CBD having the same binding pattern as known binders THC, AM11542, and CP55940, its binding energy calculated by MM-GBSA was much lower (Figure 1c). It should be noted that for all five known binders Gibbs Free Energies were calculated to be lower compared to decoys' binding energies, proving the efficiency of MM-GBSA calculation. With these values being in good agreement with literature data (Hill et al., 2013), one can conclude that the approach used here is applicable for further investigation.

Molecular dynamics simulation has shown even more reliable results (Figure 2). The majority of the time within 100 ns simulation THC, AM11542 and CP55940 retained the bonding pattern showed by MM-GBSA calculation. Meanwhile CBD was

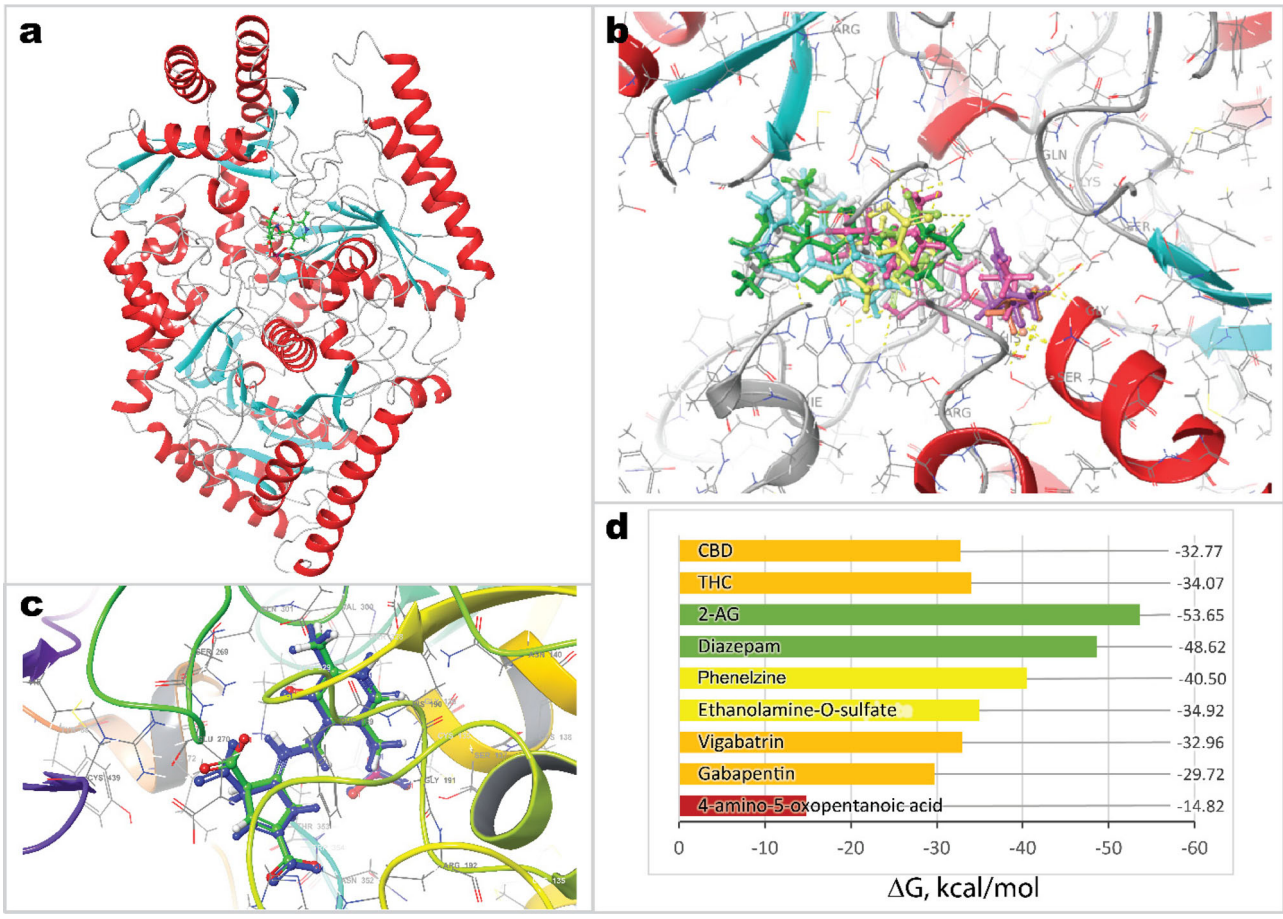

Figure 5. GABA(AT) receptor and results of molecular docking and MM-GBSA calculations: (a) protein structure with co-crystallized ligand (PDB ID: 6B6G); (b) superposition of reference and redocked co-crystallized ligand inside of GABA(AT) binding pocket; (c) superposition of all studied ligands bound to receptor (2-AG – grey; CBD – green; THC – magenta; Diazepam – cyan; EOS – orange; 4-Amino-5-oxopentanoic acid – light green; Vigabatrin – purple; Gabapentin – yellow; Phenelzine – blue); (d) Free Gibbs energies of interaction between receptor and studied compounds.

shown to interact with CB1 receptor only via weak water bridges. Root mean square fluctuation (RMSF) analyses presented in Figure S5 (Supporting Information) revealed large fluctuations of a protein when it was bound to THC and CP55940. For CBD, these fluctuations were minimal. These fluctuations can be associated with the activation of a receptor, proving once again the inability of CBD to activate the CB1 receptor. These results are validated by known experimental binding affinities. Considering the benchmark as successful, we further used the same concept for all calculations involving $GABA_A$, GABA(AT) and HCN1.

