CHAPTER «BIOLOGICAL SCIENCES»

DOCKING ANALYSIS OF THE INTERACTION OF PROPOXAZEPAM WITH BIOTARGETS THAT REGULATE ITS MECHANISM OF ACTION AND PHARMACOLOGICAL ACTIVITY

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Abstract. One of 1.4-benzodiazepine 3-alcoxy derivatives – propoxazepam, possessing high analgetic action, also effectively suppresses experimental seizures of different etiology. Unexpected combination of pharmacological spectrum components suggests its different binding sites of GABAA receptor subunits.

The aim of the work was docking analysis of the interaction of Propoxazepam with biotargets that mediate its mechanism of action and pharmacological activity (using experimental data of the propoxazepam conformation and calculated data for the three-dimensional structure of the ligand-binding site and subsequent docking to characterize its binding to this receptor)/

Materials and methods. X-ray diffraction studies of the compound were performed using Xcalibur 3 single crystal X-ray diffractometer. Calculation of the molecular docking parameters was performed using the iGEMDOCK v2.1 program for the GABA receptor (GABA (A) R-beta3 homopentamer, 4COF), the molecular structures of propoxazepam conformers were prepared using ChemAxon (MarvinSketch 17.11.0); study the binding energy of the TRPV1 receptor with the researched compounds,

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PDB: 8GFA – Cryo-EM structure of human TRPV1 in complex with the analgesic drug SB-366791 was used. The protein was modelled by using Protein Preparation of Schrodinger Suite; the protein structure was prepared by adding hydrogen atoms, optimizing hydrogen bonds. The ligands were prepared by LigPrep module of Schrödinger suite before proceeding for docking. The ligands were minimized using OPLS4 force field in Schrödinger suite. The docking and QSAR prediction were carried out with propoxazepam, SB-366791, RTX, capsazepin, capsaicin, diazepam, oxazepam, 3-hydroxopropoxazepam. To study the effect of the studied ligands on the NMDA receptor, it was used PDB:7EU7 – structure of the human GluN1-GluN2A NMDA receptor in complex with S-ketamine, glycine and glutamate.

Results. Based on the X-ray diffraction analysis, the coordinates of the atoms, bond lengths and valence angles in the propoxazepam molecule were calculated, it was found that it forms crystallographic twins as racemate. The molecular docking method showed that propoxazepam has several binding sites with the energy of complex formation from -78.64 to -85.29 kcal/mol exist on the isolated site of the GABA-receptor. The docking score of propoxazepam (-7.30 kcal/mol) indicates a stronger interaction with the TRPV1 receptor compared to oxazepam (-6.82 kcal/mol), 3-hydroxopropoxazepam (-6.49 kcal/mol), and capsazepin (-6.39 kcal/mol). Propoxazepam creates hydrogen bond with TYR 511 of the TRPV1 receptor as referent ligand SB-366791.

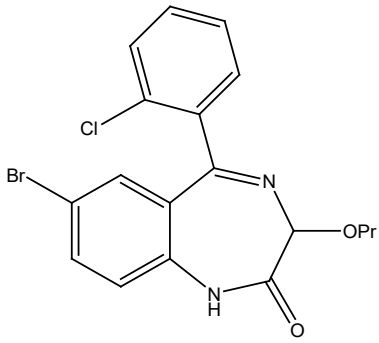
Conclusions. The highest contribution to the formation of the bond of the complex is carried out by residues of polar amino acids (serine, asparagine, methionine and arginine in polar binding sub-center). However, also for individual conformers, aromatic amino acids, predominantly phenylalanine (Phe-31, Ala-135 – hydrophobic binding sub-center) make a significant contribution. According to QSAR modelling, all studied compounds (3-hydroxopropoxazepam, diazepam, oxazepam, propoxazepam) have low pIC_{50} values, which could indicate a relatively low potency or affinity for TRPV1. The computational prediction, propoxazepam has one of the highest docking scores for (-6.77 kcal/mol), kynurenic acid (-6.60 kcal/mol), ketamine (5,34 kcal/mol) and N-methyl-D-aspartate (-4.32 kcal/mol). As a result of docking with the above ligands, it can be noted that most often a hydrogen bond was formed between Asn 616 with

the carbonyl group (H acceptor) or the secondary amine group and Phe 613 with the secondary amine group. The best pharmacophore (AHHR1) has been selected based on ketamine structure, which consist of H-bond acceptor site (A1), H-hydrophobic sites (H3, H2) and an aromatic ring (R5).

1. Introduction

Pharmacological properties of medicinal products are determined with their interactions with therapeutical targets (proteins and nucleic acids). The "drug-target" complex formation is tupical for chemical substances of various structure, which can be either agonists or antagonists (full or partial). In such complexes molecules-ligands are spatially (geometrically) complementary to binding site of the macromolecule surface and are rendered on it by the help of Coulombe`s forces, Van-der Vaal interactions, hydrogen bonds etc.

Among the different biotargets (receptors, enzymes, transporters, ione channels and others) of the drug`s action the main attention is paid to $GABA_A$-receptor complex ($GABA_A$-RC), impairments in which are associated with such disorders as schizophrenia, bipolar disorders, insomnia epilepsy alcoholism and others [1, p. 445–450; 2, p. 815–850]. In addition to GABA-binding site the receptor complex contains alosteric segments, which are capable to bind benzodiazepines, widely used for such disorders treatment [1, p. 445-450; 3, p. 2755–2775]. However, for these substances insufficient action selectivity, leading to different side effects, still remains the main problem. In this way, $GABA_A$ receptor and its subtypes selective ligands finding is still actual and this is directed on creating of new medicines for these disorders treatment, as well as for cognitive function stimulation.



Chemical structure of propoxazepam (I)

Recently our attention was attracted by 3-alcoxy substituted 1.4-benzodiazepine derivatives which, despite others members of this class, on the models of nociceptive pain shared the prominent analgesic

activity [4, p. 141–148; 5, 427–432; 6, p. 3–11]. One of them, named propoxazepam – 7-bromo-5-(o-clorophenyl)-3-propyloxy-1,2-dihydro-3H-1,4-benzodiazepine-2-one (I) is considered to be the promising analgetic.

Paying attention that such antiepileptic drugs as gabapentin and pregabalin successfully used for neuropathic pain treatment [7, p. 1475–1482; 8, p. 233–249] we have undertaken the studies of anticonvulsive action of this compound. Earlier we [9, p. 251–260] determined propoxazepam the mean effective doses ($ЕД_{50}$) on the models of chemically induced seizures by picrotoxin (1,67±0,09 mg/kg), penthylenetetrazole (0,9±0,04 mg/kg) and strychnine (14,24±0,47 mg/kg), which prove that substance high anticonvulsive activity.

