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Mendeleev Commun., 2023, **33**, 546–549

Mendeleev Communications

A novel urea derivative anticonvulsant: *in vivo* biological evaluation, radioreceptor analysis of $GABA_A$ receptors and molecular docking studies of enantiomers

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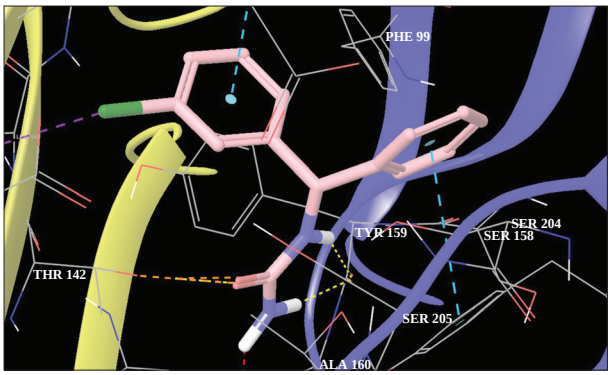
DOI: 10.1016/j.mencom.2023.06.034

It has been experimentally established that the original new generation anticonvulsant Galodif, *N*-[(3-chlorophenyl)-(phenyl)methyl]urea, allosterically modulates $GABA_A$ receptor ($GABA_AR$). Binding of [^{3}H]flunitrazepam and [^{3}H]Ro5-4864 to the benzodiazepine (BZD) site of $GABA_AR$ in the brain of Galodif-treated rats showes an increase in receptor affinity in Scatchard Plot for Ligand Receptor binding analysis. The results of molecular docking (Schrödinger program Glide) reveal that the enantiomers of Galodif are complementary to the BZD binding site of $GABA_AR$; binding energy of *R*-Galodif is lower than that of *S*-Galodif (scoring GScore being –11.14 and –10.7 kcal mol^{-1}, respectively); *R*-Galodif interacts with key amino acids at the α1γ2 interface: Tyr159, Tyr209, H101 Phe77 with high model fit – dG of insert: 7.41.



The binding modes of Galodif in the BZD-binding pocket of $GABA_A$ receptor (α1β2 γ2)

Keywords: anticonvulsants, γ-aminobutyric acid, molecular docking, $GABA_A$ receptor, enantiomers.

Development of a new generation of anticonvulsants, $GABA_A$ receptor ($GABA_AR$) modulators, is needed to increase the effectiveness of the treatment of many neurological and mental disorders.[1] Dysfunction of $GABA_AR$ leads to the development of neuropsychiatric and addiction disorders.[2–4] Modern studies have established that one of the leading components in the development of alcoholic neuroplasticity of the brain is a neuroadaptive change in $GABA_AR$.[4–6] The crystal structure of the $GABA_AR$ α1β2γ2 heteropentamer is a receptor model optimized for studying interactions with agonists and allosteric modulators that bind to targets, *i.e.*, $GABA_AR$ sites of the multireceptor complex.[7] A modern approach to molecular modeling using computer design and computing platforms in combination with molecular and quantum mechanics (molecular docking) makes it possible to evaluate the geometry of ligand–receptor interactions of new chemical compounds based on their mechanism of action and structure of the target under study.

The innovative molecule *N*-[(3-chlorophenyl)(phenyl)-methyl]urea (Galodif), a non-cyclic derivative of benzhydrylurea (Figure 1), is an anticonvulsant with low toxicity, which was developed for the treatment of epilepsy, paroxysmal disorders and addiction diseases.[8,9] The pharmacological activity of a drug depends on the degree of compliance (complementarity) with the target receptor of its structure. To ensure optimal pharmacological activity, 'ideal' drugs should be enatiomerically pure. Enantiomers of Galodif (*S* and *R*) were obtained by HPLC chiral separation of racemic mixture and quantitatively characterized by polarimetry according to correlation equation, and by NMR spectroscopy of diastereomeric derivative of Galodif precursor, (3-chlorophenyl)(phenyl)methylamine, with chiral camphorsulfonic acid. According to quantum calculations of specific rotation, the absolute configuration of (+)- and (–)-Galodif was determined as *S* and *R*, respectively.[10]