3.2. HCN1 receptor

Known blockers of the HCN1 receptor and studied cannabinoids were docked into the open pore the of HCN1 receptor (Figure 3a,b). Complexes produced by molecular docking were subjected to MM-GBSA calculations. 2 D interaction diagrams obtained after MM-GBSA complexes (Figure 3c) were collected in Figure S2 (Supporting Information). Cannabinoids (CBD and THC and imidazole derivatives (Alinidine and Clonidine) showed no interactions except for hydrophobic, while Ivabradine, Lamotrigine and Lidocaine all contained a H-bond acceptor interacting with TYR386. Additional $\pi-$cation interactions were observed for Ivabradine with TYR386 residue of the neighboring proteins subunit.

Calculated free binding energies (Figure 3c) revealed a relatively weak binding affinity of both CBD and THC toward the HCN1 receptor. These cannabinoids illustrated higher values of binding energy, compared to potent inhibitors, surpassing only lidocaine and clonidine by its binding affinity. Interestingly the lowest binding energy was exhibited by endocannabinoid 2-AG and Ivabradine. Even though cannabinoids are assumed to treat limbic epilepsy, recent study showed that endocannabinoid 2-AG can activate HCN receptor through the cascade pathway involving CB1 receptor, series of kinases, nitric oxide synthase and guanyl cyclase (Maroso et al., 2016). Dendritic current (Ih) increases because of HCN activation. It is possible that while 2-AG triggers a cascade which activates HCN, it can also block it directly when in abundance, thus controlling an unnecessary activation. Though, this hypothesis is just a presumption, and it must be verified by experimental studies.

Interestingly, the results of molecular dynamics simulation partially contradicted the ones obtained by molecular mechanics. Figure 4 shows the results of 100 ns MD simulations for complexes of studied compounds with HCN1. While according to MM-GBSA 2-AG and Ivabradine showed the highest affinity toward the target, MD simulation did not

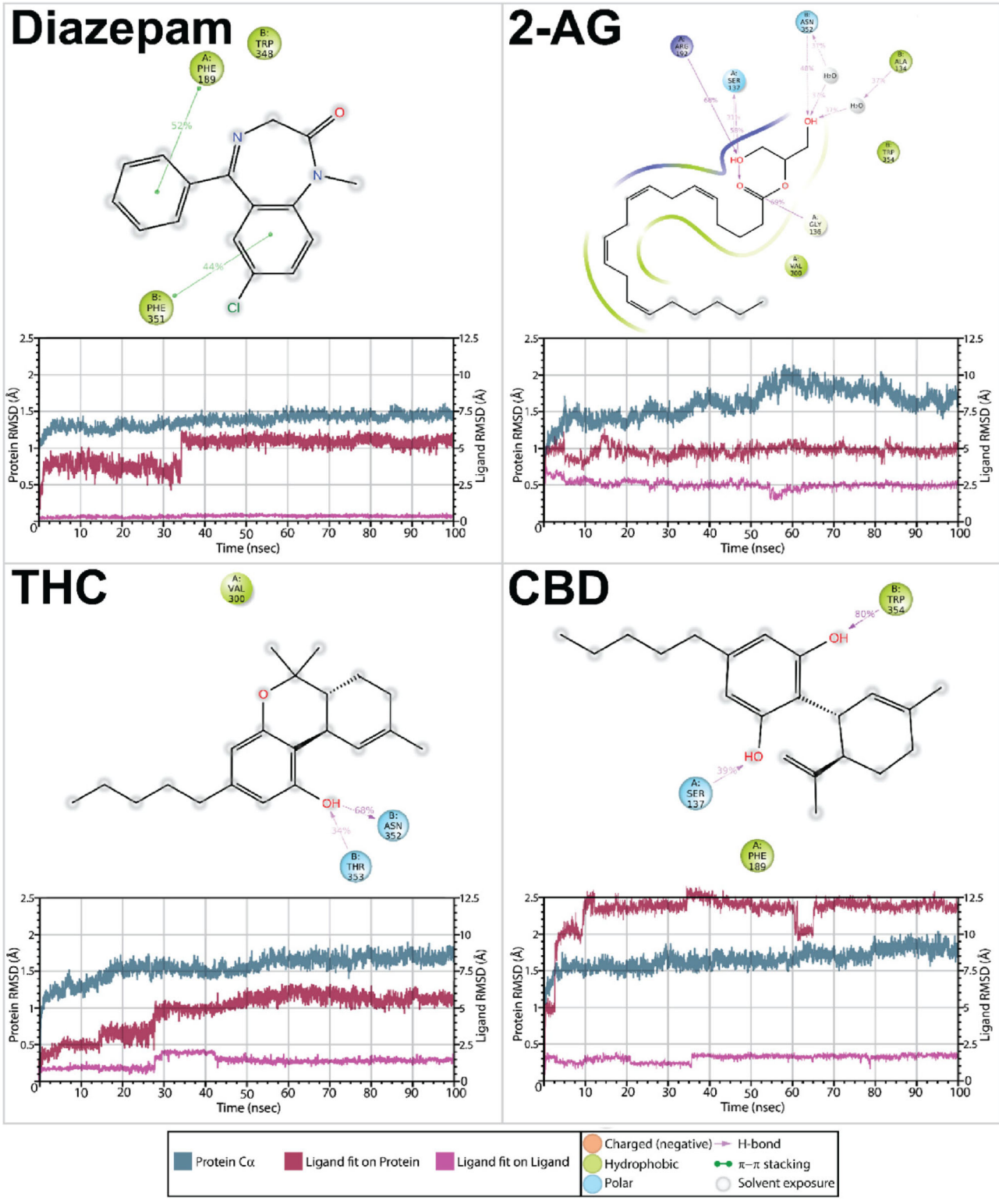

Figure 6. Results of 100 ns molecular dynamics simulation for selected complexes of studied compounds with GABA(AT).

confirm the formation of extremely stable complexes. According to simulation, Lamotrigine, which had slightly higher free energy comparing to 2-AG and Ivabradine, must be the most potent binder among compounds studied here, retaining H-bonds with TYR386 of subunits A and B within 93%–94% of a simulation time. It also showed the least deviations for the ligand fit on a protein and itself as can be seen from root mean square deviation (RMSD) plots. Surprisingly stable appeared to be CBD inside of the binding pocket of the HCN1 receptor. Similarly, to Lamotrigine it formed two H-bonds with TYR386 of subunits A and B (64% and 83% of a simulation time, respectively), with additional stabilization by $\pi - \pi$ stacking between its phenyl ring and TYR386 of a subunits D during 53% of a simulation time. Along with Lamotrigine, CBD showed the least ligand RMSF Figure S6 (Supporting Information).