On the base of "dose-effect" curves shapes there were demonstrated different stages of propoxazepam interaction with GABA and glycine receptors in vivo. It is assumed that obtained data prove the preferential propoxazepam anticonvulsive effect realization through GABA-ergic mechanisms. Glycine-ergic components, participating in strychnine-induced seizures suppression, are involved in the process when propoxazepan is administered in doses higher than $ED5_0$ and obviously serve as additional anticonvulsive protection mechanism.

On the model of thiosemicarbazide-induced GABA-deficient seizures propoxazepam had shown the high activity and on the base of "dose-response" curve shape one can assume the antagonistic interaction with GABA synthesis inhibitor – thiosemicarbazide [10, p. 34–39].

The use of mentioned methods of "pharmacological probing" for propoxazepam action let reliably reveal the possible structural-functional sites of GABA-RC, which are responsible for neuronal effects realization on the whole organism level. On the stage of new (original) medicines the different approaches with computation technologies are used. Recently the most effective one is docking procedure, which estimates energetic parameters of molecular matching of ligand (pharmacophore groups separately or the whole structure) to functionally important protein (receptor) sites. Using the docking mechanism one can suggest the molecules interactions, determine spatial structure of complexes and affinity of conformation-dependent interactions. The docking algorithms of low-molecular weights ligands to receptor molecules are essential instrument for rationale drug design and are applied on different stages of medicines R&D process (screening, ligand

action mechanism clarification, identification of receptor sites, involved in intermolecular interactions). In this case are necessary both the data about spatial ligand structure and three-dimension structure of target protein binding site, obtained by X-ray diffraction methods.

Also a founding member of the vanilloid subfamily of TRP channels, TRPV1, represents one of the most sought-after pain therapy targets. The need for selective TRPV1 inhibitors extends beyond pain treatment, to other diseases associated with this channel, including psychiatric disorders [15]. TRPV1 has been identified as a promising therapeutic target to reduce pain perception and itch sensation under pathological conditions. It is also involved in the regulation of several physiological and pathological processes; therefore, it has been also considered in the development of therapies against schizophrenia, epilepsy, diabetes, ischaemia, chronic cough, etc. TRPV1 ligands can be classified to agonists and antagonists. The therapeutic role of TRPV1 agonists is based on the desensitisation of pain-conducting nerve fibres, which contributes to analgesic effects. In general, the majority of the potent TRPV1 agonists reported until today contain the vanillyl group; however, several examples show that this group can be replaced by similar chemical groups [16, p. 2169–2178].

In turn the NMDA receptor (NMDAR) has become known as a potential target for the treatment of neurodegenerative diseases. Thus, the discovery of NMDA antagonists has attracted much attention in recent years [17, p. 125–145].

Blockers of NMDAR channels are of medical interest because of their potential to treat depression, Alzheimer's disease, and epilepsy. However, the precise mechanisms underlying channel binding and gating remain limited due to challenges in obtaining high-resolution binding site structures within transmembrane domains [18, p. 507–518].

NMDA have been implicated as potential mediators of pain-related neuroplasticity in the peripheral nervous system (PNS), and mediate excitatory synaptic transmission and synaptic plasticity in the central nervous system (CNS) [19, p. 301–305].

The aim of the work was docking analysis of the interaction of Propoxazepam with biotargets that regulate its mechanism of action and pharmacological activity (based on experimental data of conformations of Propoxazepam and calculated data of the three-dimensional structure of the

ligand binding site, as well as subsequent ligand-receptor docking with a description of its process).

2. Materials and methods

The X-ray diffraction study of the substance was performed on monocrystal X-ray difractometer Xcalibur 3 (MoKα radiation, CCD-detector, graphite monochromator, ω-scanning, $2\Theta_{max}$ = 50) using SHELXTL-97 software [11, p. 112–122] according to the standard method (MoKα-radiation, *T* 130(2) K, ω-scanning with step 1°). 0.882). The sample was decoded in two spatial groups: three-wedged with cell parameters *a* = 10.434(1), *b* = 10.873(1), *c* = 17.837(2) Å,α = 74.810(9), β = 77.22(1), γ = 66.06(1), V = 1769.6 Å^3, spatial group P$\bar{1}$. Monoclinic with cell parameters *a* = 11.695(2), *b* = 21.507(2), *c* = 14.506(2) Å, β = 92.82(1), V = 3644.2) Å^3, spatial group $P2_1/c$. Minimal divergence factor for triclinic structure was 38 %, while for monoclinic 25 %.

Compound structure was decoded by direct method in isotropic approximation and specified in anisotropic approximation for non-hydrogen atoms. Hydrogen atoms were placed in geometrically calculated sites and included in the refinement on the "rider" model in isotropic approximation with $U_{iso} = nU_{eq}$. Of the non-hydrogen atom, connected with the given hydrogen (n=1.5 for methyl groups and n=1.2 for other hydrogen atoms). The structure was refined using F^2 polymatrix LSM in the anisotropic approximation for non-hydrogen atoms to wR_2 = 0.289 on the base of 6284 reflections (R_1 = 0.106 on the base of 2142 reflections c $F > 4\sigma(F)$, S = 0.882).

Molecular docking parameters calculation was made using iGEMDOCK v2.1 software [12, p. 288–304; 13, p. 1455–1474] (freeware, http://gemdock.life.nctu.edu.tw/dock/download.php). As macromolecule the GABA-receptor (crystalline structure, GABA(A)R-beta3 homopentamer, 4COF) was chosen, being received from biological macromolecules database (http://www.rcsb.org/) as *.pdb file. At the same file extension the ligand structure was also prepared. Propoxazepam conformers molecular structures were prepared using ChemAxon (MarvinSketch 17.11.0) software, conformers internal energy calculations were carried out on Avogadro (v 1.2.0) software, cavities analysis and mutual amino acids residues in active centers – on the base of Mole 2.13.9.6. Docking parameters calculation for ligand and receptor was performed using force field data

on 100 generations of flexible ligand conformations (300 states for each population size); from 20 number of solutions the most optimal was chosen. Automatic binding site detection was determined by the referent ligand localization (benzamidine) [14, p. 270–275].

Binding site radius was enlarged to 30 Å for substance binding visualization with simultaneous ligand excluding. Docking results were grouped according to the hierarchic clustering procedure. Clustering was performed using K-means after previous estimation of binding localization topography due to total interaction energy, as well as hierarchic clustering.