In this work we studied the mechanism of interaction of Galodif enantiomers with the $GABA_A$ receptor based on molecular modeling and radioreceptor analysis of the binding

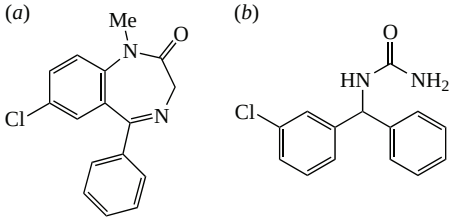


Figure 1 Structural formulas of (*a*) Diazepam and (*b*) Galodif.

of selective benzodiazepine ligands, labeled with tritium to $GABA_AR$ in the brain cortex of Galodif-treated rats with experimental alcoholism.

Galodif (molecular formula $C_{14}H_{13}ClN_2O$, molecular weight 260.74 g mol^{-1}) is white crystalline powder with a bitter taste, practically insoluble in water, soluble in ethanol. For comparison, Diazepam, 7-chloro-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (molecular formula $C_{16}H_{13}ClN_2O$, molecular weight 284.7 g mol^{-1}), is white odorless crystalline powder, practically insoluble in water, hardly soluble in ethanol, soluble in chloroform with anticonvulsant and anxiolytic activity (see Figure 1).

In the experiment, male Wistar rats were tested for alcohol preference according to the results of screening under conditions of free choice of 15% ethanol and water. Animals that preferred alcohol were included in the experimental group, which were under conditions of chronic alcoholization for 10 months. To study the effect of Galodif on the consumption of ethanol solution, animals were intragastrically injected with Galodif in the form of a suspension of 1% starch mucus 100 mg kg^{-1} per day for 14 days. The comparison group consisted of rats that did not prefer ethanol according to the screening conditions and were without access to ethanol during the experimental period. Preparative isolation of synaptosomal membrane fractions of the cerebral rat cortex from studied groups was carried out by high-speed ultracentrifugation using a combined flotation–sedimentation sucrose density gradient. Membrane fractions obtained by centrifugation were frozen and stored at –80 °C. Radioreceptor binding of selective ligands [^{3}H]flunitrazepam and [^{3}H]Ro5-4864 (Amersham) to synaptosomes of rat brain tissue was performed during incubation. The dissociation constant of the ligand–receptor complex K_d (nM) was determined in Scatchard Plot for Ligand Receptor binding analysis.

When Galodif was administered for 14 days (100 mg kg^{-1} per day) to rats under conditions of experimental alcoholism (group 3), a decrease in the consumption of ethanol solution by animals was observed compared to untreated alcoholic rats (group 1). According to the results of radioreceptor binding, a decrease in receptor affinity ($1/K_d$) was revealed, namely, an increase in the K_d values of ligands ([^{3}H]flunitrazepam and [^{3}H]Ro5-4864) that bound to the BZD site of $GABA_AR$ in the brain of alcoholic rats that did not receive Galodif (group 1), compared with the level of K_d values of receptors in rats that were not subjected to chronic alcoholization (group 2) (Figure 2). The K_d values of ligands that bound to the BZD site of $GABA_AR$ in the brains of alcoholic rats treated with Galodif (group 3) approached the K_d values of receptors in the brains of rats that did not prefer alcohol in the control group (group 2). Binding of [^{3}H]flunitrazepam and [^{3}H]Ro5-4864 to the BZD site of $GABA_AR$ in the brains of Galodif-treated rats showed increased receptor affinity ($1/K_d$) compared to untreated alcoholic rats (see Figure 2). It has been experimentally established that Galodif was a positive allosteric modulator of $GABA_AR$, predominantly binding to the BZD site of the receptor, increasing the affinity of the site for the selective ligands [^{3}H]flunitrazepam and [^{3}H]Ro5-4864.