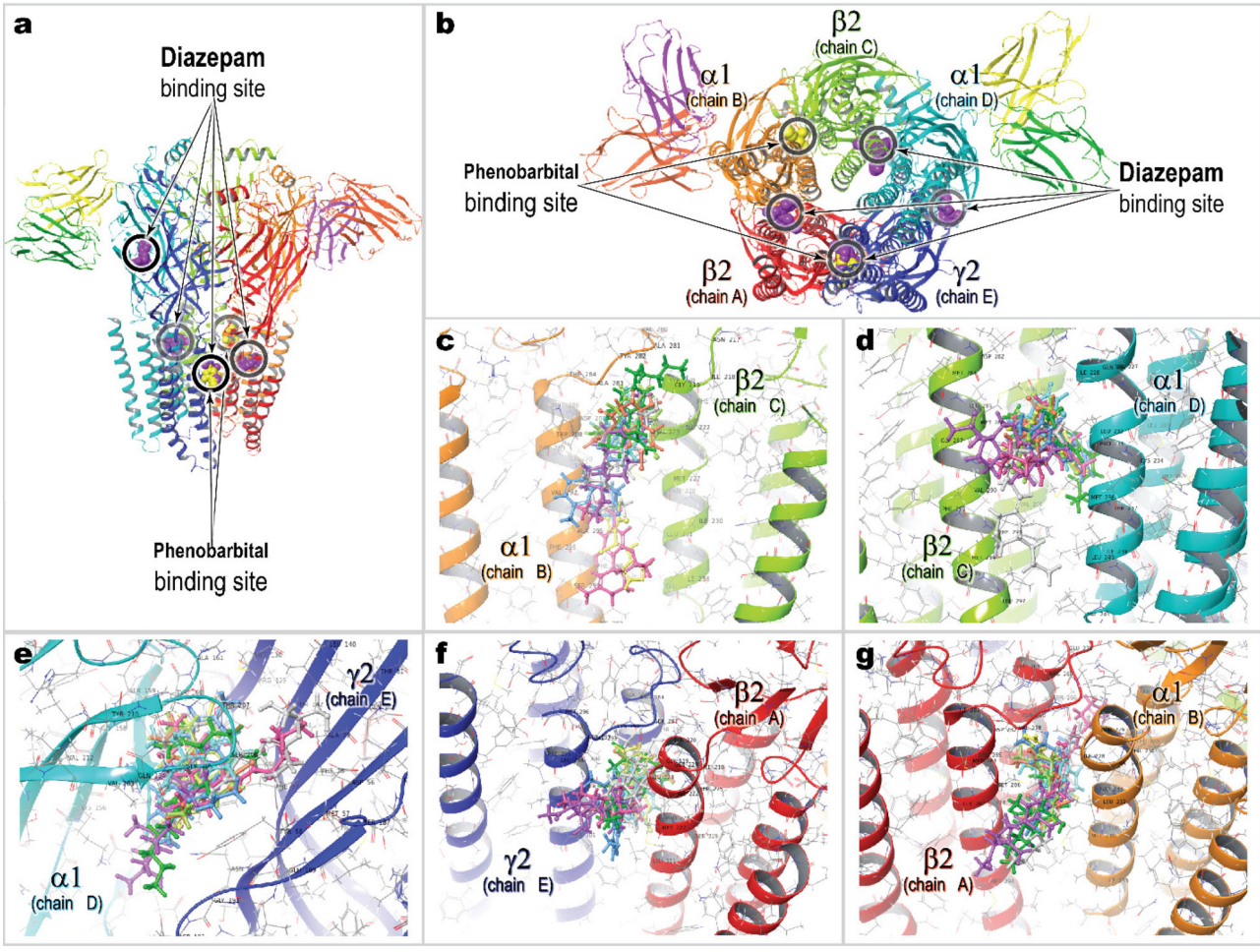

Figure 7. $GABA_A$ receptor and results of MM-GBSA calculations: (a) side view of protein structure with Diazepam (*purple space-fill*) and Phenobarbital (*yellow space-fill*) binding sites in $GABA_A$ receptor (PDB ID: 6X3W); (b) top view of $GABA_A$ protein structure; (c) superposition of all studied ligands bound to $GABA_A$ receptor in Phenobarbital binding sites α1 (chain B, orange ribbons) and β2 (chain C, green ribbons); (d) superposition of all studied ligands bound to $GABA_A$ receptor in Diazepam binding site β2 (chain C, green ribbons) and α1 (chain D, cyan ribbons); (e) superposition of all studied ligands bound to $GABA_A$ receptor in Diazepam binding site α1 (chain D, cyan ribbons) and γ2 (chain E, blue ribbons); (f) superposition of all studied ligands bound to $GABA_A$ receptor in Diazepam and Phenobarbital binding site γ2 (chain E, blue ribbons) and β2 (chain A, red ribbons); (g) superposition of all studied ligands bound to $GABA_A$ receptor in Diazepam binding site β2 (chain A, red ribbons) and α1 (chain B, orange ribbons). 2-AG – grey; CBD – green; THC – magenta; Ganaxolone – cyan; Loreclezole – orange; Zolpidem – light green; Phenobarbital – purple; Clobazam – yellow; Diazepam – blue.