In order to study the binding energy of the TRPV1 receptor with the researched compounds, 8GFA – Cryo-EM structure of human TRPV1 in complex with the analgesic drug SB-366791 was utilized. The protein was modelled by using Protein Preparation of Schrödinger Suite; the protein structure was prepared by adding hydrogen atoms, optimizing hydrogen bonds. The ligands were prepared by LigPrep module of Schrödinger suite before proceeding for docking. The binding free energy was calculated using the generalized Born surface area. The docking and QSAR prediction were carried out with propoxazepam, its possible metabolite 3-hydroxopropoxazepam, diazepam, oxazepam, SB-366791, RTX, capsazepin, capsaicin. Automatic binding site detection was determined by the referent ligand localization (the analgesic drug SB-366791). The protein- ligand complex interactions were calculated based on the quality of geometric contacts and their energy. The docking results were analysed by using docking score (calculated noncovalent three-dimensional interactions between a ligand and a protein), gscore, and other interaction type (metal-binding + rewards + penalty for freezing rotatable bonds + polar interactions in the active site). Ranking was given to the ligands based on their G-scores using the following formulae G-score = 0.05*vdW + 0.15*Coul + Lipo + + Hbond + Metal + Rewards + RotB + Site (1), where vdW was the Van der Waals energy, Coul represents the Coulomb energy, Lipo term explains the Lipophilic, Rewards describes the favorable hydrophobic interactions, Hbond means Hydrogen-bonding term, Metal gives the information about metal-binding RotB tells about penalty for freezing rotatable bonds and Site defines polar interactions in the active site. The QSAR build model using the automated QSAR panel of Maestro Schrödinger Suite. For building QSAR model we used 408 TRPV1 antagonists being comparable between

each other. List of these substances we took from Supporting Information of the article of Pharmacoinformatics Research Group Univ.-Prof. Dr. Gerhard F. Ecker [20, p. 555–562]. *MM-GBSA binding free energy calculations:* Prime module in the Schrodinger suite was used to calculate the binding free energies of the complexes. The binding energy is calculated according to the equation: DG bind = E_complex(minimized) – E_ligand(minimized) – E_receptor(minimized).

Pharmacophore model. Multiple ligand-based pharmacophore models were developed using Phase Schrodinger. The source of molecules to create the data set was the chemical database ChEMBL [21]. The model was validated against a library of decoy and active sets. To verify the pharmacophore theory, a test kit database was created consisting of the recognized NMDA inhibitors added to molecules derived from the DUDE database and treated as inactive [22, p. 6582-94].

To study the effect of the studied ligands on the NMDA receptor, it was used 7EU7 – structure of the human GluN1-GluN2A NMDA receptor in complex with S-ketamine, glycine and glutamate. The docking was carried out with ketamine, kynurenic acid, N-methyl-D-aspartate and propoxazepam on the binding site of ketamine with GluN1-GluN2A NMDA.

3. Results and discussion

Propoxazepam molecular structure

Propoxazepam crystalline sample X-ray analysis (Figure 1) had shown that it exists as crystallographic twins with different twinning degree, forming during crystallization. The highest precise resolution of twinning was made using Platon software, showing presence of more than two components.

Molecule has asymmetric center at C8 (Figure 2) and is crystallized in center-symmetric spatial group, forming racemate crystals. In the independent unit cell two molecules were found (A and B) with different conformations and asymmetric atom configurations (A molecule with R-configuration while B molecule with S-configuration).

Diazepine cycle is in the bath conformation, N1, C9, C7, N2 atoms are coplanar while C1, C6 and C8 atoms are deflected to one side of this plane on 0.70 Å, 0.65 Å and 0.75 Å in the molecule A accordingly and on -0.69 Å, -0.66 Å and -0.74 Å in molecule B accordingly. As a result chlorobenzyl

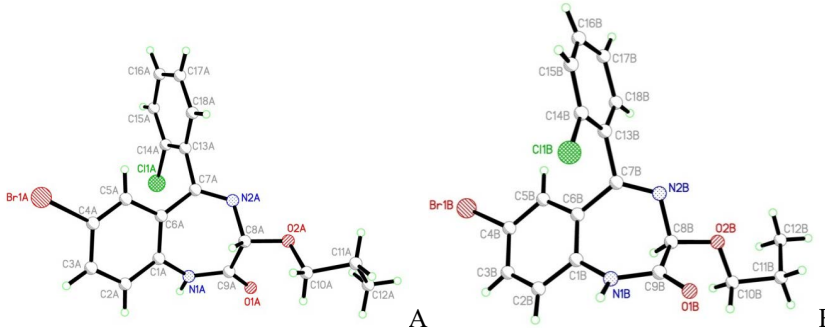


Figure 1. Common view of propoxazepam molecule (A and B) as represented by elipsoids of thermal oscillations with 50 % possibility

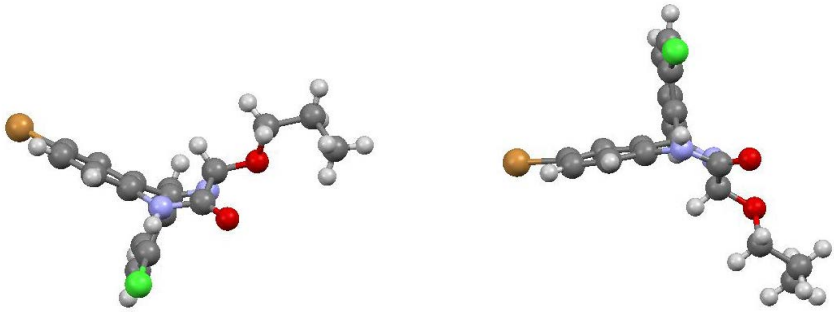


Figure 2. Molecules A and B conformations

substituent deflects from the plane bicycle fragment in the different sides and is rotated in relation to endocyclic C6-C7 bond on the equal angle, but to different directions (torsion angles C1-C6-C7-C13 and C6-C7C13-C14 are 144(1) and -73(1)o in the molecule A and -144(1) and 73(1) in the molecule B). The main difference in the unit cell structure, determining the presence of two molecules in it, is in propyl group conformation. The substituent at C8 position has equatorial orientation (torsion angle N1-C9-C8-O2 is 172.1(8)° in the molecule A and -170.4(8) in the molecule Б), propyl group in the molecule A is situated nearly orthogonally

to the cycle and has *ap* – *-sc-* conformation. Propyl group in the molecule B is in *+sc-* position in relation to endocyclic C9-C8 bond and has *ap* – *-sc-*conformation. The relevant torsion angles:

	Molecule A	Molecule B
C9-C8-O2-C10	-88(1)	75(1)
C8-C2-C2-C11	-172(1)	175.5(8)
O2-C10-C11-C12	-82(1)	-63(1)

In the crystal molecules A and B alternate forming chains along the crystallographic direction [1 0 0] because of intermolecular hydrogen bonds N1a-H…N2b' H…N 2.24 Å N-H…N 162°, N1b-H…N2a' (x-1, y, z) H…N 2.22 Å N-H…N 173°.

Atom coordinates, bonds lengths and valent angles in the propoxazepam molecule were also calculated.

Structure and properties of the $GABA_A$-receptor complex.

GABAA-receptor complex ($GABA_A$-RC) belongs to the ligand-depending ionic channels class and is the main therapeutic target, participating in the human physiological processes: education and memory formation, awaking and sleeping. The terms GABA-benzodiazepine-receptor ionophoric complex, GABA-benzodiazepine-ionophore and others are also often appear. In these terms not only complicity but tight junctions between its components are reflected.