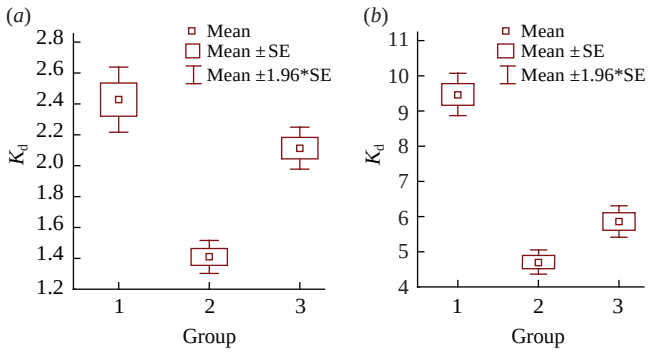


Figure 2 Statistical analysis of binding affinity (K_d) of (*a*) [^{3}H]flunitrazepam and (*b*) [^{3}H]Ro5-4864 with benzodiazepine $GABA_AR$ site of the cerebral cortex of rats in different groups. Note: group 1, rats that preferred alcohol and were in a state of experimental alcoholism (10 months under the influence of 15% ethanol); group 2, rats that did not prefer or drink alcohol during the entire experiment; group 3, rats under conditions of experimental alcoholism, treated with Galodif for 14 days (100 mg kg^{-1} per day).

Docking was performed using a unified model of the most common $GABA_AR$ subtype $\alpha1\beta2\gamma2$ as described.[11] Prior to docking, the model protein was prepared with the Maestro Protein Preparation Wizard (Maestro, version 10.2, Schrödinger, LLC, New York, NY, 2015). Galodif – ligand prepared with LigPrep (LigPrep, version 2.3, Schrödinger, LLC, New York, NY, 2009) generating possible protonation states using Epik program (Epik, version 2.0, Schrödinger, LLC, New York, NY, 2009). The ligands were placed in a box covering the benzodiazepine binding site (BZD) located at the $\alpha1\gamma2$ interface. The box had an automatic size and was located in the center of Diazepam. All ligands were docked in the form of flexible molecules. Docking was performed using Glide (Glide, version 6.7, Schrödinger, LLC, New York, NY, 2015) in Induced Fit Docking mode according to a standard protocol using the Extra Precision evaluation function. The GlideScore scoring function was used to determine the position of the ligand with the best fit.[12]

Membrane permeability of Galodif was calculated using Physics-Based ADME/Tox tool in Maestro (Maestro, version 10.2, Schrödinger, LLC, New York, NY, 2015). Logarithm of membrane permeability of the RRCK (MDCK-LE) in cm s^{-1} (Log Perm.) and the total free energy penalty for the ligand to change state the neutral form and enter the membrane (move from the high dielectric region to the low dielectric region) (dG_Insert) were calculated.

Despite the fact that no experimental crystal structure of $GABA_AR$ $\alpha_1\beta_2\gamma_2$ heteropentamer is available to date, a unified pharmacophore models summarizing the structure–activity (SAR) relationships of compounds acting as a BZD site allosteric modulators and mutational analysis data allowed for the precise positioning of Diazepam in BZD binding pocket.[11] BZD binding site is located on $\alpha_1\gamma_2$ interface of $GABA_AR$ and formed by α_1Tyr159, α_1Thr206, α_1Gly207, α_1Phe99, α_1Hid101, α_1Tyr209, and γ_2Phe77 residues. Residues α_1Gly200, α_1Val202, and γ_2Met130 line the binding pocket. Diazepam has a fused aromatic ring system, which is a lipophilic pharmacophoric feature supposed to be an essential part of benzodiazepins (BZDs).[13] This moiety is located in a BZD pocket beneath the C-loop and surrounded by the hydrophobic residues α_1Val202, α_1Tyr209, and α_1Val211. It is also involved in π–π stacking with α_1Tyr209. The carbonyl moiety of Diazepam is also located under the C-loop and forms two hydrogen bonds with γ_2Thr142 and α_1Thr206 residues. The α_1Tyr209 and α_1Thr206 residues are strongly required for ligand binding in BZD site, which was previously demonstrated by the mutational analysis.[14] The phenyl ring of Diazepam is located in a hydrophobic box formed by α_1Phe99, α_1Hid101, α_1Tyr159, γ_2Phe77 and γ_2Asn128 and act as a strong link between α_1 and γ_2 subunits (Figure 3).