According to obtained MD results one can see that HCN1 receptor can presumably be one of the targets for CBD treatment of epilepsy, however, CBD did not appear to be more potent than other AEDs (Lamotrigine in particular).

3.3. GABA-transaminase

For GABA(AT) receptor (Figure 5a) its co-crystallized ligand (S)-3-Amino-4-(difluoromethylenyl)cyclopent-1-ene-1-carboxylic acid was redocked into the binding site. As one can see from Figure 5b, binding mode was almost identical to the one of the reference structure. Studied compounds and the drugs targeting GABA(AT), such as Vigabatrin, Diazepam, EOS, Gabapentin, Phenelzine, and 4-Amino-5-oxopentanoic acid, were further docked into the binding site of a protein and subjected to MM-GBSA calculations (Figure 5c and Supporting Information Figure S3).

The 2-AG, EOS, 4-Amino-5-oxopentanoic acid, Vigabatrin, and Gabapentin all showed to have hydrogen bonding with the THR353 residue. ARG192 residue formed additional hydrogen bonds with THC, CBD, and 4-Amino-5-oxopentanoic acid. While the majority of compounds were bound through H-bonding and salt bridges, stabilization through $\pi-\pi$ stacking was only observed in the case of Phenelzine. The strongest binding affinity according to MM-GBSA (Figure 5d) was calculated for 2-AG and Diazepam, while the least binding affinity among studied compounds possessed 4-Amino-5-oxopentanoic acid. CBD and THC showed decent binding affinity.

Molecular dynamics simulation (Figure 6) provided a significantly different binding mode. RMSD of a ligand fit on a protein for CBD, THC, and Diazepam showed several jumps with further stabilization, illustrating a switch of the binding mode. As a result of this switch, Diazepam showed to be stabilized only by $\pi-\pi$ interactions between both phenyl rings and phenylalanine amino acid residues PHE189 and PHE351. 2-AG showed more interactions forming hydrogen bonds with GLY 136, SER137, ASN152, and ARG192 and having its hydrophobic chain being partially stabilized inside of the protein's hydrophobic pocket. THC had only contact with protein through its hydroxyl group, accepting H-bond from