$GABA_A$-RC is built with five subunits, which belong to different classes (α, β, γ, δ, ε, π, θ, ρ), forming the symmetrical ion channel, posing with second transmembrane domain to each other. At present from the mammals nervous system there were cloned and sequined six α-, three β-, three γ-, one δ-, one ε-, one π-, one θ-, and three $GABA_A$-PC ρ-subunits, as well as forms which form as a result of alternative splicing of some of these subunits [1–4]. The most common subunits combination in the CNS (about 40 % of GABAA-RC) is formed from two α1, two β2 and one γ2s, surrounding the chloride-transporting pore. When binding two GABA molecules complex changes its conformation, opens pore for anions transport and as a result hyperpolarization develops leading the cell to be less sensitive to excitement signals of other neurons – the process accompanied with postsynaptic inhibitory potential development.

The main ligand-binding of the $GABA_A$-RC is that of GABA (agonist) binding site which is situated in the area between α-and ρ- subunits contact. On the surface of α and γ subtypes contacts the benzodiazepine binding site is located. Barbiturates and ethanol binding sites are supposed to be located on the transmembrane domains in the deep of the channel. In the first case, perhaps, the main role is played by β-subunit while ethanol interacts with different subunits, including ρ and δ, however with different sensitivity.

Subunits combination in the pentamer determine ligand pharmacological profile. It was found that benzodiazepines pharmacological action as well as their analogs is primarily determined by α-subunits subtype. Particularly, α1-selective ligands usually posses tranquilizing, anticonvulsive amnesic action; α2 and α3 – anxyolytic hypnotic, anticonvulsive and muscle relaxation, though α5-selective – stimulate education and memory processes [2]. Thus new ligands have to be highly selective to the certain $GABA_A$-RC subtype for sharing unique therapeutic properties simultaneously lucking side effects, inherent to classical benzodiazepines.

The one of main molecular docking aims is new possible binding sites search. Correct screening algorithm have to determine and estimate as much as possible the modes of two molecules interaction. However this process can be too calculation- and time consuming. Due to this there have to be balance between computer process costs and screening space. As a screening algorithm the method of energy interaction estimating, calculated in accordance to ligand and receptor cavity fields, was used.

Despite the other 1.4-benzodiazepine derivatives, propoxazepam, as alcoxy derivative, possess mainly analgesic action in its pharmacological spectrum while inhibitory action (muscle relaxation, hypnotic and tranquilizing) is markedly reduced. As the alcoxy radical is not the dramatic change in the substance structure, using molecular docking results there was made the attempt to analyze this flexible substituent influence on the ability to be bind to the GABA receptor. Detailed docking with this receptor was analyzed on the base of 20 number of solutions (19 conformations and one non-optimized structure, excluded from further analysis) for each the optimal conformation 100-times generated in 500 approaches. The most optimal from the energetic point of view (minimal energy) conformers further were estimated as the most favourable and effective (according to the binding energy).

It was rather unexpected that generated propoxazepam conformers had not the only one binding site (Figure 3). Maximal biding energies difference for all the conformers is 6,65 kcal/mole (from -78,64 to -85,29 kcal/mole), that is not a big value but can be significant in the substance low concentrations in brain in vivo. Cluster analysis revealed six binding sites with one only conformer (№ 5) separate binding, which can be explained as non-specific binding with surface hydrophobic regions. This suggestion is supported also with the low complex formation energy (-79,76 kcal/mole, Table 1).

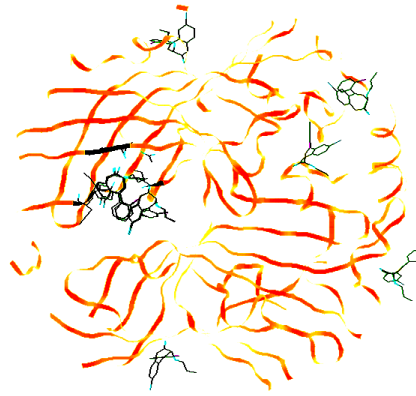


Figure 3. Schematic localization of propoxazepam conformers preferred binding sites

The rest conformers form five classes (clusters) second of them is the most numerous (Table 1) with similar binding energy values. Though one have to mention that similar values of complex formation total energy consist of Van-der-Vaal interaction energies and hydrogen bond energies with quite different impacts. As the benzodiazepine "bath" conformation is very rigid and changes negligibly, the difference can be due to conformation mobility/flexibility of alcoxy (propyl) radical. In is confirmed by the calculated every conformer intrinsic energy with nearly similar for each representative (Table 1).

As the alcoxy radical flexibility plays such a big role for binding site preference, the main amino acids residues, participating this process were also determined. For data reduction to the most significant in a z-normalization procedure the binding energy values, nearest to the representative mean, for each amino acid residue were selected (which equals to z=0, Table 2). For conformer № 5 with low binding energy value there haven't been reveled amino acid residues with energy, near to mean values of other conformers. As it was found earlier, the main impact in the complex formation residues of polar amino acids which can fix polarization-

Table 1

Calculated propoxazepam conformers binding energy and their internal energy

Cluster number	Ligand conformer (№)	Total binding energy, kcal/mole	Van-der-Vaal interaction	Hydrogen bonds	Internal conformer energy, kcal/mole
1	5	-79,76	-61,50	-18,25	91,48
2	2	-80,01	-76,51	-3,50	91,50
2	3	-78,64	-61,72	-16,92	91,39
2	4	-81,96	-62,99	-18,97	91,41
2	9	-82,00	-63,03	-18,98	91,44
2	11	-79,98	-76,48	-3,50	91,53
2	12	-80,05	-76,55	-3,50	91,42
2	16	-80,04	-76,54	-3,50	91,47
2	19	-81,99	-63,00	-19,00	91,45
3	1	-83,75	-69,59	-14,17	91,45
3	8	-83,76	-69,59	-14,17	91,49
4	13	-83,49	-67,85	-15,63	91,45
4	14	-83,49	-67,75	-15,74	91,47
4	18	-83,49	-67,74	-15,75	91,44
5	6	-85,29	-74,91	-10,38	91,41
5	10	-85,29	-74,83	-10,46	91,51
5	15	-85,28	-74,86	-10,42	91,50
6	7	-82,29	-68,93	-13,36	91,48
6	17	-82,29	-68,95	-13,34	91,47

able parts and groups of ligand (M-Ser-10, M-Asp-30, S-Asn-100, S-Met-137, S-Lys-13, M-Asp-30, S-Arg-71, M – main chain, S – side chain). Though for some conformers large contribution have aromatic amino acids, mainly phenylalanine (M-Phe-31, S-Phe-31, M-Ala-135) and even glycine (M-Gly-32). Because propoxazepam conformers are bind with the polar amino acids residues, one can suggest that flexibility of the alcoxy radical can acquire conformations with ether oxygen more available for binding.