A docking of Galodif enantiomers to BZD binding site reveals that Galodif binds to the receptor pocket in similar fashion as Diazepam. There are two factors contributing to such an interaction. First one is fairly close size of the Galodif and Diazepam molecules. The second one is the presence of two phenyl rings in Galodif acting as a lipophilic pharmacophoric feature mentioned above. Both factors allow Galodif successfully enter the hydrophobic region of the binding pocket.

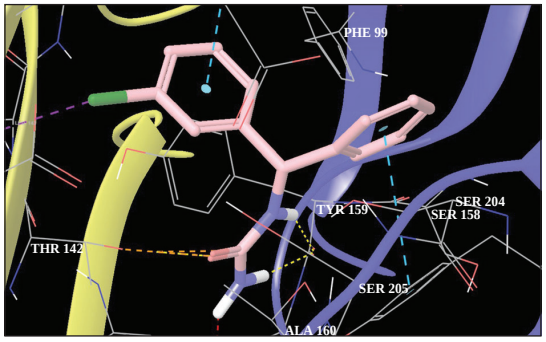


Figure 3 The binding modes of Galodif in the BZD-binding pocket of $GABA_A$ receptor (α1β2 γ2).

It can be noted that Galodif enantiomers interact with BZD binding site differently. *R*-Galodif geometrical position partially overlaps with Diazepam one: it also has one phenyl ring located in a hydrophobic box formed by α_1Phe99, α_1Hid101, α_1Tyr159, γ_2Phe77, and γ_2Asn128 and involved in π–π stacking with α_1Tyr209 and other buried beneath the C-loop in a hydrophobic region of the binding pocket [Figure 4(*a*)]. However, the position of carbonyl moiety of *R*-Galodif is different, thus it does not form an essential hydrogen bond with α_1Thr206. In contrast, the urea moiety of *S*-Galodif is largely involved in a formation of hydrogen bonds network with γ_2Thr142, α_1Thr206, and α_1Tyr159 residues [see Figure 4(*b*)]. Though, the phenyl groups of *S*-Galodif directed oppositely to Diazepam ones and, thus, do not provide the contact between α_1 and γ_2 subunits through interaction with α_1Tyr209. However, taking the interaction of Galodif and Diazepam with BZD binding site is very similar, thus, it could be concluded that Galodif might act as a BZD site

Table 1 Summary of Galodif enantiomers interaction with $GABA_AR$.

Ligand	Log Perm.[a]	Perm./cm s^{-1}	dG Insert	GScore (BZD pocket)
R-Galodif	–4.595	25.4×10^{-6}	7.41	–11.1
S-Galodif	–4.591	25.6×10^{-6}	7.32	–10.7

[a] Calculated logarithm of membrane permeability of the RRCK (MDCK-LE) in cm s^{-1}.

allosteric modulator. It was previously[15] demonstrated that positive BZD modulators triggered specific conformational changes of $GABA_AR$, moreover modulation of I_{GABA} by different BZDs required specific residues in Loop F.

To assess RRCK (MDCK-LE) membrane permeability of *R*,*S*-Galodif, the ADME/Tox calculations were performed. Membrane permeability slightly differs between enantiomers, which might be caused by different energy penalty for tautomerization. Both Galodif enantiomers are characterized by high absorption potential (>10×10^{-6} cm s^{-1}).[16] This is in compliance with calculated total free energy penalty for the ligand to enter the membrane. It should be noted that the binding energy of $GABA_AR$ with *R*-Galodif is lower than that with *S*-Galodif (see Table 1); accordingly, there is a predominant energy gain during the formation of the $GABA_AR$ complex with *R*-Galodif as a selective ligand.