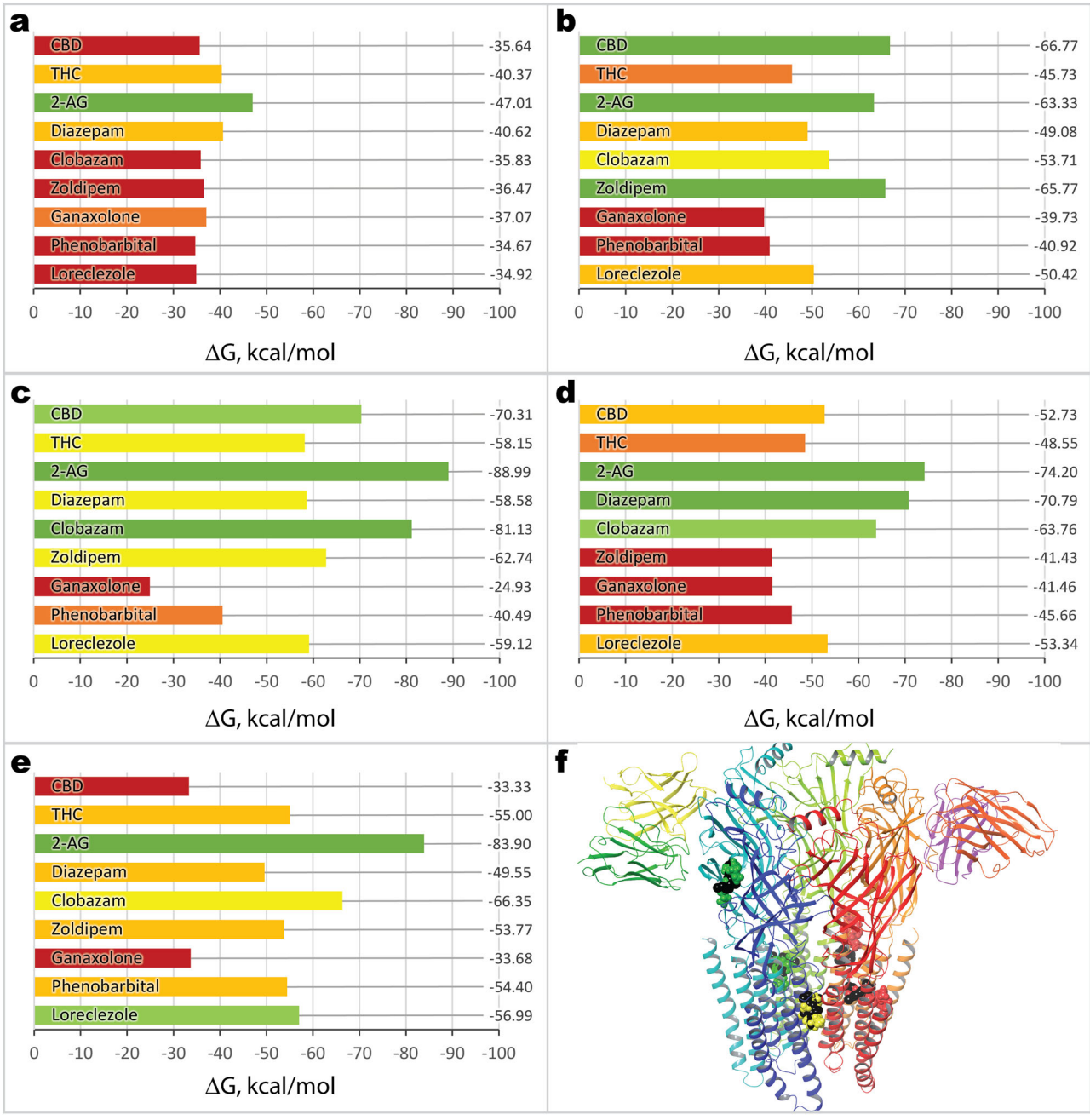

Figure 8. Free Gibbs energies of interaction between $GABA_A$ receptor and studied compounds as a result of MM-GBSA calculations: (a) studied compounds bound to Phenobarbital binding site α1 (chain B) and β2 (chain C); (b) studied compounds bound to Diazepam binding site β2 (chain C) and α1 (chain D); (c) studied compounds bound to Diazepam binding site α1 (chain D) and γ2 (chain E); (d) studied compounds bound to Diazepam and Phenobarbital binding site γ2 (chain E) and β2 (chain A); (e)studied compounds bound to Diazepam binding site β2 (chain A) and α1 (chain B); (f) 3 D structure of CBD bound to $GABA_A$ receptor within five known binding sites (reference ligands – black space-fill; CBD – red, yellow and green space-fill in order of increasing the binding energy).

THR353 and donating it to ASN352. CBD, meanwhile, served purely as an acceptor of hydrogen bonds from SER137 (39% of a simulation time) and TRP354 (80% of a simulation time). One can see that the CBD complex with GABA(AT) showed overall lesser stability compared to its complex with HCN1 receptor (stabilized by two H-bonds and $\pi - \pi$ stacking during 64%, 83%, and 53% of the simulation time, respectively).

3.4. $GABA_A$ receptor

$GABA_A$ receptor has several binding sites. For the α1β2α1γ2β2-type pentamer, like the one studied here, there are four Diazepam and two for Phenobarbital binding sites (Richter et al., 2012; Kim & Hibbs, 2021; Figure 7a,b). Cannabinoids and selected AEDs were docked into all five binding sites followed by MM-GBSA calculation. The superposition of all studied ligands bound to $GABA_A$ was illustrated in Figure 7c–g. Similarly to all previous ligand-receptor complexes, the lowest energy was calculated for ones associated with 2-AG (Figure 8). The highest energy in the majority of binding sites was observed for neurosteroid ganaxolone. According to the literature, it should share the diazepam binding site. Here, it showed the highest affinity to the diazepam binding site between subunits β2 (chain C) and α1

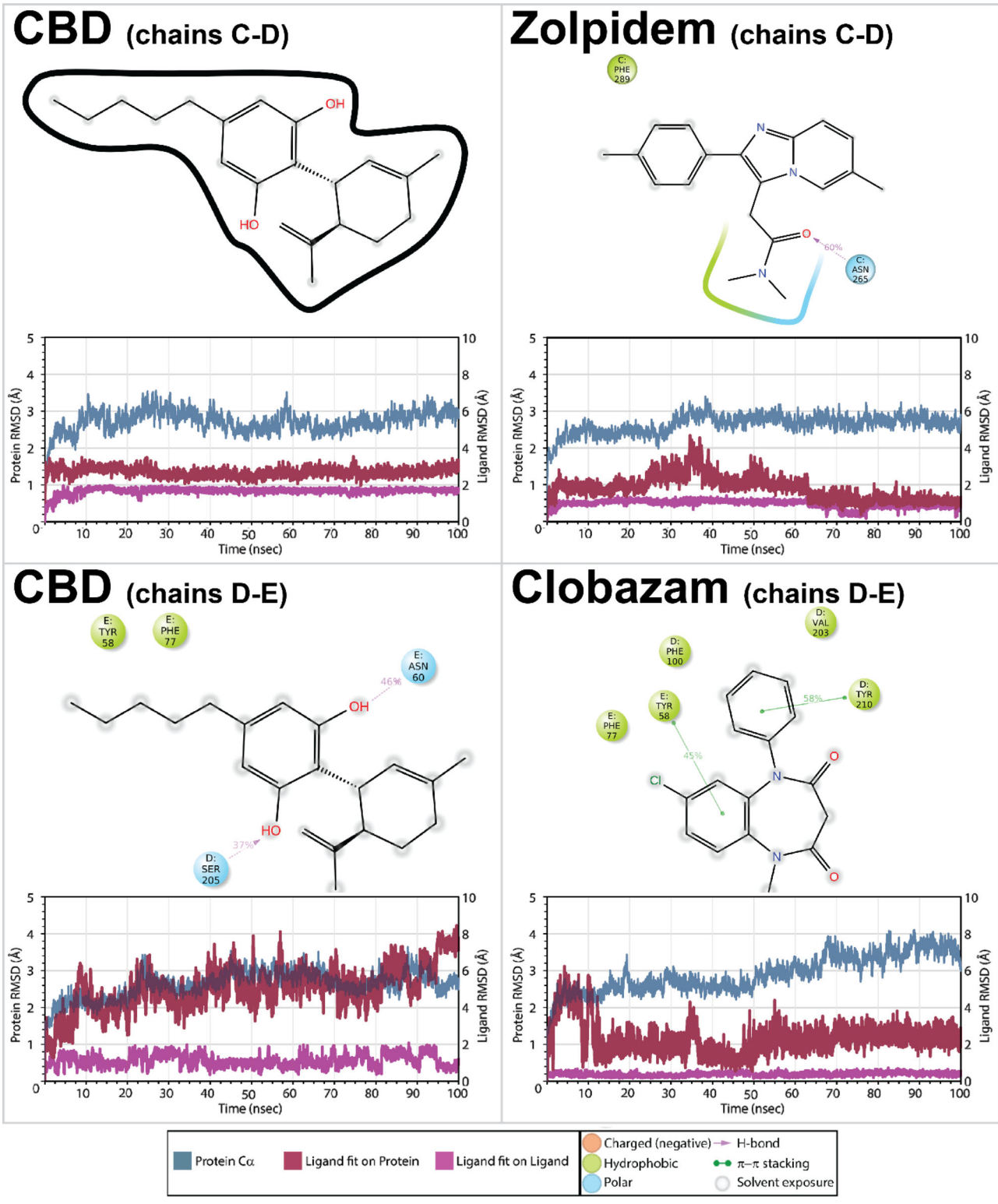

Figure 9. Results of 100 ns molecular dynamics simulation for selected complexes of studied compounds with $GABA_A$.

