https://doi.org/10.36290/csf.2025.021

Anticonvulsant biotargets of digoxin: in silico study and in vivo verification

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One of the promising ways to control multidrug-resistant epilepsy may be to use, in addition to traditional antiepileptic drugs (AEDs), medicines from other pharmacological groups – the so-called „non-antiepileptic" drugs. Among such drugs, the cardiac glycoside digoxin deserves special attention. It was established that sub-cardiotonic doses of digoxin exhibit both their own anticonvulsant effect and have been shown to enhance the anticonvulsant potential of classical AEDs. However, the mechanisms of this action and the probable cerebral biotargets with which these properties of digoxin may be associated, remain completely unknown. Molecular docking of digoxin to the following cerebral biotargets was performed: $GABA_AR$, GlyR, mGluR5, AMPAR, hBCATc, mGlu8R, NMDA-GluN1, KCNQ2, COX-1 and COX-2. To confirm the *in silico* results *in vivo* pharmacological studies under conditions of acute bicuculline-induced seizures and pentylenetetrazole kindling in mice have been carried out. It was established that digoxin shows high affinity to GABAergic biotargets and an identical to retigabine affinity to voltage-gated potassium channels KCNQ2 *in silico*. *In vivo* results fully confirm the established by molecular docking GABAergic properties of the cardiac glycoside: digoxin provides a potent anticonvulsant activity on the model of acute primary generalized bicuculline-induced seizures and a moderate anticonvulsant effect under pentylenetetrazole kindling. At the same time, the ability of digoxin to maximally enhance the anticonvulsant potential of sodium valproate has been revealed. Thus, it has been proven that the anticonvulsant properties of digoxin are most likely related to the ability to enhance the inhibitory properties of GABA and GABAergic agents.

Key words: digoxin, anticonvulsant activity, molecular targets, docking, experimental seizures.

Antikonvulzivní biotargety digoxinu: studie in silico a ověření in vivo

Jednou ze slibných možností, jak zvládnout multirezistentní epilepsii, může být kromě tradičních antiepileptik (AED) také použití léků z jiných farmakologických skupin - tzv. "neantiepileptik". Mezi takovými léky si zvláštní pozornost zaslouží srdeční glykosid digoxin. Bylo zjištěno, že subkardiotonické dávky digoxinu vykazují jednak vlastní antikonvulzivní účinek, jednak se ukázalo, že zvyšují antikonvulzivní potenciál klasických AED. Mechanismy tohoto účinku a pravděpodobné mozkové biotargety, s nimiž mohou tyto vlastnosti digoxinu souviset, však zůstávají zcela neznámé. Bylo provedeno molekulární dokování digoxinu k následujícím cerebrálním biotargetům: $GABA_AR$, GlyR, mGluR5, AMPAR, hBCATc, mGlu8R, NMDA-GluN1, KCNQ2, COX-1 a COX-2. Pro potvrzení výsledků in silico byly provedeny farmakologické studie in vivo v podmínkách akutních záchvatů vyvolaných bikukulinem a pentylenetetrazolem u myší. Bylo zjištěno, že digoxin vykazuje vysokou afinitu ke GABAergním biotargetům a identickou afinitu jako retigabin k napěťově řízeným draslíkovým kanálům KCNQ2 in silico. Výsledky in vivo plně potvrzují molekulárním dokováním zjištěné GABAergní vlastnosti srdečního glykosidu: digoxin poskytuje silnou antikonvulzivní aktivitu na modelu akutních primárně generalizovaných záchvatů vyvolaných bikukulinem a mírný antikonvulzivní účinek při pentylenetetrazolovém rozněcování. Zároveň byla odhalena schopnost digoxinu maximálně zvýšit antikonvulzivní potenciál valproátu sodného. Bylo tedy prokázáno, že antikonvulzivní vlastnosti digoxinu s největší pravděpodobností souvisejí se schopností zvyšovat inhibiční vlastnosti GABA a GABAergních látek.

Klíčová slova: digoxin, antikonvulzivní aktivita, molekulární cíle, dokování, experimentální záchvaty.