The model and subsequent validation with the available experimental data proves that reliable models of the $GABA_A$ receptor can be generated using new full-length receptor arrays. In addition to being used as a model for how agonists and modulators can bind to $GABA_AR$, this model can help in future studies revealing the mechanism of action of agonists, benzodiazepines and other allosteric modulators, in particular, Galodif. The obtained data on the difference between its *R*- and

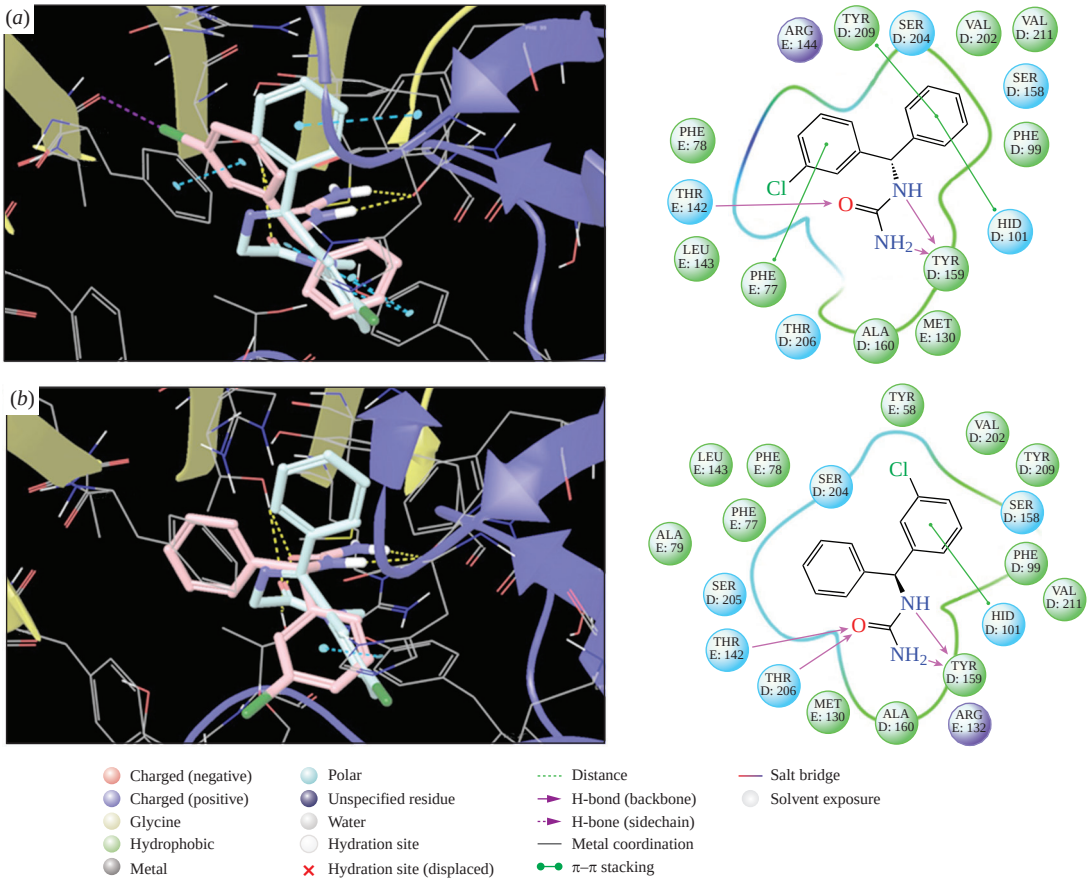


Figure 4 The position of Galodif (pink) in the benzodiazepine binding site of $GABA_AR$ overlapped with Diazepam (light blue) and 2D map reflecting the interaction of the Galodif with the residues in the binding pocket (D – α_1, E – γ_2): (*a*) *R*-Galodif (GScore –11.1 kcal mol^{-1}); (*b*) *S*-Galodif (GScore –10.7 kcal mol^{-1}).

S-enantiomers will be taken into account in the future to ensure the optimal pharmacological action of the developed drug.

The study was carried out at the expense of budget financing under the main plan of research for 2022–2026 'A multidisciplinary study of clinical heterogeneity and pathobiological mechanisms of the progressive development of addictive disorders with the development of innovative therapy programs and differentiated prevention' (State registration no. 122020200053-1).

The work complies with the ethical standards of the Helsinki Declaration of the Military Medical Academy and was approved by the local ethics committee at the Research Institute of Mental Health of the Tomsk National Research Medical Center (Minutes no. 147 dated November 22, 2021, Case no. 147/5.2021).

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Received: 31st March 2023; Com. 23/7136