(chain D), and the diazepam and phenobarbital binding site between subunits γ2 (chain E) and β2 (chain A). CBD showed little to no affinity to phenobarbital binding site α1-β2 and diazepam binding site β2 – α1 (chains B-C and chains A-B, respectively). Interestingly, in these binding sites, the binding mode of CBD differs significantly from the one of the reference ligands, diazepam, and phenobarbital (Figure 8f; CBD colored in red). The most efficiently CBD bound other diazepam binding sites: β2 – α1 (chain C-D) and α1 – γ2 (chains D-E) (Figure 8f, CBD colored in green). While inside of the α1 – γ2 binding site it showed to be less efficient compared to 2-AG and Clobazam, in the case of β2 – α1 interface CBD showed the highest affinity compared to all other AEDs and even endocannabinoid 2-AG.

Of two possible binding sites suggested by MM-GBSA calculations, only one was verified by molecular dynamics simulation (Figure 9). Soon after the beginning of the simulation, CBD left the binding pocket of the diazepam binding site inside of subunits $\beta 2-\alpha 1$ (chain C-D). AED Zolpidem, being placed inside of this binding pocket, formed a stable hydrogen bond with amino acid ASN265 (60% of a simulation time) and had its amide group being tightly placed inside of the protein interface. Firmer binding was observed for the diazepam binding site between subunits $\alpha 1-\gamma 2$ (chains D-E). In this case, both hydroxyl groups of CBD were participating in binding by forming H-bonds with SER205 and ASN60 (varying from 37% to 46% of a simulation time). Nonetheless, looking at ligand's RMSD and RMSF illustrated in Figure S12 (Supporting Information), one can conclude that CBD has significantly higher flexibility, thus lesser binding strength compared to Clobazam. According to molecular dynamics simulation, Clobazam retained $\pi-\pi$ interactions with TYR210 and TYR58 within 58% and 45% of a simulation, respectively. Moreover, after 50 ns ligand was stabilized inside of the binding pocket and formed the interaction with TYR210 for the remaining 80% of the simulation time.

Summarizing the results of molecular dynamics for all the proteins studied here, it can be assumed that the most prominent target for CBD as an antiepileptic agent is not the $GABA_A$ but rather the HCN1 receptor. The complex of CBD with $GABA_A$ was formed by only two H-bonds, which sustain its binding for less than half of the 100 ns simulation time. Meanwhile, forming similar interactions as the potent Lamotrigine, CBD was held tightly inside of the open pore of the HCN1 receptor, stabilized by two hydrogen bonds and $\pi-\pi$ interactions for up to 83% of the simulation time. The binding of CBD to GABA(AT) was not as strong as in the case of the HCN1 receptor, CBD also formed strong interactions with it, stabilized by two H-bonds, which remained for up to 80% of the simulation time.

4. Conclusions

Up to date, there is not much known about the mechanism of action of cannabinoids as AEDs. Herein, the comprehensive computational investigation was performed to evaluate the affinity of CBD toward selected limbic seizure treatment targets. HCN1, GABA-AT, and $GABA_A$ were used to model its interactions with some cannabinoids and to compare its binding strength with potent AEDs. The benchmark model targeting the CB1 receptor showed a good agreement with the literature data, thus, enabling further implementation of the proposed here methodology.

As a result of extensive molecular docking, molecular mechanics, and 100 ns molecular dynamics simulation, it was shown that CBD can bind all receptors of interest with a decent affinity. The affinity of CBD for the $GABA_A$ receptor, shown earlier in the literature, was confirmed here. The CBD bound efficiently only inside of one of the four diazepam binding sites. Curious results were obtained when investigating the interactions of the CBD complex with the open-pore HCN1 receptor. MM/GBSA calculations showed that CBD was not as effective as potent Ivabradine, Lamotrigine, ZD7288 and even as cannabinoids 2-AG and THC. Meanwhile, a more accurate MD simulation revealed that CBD was only inferior to Lamotrigine. According to the 100 ns simulation results, CBD bound to the HCN1 receptor in a similar manner to this potent FDA-approved inhibitor, interacting with the same residues. None of the investigated ligands (even potent binders) could bind a close-poreHCN1 receptor, which suggested that only an open-pore conformation can be used for such simulations.

When comparing the MD trajectories of CBD complexes with all three target proteins (Figures 4, 6, and 9), it can be seen that the interactions between CBD and the HCN1 receptor remained strong during most of the simulation time. Thus, it can be hypothesized that the HCN1 receptor may serve as the most important target for the CBD treatment of epilepsy. Proposed here models must be verified experimentally before making any solid conclusions as for the mechanism of CBD's antiepileptic activity. Nonetheless, proposed here hypothesis can lead to a better understanding of how CBD-rich plant extracts can help in treating drug-resistant seizures. Being natural and less harming compared to synthetic AEDs, cannabinoids can open new paths for formulating drugs that will reduce frequency and depth of epileptic seizure and overtime improve the general wellbeing of people suffering from drug-resistant epilepsy.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Science Foundation under Grants: NSF/CREST HRD-1547754 and NSF EPSCoR OIA – 1757220.