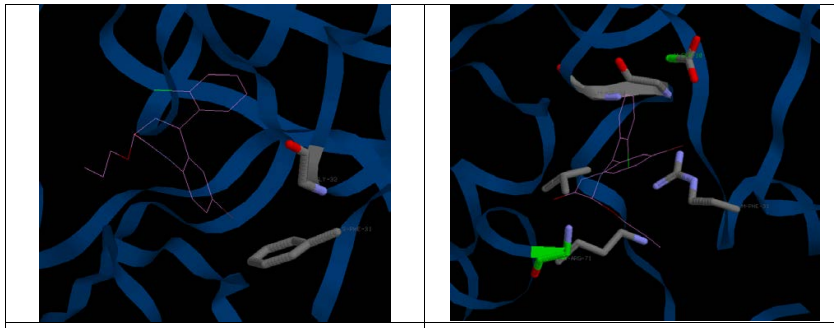
Table 2

Amino acids residues which participate bonds formation with different propoxazepam conformation (bond energies normalization at z=0, M – main chain, S – side chain)

Conformer number	6	10	15	1	8	4	9	19	2	11	12	16	3	13	14	18
Energy, kcal/ mole	-85,3	-85,3	-85,3	-83,8	-83,8	-82,0	-82,0	-82,0	-8,0	-82,0	-82,0	-82,0	-78,6	-83,5	-83,5	-83,5
M-LEU-99									-3,5	-3,5	-3,5	-3,5				
S-ASN-100						-3,5	-3,5	-3,5					-1,9			
M-ALA-135						-7,0	-7,0	-7,0					-6,6			
M-THR-151						-3,5	-3,5	-3,5					-3,5			
S-THR-151						-5,0	-5,0	-5,0					-5,0			
M-SER-10	-5,5	-5,7	-5,6													
M-LYS-13	-3,2	-3,1	-3,7													
S-ASP-30														-2,5	-2,5	-2,5
S-ASP-69														-5,5	-5,6	-5,6
M-LYS-70														-3,5	-3,5	-3,5
S-LEU-27	-4,2	-4,2	-4,2													
M-ASP-30	-5,9	-5,9	-5,9	-0,5	-0,5											
S-ASP-30	-4,4	-4,4	-4,4													
M-PHE-31	-8,2	-8,2	-8,2	-3,3	-3,3											
S-PHE-31	-6,3	-6,3	-6,3	-5,7	-5,7											
M-GLY-32	-4,7	-4,7	-4,7	-4,7	-4,7											
S-ARG-71	-6,6	-6,5	-6,5													
M-LEU-99						-0,2	-0,2	-0,2	-4,7	-5,0	-4,6	-4,6	-0,3			
S-LEU-99						-0,3	-0,3	-0,3	-5,2	-4,8	-5,0	-5,0	-0,4			

(End of Table 2)

Conformer number	6	10	15	1	8	4	9	19	2	11	12	16	3	13	14	18
M-ASN-100						-1,7	-1,7	-1,7	-11,8	-11,1	-11,9	-11,9	-2,4			
S-ASN-100						-7,0	-7,0	-6,9	-4,3	-4,4	-4,5	-4,5	-6,6			
M-ASP-101									-5,7	-5,9	-5,6	-5,6	-0,3			
M-CYS-136						-1,2	-1,3	-1,3					-8,8			
M-MET-137						-1,5	-1,5	-1,5					-9,7			
S-MET-137						-5,8	-5,8	-5,8	-0,7	-0,1	-0,7	-0,7	-5,4			
S-GLU-153						-5,5	-5,5	-5,4	-1,7	-1,8	-1,8	-1,8	-5,2			
S-LYS-13														-6,9	-6,9	-6,9
S-LYS-13	-12,6	-12,6	-12,6													
M-PRO-29														-6,5	-6,5	-6,5
M-ASP-30														-8,5	-8,6	-8,6
M-ALA-45						-0,3	-0,4	-0,3	-9,8	-9,7	-9,8	-9,8	-0,6			
S-SER-46						-1,4	-1,5	-1,5	-5,1	-5,9	-5,3	-5,3	-1,3			
M-ARG-71														-4,2	-4,3	-4,3
S-ARG-71														-14,7	-14,6	-14,6



A B

Figure 4. Spatial propoxazepam arrangement when binding with esidues of phenilalanine and glycine (A) and phenilalanine, asparagine, arginine and serine (B)

Docking results visualization had shown that when interacting with phenylalanine residues propoxazepam conformer is situated in the way of the main influence to be fulfilled through bromine atom (Figure 4, A).

On the contrary the more polar subcentre carries out the binding not only via phenylalanine residue (through bromine atom), but also with more polar amino acids – asparagine and arginine (Figure 4, B).

Docking analyses of propoxazepam with TRPV1 receptor using GLIDE module.

Based on the results of docking, the values of the gscore of interaction, as well as its components – hydrophobic interactions and hydrogen bonding for researched ligands (propoxazepam, its possible metabolite 3-hydroxopropoxazepam, diazepam, oxazepam, SB-366791, RTX, capsazepin, capsaicin) in the binding site of TRPV1 receptor were determined. Molecular docking was provided for each molecule of ligand per subunit of hTRPV1 tetramer (Table 3).

The findings indicate that the reference compound SB-366791 has the lowest docking scores and MMGBSA free energy of binding across binding sites on all four chains, which means that this compound has the best affinity for the TRPV1 receptor than the other ligands. Specifically, the docking score of propoxazepam (-7.30 kcal/mol) indicates a stronger interaction with the TRPV1 receptor compared to oxazepam

Table 3

Docking scores using GLIDE module chain A of TRPV1 receptor

Ligand	docking score	gscore	lipo[1]	hbond[2]	Evdw[3]	ecoul[4]	other interaction types5
Capsaicin	-7.71	-7.71	-3.73	-0.30	-39.98	-9.01	-0.33
Capsazepin	-6.39	-6.40	-3.18	0	-32.18	-4.09	-0.99
3-hydroxo-propoxazepam	-6.49	-6.49	-2.43	0	-29.38	-6.11	-1.68
Diazepam	-7.66	-7.66	-3.05	-0.21	-28.39	-4.67	-2.29
Oxazepam	-6.82	-6.82	-2.60	0	-26.09	-5.17	-2.41
Propoxazepam	-7.30	-7.30	-3.15	-0.57	-35.73	-4.52	-1.12
RTX	-8.23	-8.23	-4.02	-0.32	-46.03	-4.67	-0.9
SB-366791	-9.54	-9.54	-4.48	-0.32	-42.82	-6.50	-1.64

[1]**lipo** (lipophilic contact), [2]**hbond** (hydrogen bond), [3]**evdw** (Van der Waals energy), [4]**ecoul** (Coulomb energy), [5]**other interaction type** (metal-binding + rewards + penalty for freezing rotatable bonds + polar interactions in the active site)

(-6.82 kcal/mol), 3-hydroxopropoxazepam (-6.49 kcal/mol), and capsazepin (-6.39 kcal/mol). The docking score of propoxazepam in chain C and B is lower than that of diazepam, resulting in stronger interaction than that of diazepam (Table 3).