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Cit. zkr: Čes. slov. Farm. 2025;74(2):E1-E8
Článek přijat redakcí: 6. 1. 2025
Článek přijat po recenzích: 2. 5. 2025

Introduction

The problem of multidrug-resistant epilepsy not only remains relevant even in the 2020s, but also becomes more and more important every year, which is definitely a serious challenge for modern pharmacology, pharmacotherapy and neurology (1). One of the possible ways, if not to overcome the resistance of epilepsy, then at least conditionally stable control of seizures due to a decrease in the activity of the disease can be considered to be the use of drugs from other pharmacological groups – the so-called "non-antiepileptic" medicines – rather than classical antiepileptic drugs (AEDs). A lot of antihypertensive, anti-inflammatory and metabolitotropic agents show a pronounced anticonvulsant effect, established both in preclinical studies and in the clinic, including in resistant epilepsy. This, on the one hand, is explained by the fact that non-classical antiepileptic drugs affect other, little-known links in the pathogenesis of epilepsy, involving new biological targets, such as cyclooxygenases-1 and 2, prostaglandin receptors, sodium-glucose cotransporters, Na^+/K^+-ATPase, etc. (2–5). On the other hand, "non-antiepileptic" medicines may also affect traditional molecular targets in the brain (in particular, GABA, glycine, glutamate receptors, enzymes involved in the synthesis and degradation of neurotransmitter amino acids, ion channels) (6), which in case of resistant epilepsy are no longer sensitive to any traditional AEDs.

One of the most promising "non-antiepileptic" medicines is the well-known cardiac glycoside digoxin. A pronounced anticonvulsant effect of digoxin, as well as its ability to enhance the anticonvulsant potential of classical AEDs, was previously established in models of acute primary generalized seizures induced by the GABA antagonists pentylenetetrazole (PTZ) and picrotoxin, the blocker of glycinergic neurotransmission strychnine, as well as the inhibitor of glutamate decarboxylase and the mediated inducer of glutamate excitotoxicity thiosemicarbazide (7). However, the question of the mechanisms of digoxin's anticonvulsant action, in particular, its cerebral molecular targets, remains not fully elucidated. The hypothesis proposed by us earlier of activation of cerebral Na^+/K^+-ATPase by sub-cardiotonic doses of digoxin, which leads to the normalization of the membrane potential of neurons, was not fully confirmed in further studies: under the pentylenetetrazole kindling model in mice, the activity of this enzyme was reduced by almost half, and digoxin (0.8 mg/ kg) *per se* additionally inhibited it, but in combination with sodium valproate (150 mg/kg) digoxin really promoted the activation of Na^+/K^+-ATPase to subnormal values – significantly higher than against the background of valproate *per se* (8).

The previously established central anti-cyclooxygenase properties of digoxin (9), and, therefore, the possibility of digoxin's anticonvulsant effect realization by influencing neuroinflammation, also require a deeper understanding. Thus, digoxin, sodium valproate and especially their combination normalize pentylenetetrazole-induced changes in the content of COX-1/2 enzymes, as well as individual products of the cyclooxygenase pathway – prostaglandins (PGE2, PGI2, PGF2α) and thromboxane (TXB2) – in the brain, which, obviously, can be one of the possible mechanisms of its anticonvulsant action. At the same time, the question of the interaction of digoxin with the active sites of COX-1 and COX-2 enzymes remains unclear.

Thus, the verification of the central biotargets of cardiac glycoside, which may be associated with its antiepileptic effect, primarily receptors of neurotransmitter amino acids (GABA, glycine, glutamate), as well as key enzymes of the arachidonic acid cascade – cyclooxygenases 1 and 2, is high relevant.

Therefore, the aim of the study was to investigate the potential molecular targets of digoxin which may be associated with its anticonvulsant properties *in silico* as well as to confirm the proposed mechanism of the anticonvulsant action of the cardiac glycoside *in vivo*.

Materials and methods

In silico study

Molecular docking was performed using the AutoDock Vina and AutoDockTools 1.5.6 software packages. Macromolecules from the Protein Data Bank [PDB] were used as biotargets: GABAA – PDB ID 6X3W; GABAA (receptor isoform α1β3γ2) – PDB ID 6HUP; GABAA – PDB ID 5O8F; GlyR – PDB ID 5TIN; mGluR5 receptor – PDB ID 6FFI, AMPAR – PDB ID 5L1F; hBCATc – PDB ID 2COI; mGlu8R – PDB ID 6E5V; NMDA-GluN1 – PDB ID 4KFQ; KCNQ2 – PDBID 7CR2; COX-1 – PDB ID – 3N8Y; COX-2 – PDB ID – 3LN1.