ORCID

Karina Kapusta http://orcid.org/0000-0001-8466-4098
Wojciech Kolodziejczyk http://orcid.org/0000-0002-7754-8969
Julia Saloni http://orcid.org/0000-0003-3995-2909

References

Albertson, A. J., Yang, J., & Hablitz, J. J. (2011). Decreased hyperpolarization-activated currents in layer 5 pyramidal neurons enhances excitability in focal cortical dysplasia. *Journal of Neurophysiology*, *106*(5), 2189–2200. 10.1152/JN.00164.2011.

Bakas, T., van Nieuwenhuijzen, P. S., Devenish, S. O., McGregor, I. S., Arnold, J. C., & Chebib, M. (2017). The direct actions of cannabidiol and 2-arachidonoyl glycerol at GABAA receptors. *Pharmacological Research*, *119*, 358–370. https://doi.org/10.1016/j.phrs.2017.02.022

Blair, R. D. G. (2012). Temporal lobe epilepsy semiology. *Epilepsy Research and Treatment*, *2012*, 1–10. https://doi.org/10.1155/2012/751510

Denesyuk, A. I., Denessiouk, K. A., Korpela, T., & Johnson, M. S. (2002). Functional attributes of the phosphate group binding cup of pyridoxal phosphate-dependent enzymes. *Journal of Molecular Biology*, *316*(1), 155–172. https://doi.org/10.1006/JMBI.2001.5310.

Dhir, A. (2018). Cannabidiol in Refractory Epilepsy. *Studies in Natural Products Chemistry*, *58*, 419–438. https://doi.org/10.1016/B978-0-444-64056-7.00014-3

Elokely, K. M., & Doerksen, R. J. (2013). Docking Challenge: Protein Sampling and Molecular Docking Performance. *Journal of Chemical*

Information and Modeling, *53*(8), 1934–1945. NIH Public Access: 1934. https://doi.org/10.1021/CI400040D.

ElSohly M. A., Radwan M. M., Gul W., Chandra S., & Galal A. (2017). *Phytochemistry of Cannabis Sativa L. Phytocannabinoids* (pp. 1–36). Springer.

Epilepsy Fast Facts | CDC. (2021). Accessed November 3. https://www.cdc.gov/epilepsy/about/fast-facts.htm.

Excitatory, H., John Mihic, S., & Adron Harris, R. (1997). GABA and the GABAA Receptor. *Alcohol Health and Research World*, *21*(2).

Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., Sanschagrin, P. C., & Mainz, D. T. (2006). Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *Journal of Medicinal Chemistry*, *49*(21), 6177–6196. https://doi.org/10.1021/JM051256O/SUPPL_FILE/JM051256OSI20060602_023733.PDF.

Galanopoulou, A. (2008). GABAA receptors in normal development and seizures: friends or foes? *Current Neuropharmacology* 6 (1), 1–20. https://doi.org/10.2174/157015908783769653.

Greenidge, P. A., Kramer, C., Mozziconacci, J. C., & Wolf, R. M. (2013). MM/GBSA binding energy prediction on the PDBbind data set: successes, failures, and directions for further improvement. *Journal of Chemical Information and Modeling*, *53*(1), 201–209. https://doi.org/10.1021/CI300425V.

Guo, Z., Mohanty, U., Noehre, J., Sawyer, T. K., Sherman, W., & Krilov, G. (2010). Probing the α-helical structural stability of stapled P53 peptides: molecular dynamics simulations and analysis. *Chemical Biology & Drug Design*, *75*(4), 348–359. https://doi.org/10.1111/J.1747-0285.2010.00951.X.

Hill, T. D. M., Cascio, M. -G., Romano, B., Duncan, M., Pertwee, R. G., Williams, C. M., Whalley, B. J., & Hill, A. J. (2013). Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism. *British Journal of Pharmacology*, *170*(3), 679–692. Wiley Online Library https://doi.org/10.1111/bph.12321

Jacobson, M. P., Pincus, D. L., Rapp, C. S., Day, T. J., Honig, B., Shaw, D. E., & Friesner, R. A. (2004). A hierarchical approach to all-atom protein loop prediction. *Proteins*, *55*(2), 351–367. https://doi.org/10.1002/PROT.10613.

Jeremiah, S., & Povey, S. (1981). The biochemical genetics of human gamma-aminobutyric acid transaminase. *Annals of Human Genetics*, *45*(3), 231–236. https://doi.org/10.1111/J.1469-1809.1981.TB00334.X.

Karaźniewicz-Łada, M., Główka, A. K., Mikulska, A. A., & Główka, F. K. (2021). Pharmacokinetic drug–drug interactions among antiepileptic drugs, including CBD, drugs used to treat COVID-19 and nutrients. *International Journal of Molecular Sciences*, *22*(17), 9582. https://doi.org/10.3390/ijms22179582

Kim, J. J., & Hibbs, R. E. (2021). Direct structural insights into GABAA receptor pharmacology. *Trends in Biochemical Sciences*, *46*(6), 502–517. https://doi.org/10.1016/j.tibs.2021.01.011.

Kim, J. J., Gharpure, A., Teng, J., Zhuang, Y., Howard, R. J., Zhu, S., Noviello, C. M., Walsh, R. M., Lindahl, E., & Hibbs, R. E. (2020). Shared structural mechanisms of general anaesthetics and benzodiazepines. *Nature*, *585*(7824), 303–308. https://doi.org/10.1038/s41586-020-2654-5.

Klein, T. W., Newton, C., Larsen, K., Lu, L., Perkins, I., Nong, L., & Friedman, H. (2003). The cannabinoid system and immune modulation. *Journal of Leukocyte Biology*, *74*(4), 486–496. Wiley Online Libraryhttps://doi.org/10.1189/jlb.0303101

Kole, M. H. P., Hallermann, S., & Stuart, G. J. (2006). Single Ih channels in pyramidal neuron dendrites: properties, distribution, and impact on action potential output. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(6), 1677–1687. https://doi.org/10.1523/JNEUROSCI.3664-05.2006.