Furthermore, propoxazepam demonstrates a lower value of MMGBSA free energy of binding compared to oxazepam and 3-hydroxopropoxazepam. When considering the increase in the free energy of interactions, the ligands can be ranked as follows: SB-366791 > Capsaicin > RTX > Capsazepin > > Propoxazepam > Diazepam > 3-hydroxopropoxazepam > Oxazepam. However, in chains B, C, and D, propoxazepam has a better MMGBSA free energy value than capsazepin (table. 4).

Propoxazepam establishes two hydrogen bonds: one involving the NH group of the amide (resulting in a hydrogen bond interaction with the linker-neck) and THR 550 of the protein, and another between oxygen of the alkoxy group (hydrophobic tail) and TYR 511 of the TRPV1 receptor. Ligands with confirmed effects on the TRPV1 receptor also engage in interactions with the protein by forming hydrogen bonds with the same amino acids as the benzodiazepines, namely THR 550 and TYR 511. Capsaicin uses oxygen of the amide group to form a hydrogen bond with THR 511 of the receptor and the hydroxyl group of the benzene ring with

Table 4

Energy of binding of receptor ligand complex calculated using Prime MMGBSA method chain A of TRPV1 receptor

Ligand	MMGBSA_ dG_Bind	Cou-lomb[1]	Cova-lent[2]	H-bond[3]	Lipo[4]	Pack-ing[5]	Solv_GB[6]	vdW[7]
Capsaicin	-61.98	-26.45	2.09	-1.53	-29.45	-0.44	37.67	-43.88
Capsazepin	-48.45	-15.09	-0.46	-0.83	-25.12	-2.39	27.59	-32.15
3-hydroxo-propoxazepam	-38.01	-18.48	7.67	-1.01	-15.89	-0.63	20.97	-30.65
Diazepam	-43.14	-13.40	4.54	-0.42	-21.35	-0.72	16.18	-27.95
Oxazepam	-37.51	-16.81	4.18	-1.02	-18.44	-0.52	23.46	-28.37
Propoxazepam	-40.96	-15.60	8.51	-1.27	-23.59	-0.59	30.53	-38.95
RTX	-61.12	-13.09	4.96	-0.70	-36.81	-0.25	41.22	-56.45
SB-366791	-71.63	-18.84	0.799	-0.77	-29.01	-1.22	24.22	-46.83

[1]**Coulomb** (Coulomb energy), [2]**Covalent** (Covalent binding energy), [3]**H-bond** (Hydrogen-bonding correction), [4]**Lipo** (Lipophilic energy), [5]**Packing** (Pi-pi packing correction), [6]**Solv_GB** (Generalized Born electrostatic solvation energy), [7]**vdW** (Van der Waals energy)

GLU 570. Capsazepin establishes a hydrogen bond via its OH group with THR 550. In the interaction between RTX and the TRPV1 receptor, TYR 511 plays a crucial role as this amino acid forms a hydrogen bond with the ester group of region B. Regarding the reference compound SB-366791, it forms a single hydrogen bond between the oxygen of its amide group and TYR 511 (Figure 5).

QSAR analyse of the interaction propoxazepam with TRPV1. In the research, the model kpls_desc_19 was chosen because it demonstrates the highest score 0,63. This model has an R-squared value for the regression (the coefficient of determination) is 0.6445, which is the second-highest among the models. R-squared measures how well the model fits the training data, and a value of 0.6445 indicates a reasonably good fit. "kpls_desc_19" has an RMSE of 0.6601, which is not the lowest but is still competitive. RMSE measures the average prediction error, and lower values are preferred. While it's not the lowest RMSE, it's still within an acceptable range. "kpls_desc_19" has a Q^2 value of 0.6430, indicating good predictive performance on new, unseen data. This suggests that the model is likely to make accurate predictions beyond the training dataset. Q^2 MW (Null hypothesis): Chosen model has a Q^2 MW value of 0.0042, which is positive and suggests that it performs significantly better than a null hypothesis model. Propoxazepam

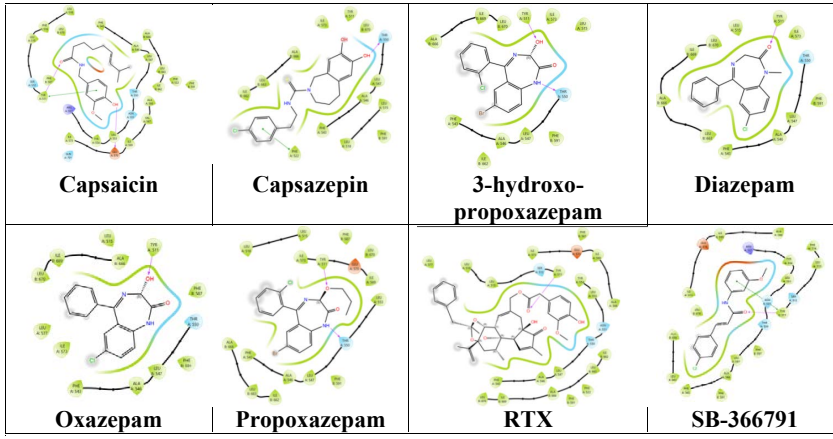


Figure 5. Visualization of location in specific binding sites of investigated ligand in the binding site of TRPV1

has the highest predicted pIC_{50}, it means that the model predicts them to be the most potent among the compounds. Diazepam and Oxazepam have the lowest predicted pIC_{50} values, indicating that the model predicts them to be less potent among the compounds.

Because of the minus sign, higher pIC_{50} values indicate exponentially more potent inhibitors. Diazepam and Oxazepam have the lowest predicted pIC_{50} values, indicating that the model predicts them to be less potent among the compounds. Propoxazepam has the highest predicted pIC_{50}, it means that the model predicts it to be the most potent among the compounds (Table 5).

Table 5

Predicted IC_{50} of investigated compounds using best model kpls_desc_19

	3-hydroxo-propoxazepam	Diazepam	Oxazepam	Propoxazepam
Pred pIC_{50}	-1.958	-2.283	-2.253	-1.115

Lower pIC_{50} values (closer to negative infinity) suggest lower potency, meaning that the compound has a weaker affinity for the target and is less likely to affect the target's activity significantly. In our case, with a pIC50 value of -1.115, propoxazepam is predicted to have relatively low potency or affinity for TRPV1, but this value is higher than other ligands.

Docking analyses of propoxazepam with N-methyl-D-aspartate receptor using GLIDE module.