Construction of ligand structures was carried out using the BIOVIADraw 2021R2 program and saved in mol format. Structures were optimized by Chem3D program using the MM2 molecular mechanics algorithm, saved in .pdb format and converted to .pdbqt using AutoDockTools-1.5.6. Discovery Studio Visualizer 2021 was used to remove solvent and native protein ligand. The prepared macromolecules were saved in .pdb format. In AutoDockTools-1.5.6, polar hydrogen atoms were added to the protein structure and saved in .pdbqt format. The size of the Grid box and its center was determined by the native ligand:

- GABAA (PDB ID 6X3W): x = 109.83, y = 93.68, z = 105.46; size x = 30, y = 32, z = 84;
- GABAA (PDB ID 6HUP): x = 117.44, y = 157.46, z =110,46; size x = 26, y = 28, z = 20;
- GABAA (PDB ID 5O8F): x = 74.20, y = 312.32, z =408.09; size x = 20, y = 12, z = 22;
- GlyR (PDB ID 5TIN): x = -58.99, y = -41.32, z =-3.29; size x = 10, y = 10, z = 14;
- hBCATc (PDB ID 2COI): x = -23.53, y = 4.14, z =-12.64; size x = 32, y = 26, z = 24;
- mGluR5 (PDB ID 6FFI): x = -24.14, y = - 5.28, z = 42.63, size x = 20, y = 24, z = 22;
- mGluR8 (PDB ID 6BT5) DCPG: x = 6.66, y = -36.61, z = -12.06; size x = 22, y = 22, z = 22;
- AMPAR (PDB ID 5L1F): x = -34.85, y = - 6.07, z = -39.15, size x = 32, y = 30, z = 26;
- NMDA-receptor GluN1 subunit (PDB ID 4KFQ): x = 26.91, y = 34.35, z =46.85; size x = 22, y = 24, z = 20;
- KCNQ2 (PDB ID 7CR2): x = 118.52, y = 135.5, z =102.93; size x = 22, y = 18, z = 16;
- COX-2 (PDB ID 3LN1): x = 18.84, y = -52.89, z = 53.81, size x = 22, y = 24, z = 24;
  COX-1 (3N8Y): x = 33.14, y = -44.49, z = -3.76, size x = 24, y = 22, z = 20.

Docking methods for all biotargets were validated by the re-docking procedure of the native ligand and comparison of its conformational placement with the experimentally determined and described position in the literature [PDB]. The calculation of the root mean square deviation during the validation of the technique was carried out using the ProFit Results online resource. The root mean square deviation (RMSD) between the two conformations did not exceed 2 Å, which confirms the reproducibility of the experimental data and the validity of the methodology (10). The validation of the docking methodology for COX-1 and COX-2 was previously described by us (11).

Binding energy (kcal/mol) was used to quantify the affinity to the active site of the receptor or enzyme. Visualization and analysis of the obtained docking results were carried out using Discovery Studio Visualizer.

In vivo study

Pharmacological verification of potential anticonvulsant targets of digoxin was carried out using 80 random-bred albino mice of both sexes weighing 20–24 g. The animals were kept in the vivarium of the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy (Kharkiv, Ukraine) on a standard diet with free access to water at a constant humidity of 60 % and temperature +20–22 °C under 12 h/12 h of light/dark ratio. The research protocol does not contradict the provisions of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and has been approved by the local Bioethics Committee (protocol No. 3 of 10 September 2020).

Two experimental seizure models associated with a deficiency of GABAergic inhibition have been used: acute and chronic. Acute seizures have been simulated using a single injection of bicuculline; chronic epileptogenesis has been simulated by regular repeated administration of subthreshold doses of pentylenetetrazole.

Bicuculline-induced seizures is a model of acute primary-generalized convulsions based on antagonism to the GABAA receptor complex (12). The mechanism of action of bicuculline is due to competitive antagonism with the GABA-binding site of GABAA receptors (12). Classic AEDs have a pronounced effect on bicuculline-induced seizures, while drugs with GABAergic properties, in particular, benzodiazepines, show maximal activity.