Kwan, P., Schachter, S. C., & Brodie, M. J. (2011). Drug-resistant epilepsy. *The New England Journal of Medicine*, *365*(10), 919–926. https://doi.org/10.1056/NEJMra1004418

Larivière, S., Bernasconi, A., Bernasconi, N., & Bernhardt, B. C. (2021). Connectome biomarkers of drug-resistant epilepsy. *Epilepsia*, *62*(1), 6–24. Wiley Online Libraryhttps://doi.org/10.1111/epi.16753

Maroso, M., Szabo, G. G., Kim, H. K., Alexander, A., Bui, A. D., Lee, S.-H., Lutz, B., & Soltesz, I. (2016). Cannabinoid control of learning and memory through HCN channels. *Neuron*, *89*(5), 1059–1073. Elsevierhttps://doi.org/10.1016/j.neuron.2016.01.023

Mathern, G. W., Beninsig, L., & Nehlig, A. (2015). Fewer specialists support using medical marijuana and CBD in treating epilepsy patients compared with other medical professionals and patients: result of epilepsia's survey. *Epilepsia*, *56*(1), 1–6. https://doi.org/10.1111/EPI.12843.

McClure, K. J., Maher, M., Wu, N., Chaplan, S. R., Eckert, W. A., Lee, D. H., Wickenden, A. D., Hermann, M., Allison, B., Hawryluk, N., Breitenbucher, J. G., & Grice, C. A. (2011). Discovery of a novel series of selective HCN1 Blockers. *Bioorganic & Medicinal Chemistry Letters*, *21*(18), 5197–5201. https://doi.org/10.1016/J.BMCL.2011.07.051.

Meldrum, B. S., & Rogawski, M. A. (2007). Molecular targets for antiepileptic drug development. *Neurotherapeutics: The Journal of the American Society for Experimental Neurotherapeutics*, *4*(1), 18–61. https://doi.org/10.1016/J.NURT.2006.11.010.

Napolitano, C. E., Magunacelaya, P., & Orriols, M. (2021). Absolute Spike Frequency and Different Comorbidities in Temporal Lobe Epilepsy. *Epilepsy & Behavior*, *116*, 107730. Elsevierhttps://doi.org/10.1016/j.yebeh.2020.107730

Nuutinen, T. (2018). Medicinal properties of terpenes found in Cannabis Sativa and Humulus Lupulus. *European Journal of Medicinal Chemistry*, *157*, 198–228. https://doi.org/10.1016/j.ejmech.2018.07.076

RCSB PDB: Homepage. (2021). Accessed November 4. https://www.rcsb.org/.

Richter, L., de Graaf, C., Sieghart, W., Varagic, Z., Mörzinger, M., de Esch, I. J. P., Ecker, G. F., & Ernst, M. (2012). Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands. *Nature Chemical Biology*, *8*(5), 455–464. https://doi.org/10.1038/nchembio.917.

Roos, K., Wu, C., Damm, W., Reboul, M., Stevenson, J. M., Lu, C., Dahlgren, M. K., Mondal, S., Chen, W., Wang, L., Abel, R., Friesner, R. A., & Harder, E. D. (2019). OPLS3e: extending force field coverage for drug-like small molecules. *Journal of Chemical Theory and Computation*, *15*(3), 1863–1874. https://doi.org/10.1021/ACS.JCTC.8B01026/SUPPL_FILE/CT8B01026_SI_002.ZIP.

Sastry, M., Matvey Adzhigirey, G., Day, T., Annabhimoju, R., & Sherman, W. (2013). Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. *Journal of Computer-Aided Molecular Design*, *27*(3), 221–234. https://doi.org/10.1007/S10822-013-9644-8.

Silverman, R. B. (2018). Design and mechanism of GABA aminotransferase inactivators. Treatments for epilepsies and addictions. *Chemical Reviews*, *118*(7), 4037–4070. https://doi.org/10.1021/ACS.CHEMREV.8B00009.

Salpekar, J. A., & Mula, M. (2019). Common psychiatric comorbidities in epilepsy: how big of a problem is it? *Epilepsy & Behavior 98*(Pt B), 293–297. https://doi.org/10.1016/j.yebeh.2018.07.023

Sampson, P. B. (2021). Phytocannabinoid pharmacology: medicinal properties of cannabis sativa constituents aside from the big two. *Journal of Natural Products*, *84*(1), 142–160. https://doi.org/10.1021/acs.jnatprod.0c00965

Shelley, J. C., Cholleti, A., Frye, L. L., Greenwood, J. R., Timlin, M. R., & Uchimaya, M. (2007). Epik: A software program for PK (a) prediction and protonation state generation for drug-like molecules. *Journal of Computer-Aided Molecular Design*, *21*(12), 681–691. https://doi.org/10.1007/S10822-007-9133-Z.

Sigel, E. (2002). Mapping of the benzodiazepine recognition site on GABA-A receptors. *Current Topics in Medicinal Chemistry*, *2*(8), 833–839. https://doi.org/10.2174/1568026023393444.

Tang, F., Hartz, A., & Bauer, B. (2017). Drug-resistant epilepsy: multiple hypotheses, few answers. *Frontiers in Neurology*, *8*, 301.

Tanguay, J., Callahan, K. M., & D'Avanzo, N. (2019). Characterization of drug binding within the HCN1 channel pore. *Scientific Reports*, *9*(1), 1–14. https://doi.org/10.1038/s41598-018-37116-2

Wongsamitkul, N., Maldifassi, M. C., Simeone, X., Baur, R., Ernst, M., & Sigel, E. (2017). α Subunits in GABAA receptors are dispensable for GABA and diazepam Action. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-15628-7

World Health Organization. Epilepsy. Key Facts. (2019). https://www.who.int/en/news-room/fact-sheets/detail/epilepsy.