Pharmacophore model of the ligand of NMDA receptor. The dataset was divided into active and inactive sites. AHHR1 was chosen as the best pharmacophore model for the aforesaid dataset of compounds. As shown in Figure 6 AHHR1 consists of four features: H-bond acceptor site (A1), H-hydrophobic sites (H3, H2) and an aromatic ring (R5).

Based on the results of docking, the values of the gscore and MMGBSA dg Bind, as well as its components – hydrophobic interactions and hydrogen bonding for ketamine, kynurenic acid, N-methyl-D-aspartate and propoxazepam with the NMDA site were determined.

The interaction in each of the binding sites is determined by the contribution to the overall process of certain amino acid residues, which determine the type and strength of the interaction. For their characterization, the amino

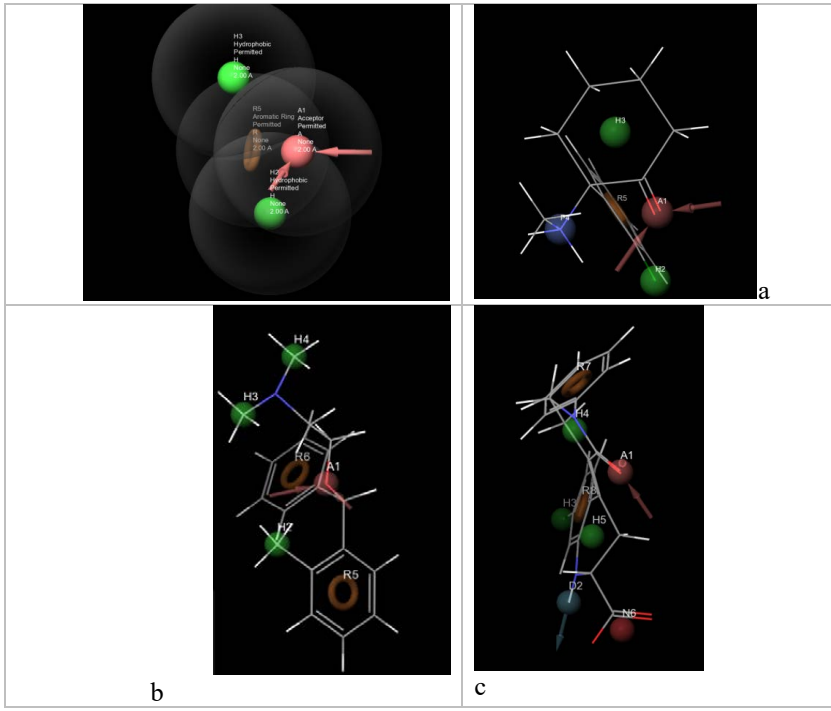


Figure 6. Geometry of the pharmacophore hypothesis with highest score:
a – Ketamine, chembl742; b – Orphenadrine, chembl900; c – GW468816, chembl1207366

acid residues with the greatest contribution to the overall interaction effect of each of the compounds were identified. Amino acid residues capable of interaction due to both strong hydrogen bonds and weaker van der Waals forces are marked separately, since this ability contributes to more effective interaction and the formation of a macromolecule-ligand complex.

Ketamine seems to interact moderately to strongly with the binding pockets of NMDA.

The table 6 showed that in the computational prediction, propoxazepam has one of the highest docking scores for (-6.77 kcal/mol), kynurenic acid

(-6.60 kcal/mol), ketamine (5,34 kcal/mol) and N-methyl-D-aspartate (-4.32 kcal/mol).

Table 6

Docking scores using GLIDE module

Maestro properties	Ketamine	Kynurenic acid	N-methyl-D-aspartate	Propoxazepam
gscore	-5.34	-6.60	-4.32	-6.77
Lipo[1]	-2.11	-1.86	-0.38	-2.81
Hbond[2]	-0.12	-0.32	-0.29	-0.57
Evdw[3]	-23.07	-22.84	-14.73	-35.60
Ecoul[4]	-2.29	-5.78	-14.15	-5.82
other interaction types[5]	-1,61	-2,42	-0,79	-0,72

[1]**lipo** (lipophilic contact), [2]**hbond** (hydrogen bond), [3]**evdw** (Van der Waals energy), [4]**ecoul** (Coulomb energy), [5]**other interaction type** (metal-binding + rewards + penalty for freezing rotatable bonds + polar interactions in the active site)

The binding free energy was calculated using the generalized Born surface area (MM-GBSA – Molecular Mechanics, the Generalized Born model and Solvent Accessibility) calculation of molecular mechanics (Table 7).

Table 7

Energy of binding of receptor inhibitor complex calculated using Prime MMGBSA method

	Ketamine	Kynurenic acid	N-methyl-D-aspartate	Propoxazepam
MMGBSA dg Bind	-29.45	-32.59	-12.79	-45.82
[1]Coulomb	-7.68	-14.36	-3.82	-14.74
[2]Covalent	-2.02	2.27	0.41	0.84
[3]Hbond	-0.58	-1.08	-1.99	-1.26
[4]Lipo	-9.93	-6.35	-2.03	-13.98
[5]Solv_GB	19.32	12.19	13.43	23.59
[6]vdW	-27.18	-22.23	-18.77	-39.53

[1]**Coulomb** (Coulomb energy), [2]**Covalent** (Covalent binding energy), [3]**H-bond** (Hydrogen-bonding correction), [4]**Lipo** (Lipophilic energy), [5]**Packing** (Pi-pi packing correction), [6]**Solv_GB** (Generalized Born electrostatic solvation energy), [7]**vdW** (Van der Waals energy)

A more negative Prime MMGBSA value suggests that the ligand and receptor have a stronger binding affinity, meaning they are more likely to form a stable complex and have a higher likelihood of interacting favourably in a biological context. Docked complexes were minimized using the local optimization function in the Prime wizard of Maestro. MMGBSA of propoxazepam is -45.82 kcal/mol, kynurenic acid is -32.59 kcal/mol, ketamine is -29.45 kcal/mol and N-methyl-D-aspartate is -12.79 kcal/mol.

Peculiarities of binding of ligands to N-methyl-D-aspartate receptor in the binding site (the referent ligand localization (S-ketamine)) (Table 8):

Ketamine:

– Hydrogen bond (ligPlot+): the secondary amine group with polar Asn(C) 616 (distance 3,12); (Discovery studio visualizer): the carbonyl group with Asn(C) 616;

– Hydrophobic interaction:

van der Waals: LeuD 642, AsnA 616, AsnD 614, AsnB615, AlaB638, LeuB611, ValB 639, AsnB 614, MetC 641, ValC 644;

pi-alkyl: LeuB 642.

Kynurenic acid:

– Hydrogen bond (LigPlot+): the secondary amine group with PheB 613 (distance 2,75); (Discovery studio visualizer): the carbonyl group with AsnC 616, the secondary amine with PheB 613;

– Hydrophobic interaction:

van der Waals: MetA 641, ValA 644, ValB612, AlaB 638, AlaB 635, AsnB 614, AsnB 615, LeuB611, LeuC 615;

pi-alkyl: LeuB 642, ValB 639.

N-methyl-D-aspartate:

– Hydrogen bond (LigPlot+): the secondary amine group with PheB 613, OH group of the carboxyl group with PheB 613, OH of carboxyl group with AsnB 614; (Discovery studio visualizer): OH group of the carboxyl group with AsnA 616 and AsnB 614, the carboxyl group with AsnC 616, the secondary amine with PheB 613, OH group of the carboxyl group with PheB 613;

– Hydrophobic interaction:

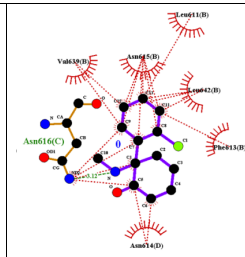
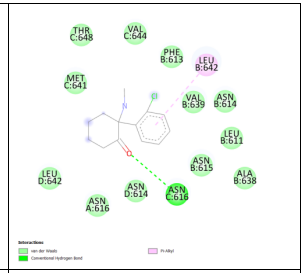
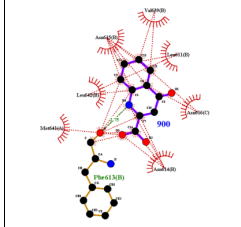
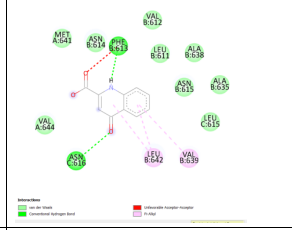
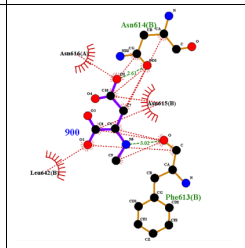
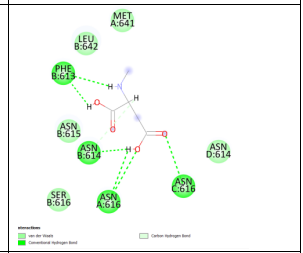
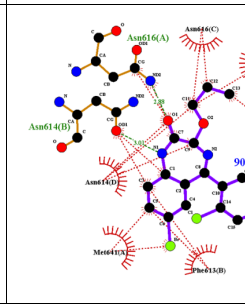
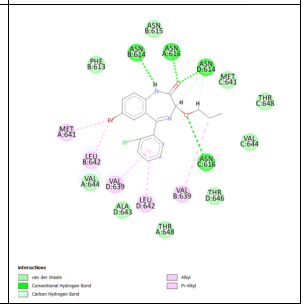
van der Waals: MetA 641, LeuB 642, SerB 616, AsnB 615, AsnD 614.

Propoxazepam:

– Hydrogen bond (LigPlot+): the carbonyl group with AsnA 616 (distance 2,88, Asn 616 H donor); the secondary amine with AsnB 614

Table 8

Visualization of location in specific binding sites of ketamine, kynurenic acid, N-methyl-D-aspartate and propoxazepam with NMDA receptor

Ketamine		
Kynurenic acid		
N-methyl-D-aspartate		
Propoxazepam		

(distance =3,01, Propoxazepam H donor); (Discovery studio visualizer): the ether group with AsnC616, the carbonyl group with AsnD 614 and AsnA616, the secondary amine with AsnB 614;

– Hydrophobic interaction:

van der Waals: MetC 641, ValC 644, AlaD 643, ValA 644, PheB 613, ThrD 646, ThrC 648, AsnB 615, ThrA 648;

pi-alkyl: LeuD 642 (distance 4,99), ValD 639 (distance 5,38);

alkyl: ValB 639 (distance 4,88);

alkyl with halogen Br and C of benzoic ring: MetA 641(distance 4,51), LeuB 642(distance 4,08).

As a result of docking with the above ligands, it can be noted that most often a hydrogen bond was formed between Asn 616 with the carbonyl group (H acceptor) or the secondary amine group and Phe 613 with the secondary amine group.

Hydrophobic bonds are formed between Leu 642 (pi-alkyl or alkyl with halogens), Val 639, Val 644, MetC 641.

4. Conclusions

1. On the base of X-ray analysis data the propoxazepam crystalline sample structure was described and its existence as crystallographic twins was demonstrated. The twinning appears due to crystallization in centrosymmetric spatial group (racemate crystals formation) as the compound has C8 asymmetric centre. The substituent at this position (alcoxy radical) is in equatorial +*sc*- and -*sc*-position. Atoms coordinates, bounds lengths and valence angles in the propoxazepam molecule were determined, that gives the possibility to validate the computer methods of its structure description.

2. Using the molecular docking method on the selected $GABA_A$-receptor part some binding sites were determined with complex formation energy from -78,64 to -85,29 kcal/mole.

3. The total binding energy of the most numerous propoxazepam conformers cluster and GABAA-receptor is similar though the contribution of Van-der-Vaal interactions and hydrogen bonds are not equal for different conformers. The main contribution in the complex formation make polar amino acids residues (serine, asparagine, methionine and arginine – polar binding subcenter). However for some conformers the

significant contribution have aromatic amino acids, mainly phenylalanine (Phe-31, Ala-135 – hydrophobic binding subcenter).

4. Propoxazepam has the necessary pharmacophoric features of pharmacophore model of TRPV1 ligands: aryl interaction head (benzene rings), H -bond interaction linker (the amide group (NH-C=O)), hydrophobic tail (alkoxy group). The docking score of propoxazepam with TRPV1 (-7.30 kcal/mol) indicates a stronger interaction with the TRPV1 receptor compared to oxazepam (-6.82 kcal/mol), 3-hydroxopropoxazepam (-6.49 kcal/mol), and capsazepin (-6.39 kcal/mol). Propoxazepam creates hydrogen bond with TYR 511 of the TRPV1 receptor as referent ligand SB-366791. Propoxazepam exhibits one of the largest contributions of hydrogen bonds in the energy of interaction with the receptor. According to QSAR modelling, all studied compounds (3-hydroxopropoxazepam, diazepam, oxazepam, propoxazepam) have low pIC50 values, which could indicate a relatively low potency or affinity for TRPV1.

5. The computational prediction, propoxazepam has one of the highest docking scores with NMDA receptor for (-6.77 kcal/mol), kynurenic acid (-6.60 kcal/mol), ketamine (5,34 kcal/mol) and N-methyl-D-aspartate (-4.32 kcal/mol). As a result of docking with the above ligands, it can be noted that most often a hydrogen bond was formed between Asn 616 with the carbonyl group (H acceptor) or the secondary amine group and Phe 613 with the secondary amine group. The best pharmacophore (AHHR1) has been selected based on ketamine structure, which consist of H-bond acceptor site (A1), H-hydrophobic sites (H3, H2) and an aromatic ring (R5).

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