Kindling is a model of chronic epileptogenesis that is closest to the natural conditions of a long period of disease formation. The mechanism of the convulsive action of pentylenetetrazole is based on inhibition of the GABAA receptor complex and, as a result, a decrease in GABAergic inhibitory processes (13).

To simulate acute primary-generalized bicuculline seizures, 30 animals have been divided into experimental groups (1-5) of 6 mice in each (n=6): control – mice with untreated seizures [1]; and animals treated with sodium valproate at a conditionally effective dose, ED50 [2] and a subeffective dose, ½ ED50 [3], digoxin [4], and a combination of sodium valproate, ½ ED50 and digoxin [5].

Bicuculline (Sigma-Aldrich, USA) in the form of an aqueous solution has been administered at a dose of 2.7 mg/kg subcutaneously. Immediately after the administration of the convulsant, the animals were placed in standard individual transparent plastic boxes and their behavior was observed for 60 min. The following indicators were recorded: the latency of the first attack, the number of seizures and their type (clonic/tonic), the severity of convulsions, the total duration of the convulsive period, the lifetime of the animals until death (if any) and the overall lethality rate in the experimental group. If convulsions did not occur during the entire observation period, the latency was considered equal to 60 min. Severity of convulsions was determined according to the modified Racine scale in points, where 1 is shaking, 2 is „maniac run" or „kangaroo pose", 3 is clonic convulsions, 4 is clonic-tonic convulsions with a lateral position, 5 is tonic extension of the hind limbs, 6 is tonic extension that led to the animal's death (7).

For the model of chronic epileptogenesis – pentylenetetrazole (PTZ) kindling – 50 animals were randomized into experimental groups (1–5) of 10 mice in each (n=10): control – mice with untreated seizures [1]; and animals treated with sodium valproate at a conditionally effective dose, ED50 [2] and a subeffective dose, ½ ED_{50} [3], digoxin [4], and a combination of sodium valproate, ½ ED50 and digoxin [5].

Pentylenetetrazole (PTZ, Sigma-Aldrich, USA) has been administered at a dose of 30 mg/kg intraperitoneally (in/o) daily at the same time once a day (8) for 16 days. After each administration of PTZ, the animals were observed for 60 min. The latency of the appearance of the first convulsions, the number of days with seizures, and the number of animals with convulsions in dynamics were determined.

Sodium valproate (Depakine syrup, Sanofi Aventis, France), the classic AED with proven GABAergic properties, has been administered intragastrically at doses of 300 mg/kg (ED50) and 150 mg/kg (½ ED50) 30 minutes before administration of a convulsive agent (bicuculline or PTZ). A dose of ½ ED50 usually does not provide the maximal protective effect, which makes it possible to detect a potential modulation of the anticonvulsant effect – both its weakening or strengthening.

Digoxin (DNCLZ/Zdorovya, Ukraine) has been administered subcutaneously at a previously determined effective anticonvulsant dose of 0.8 mg/kg (which is equal to 1/10 LD50) [15] 15 min before the administration of bicuculline or PTZ. Animals in the group of combined use of digoxin and sodium valproate received drugs at the above doses.

Mice of the control group received intragastrically and subcutaneously solvent (water) in a similar volume (0.1 ml/10 g) and mode of administration.

Statistical analysis of the results has been performed using the STATISTICA 12.0 software package. The results are given as mean ± standard error of mean. The significance of intergroup differences has been assessed by the parametric Student's t-test in case of normal distribution and the non-parametric Mann-Whitney U-test in its absence. Fisher's angular transformation φ has been used to assess the differences in indicators that were recorded in an alternative form (presence or absence of a feature). Differences were considered significant at $p<0.05$.

Results and discussion

The previously established effectiveness of digoxin on acute models of pentylenetetrazole- and picrotoxin-induced seizures (7) determines

the likelihood of an effect on GABAergic mechanisms of epileptogenesis. To predict the receptor effect, the docking of digoxin into the active sites of GABAergic biotargets was carried out:

- sites of positive allosteric modulators of the GABAA receptor – diazepam and phenobarbital;
- site of neurosteroids GABAA receptor modulators – pregnanolone (14).

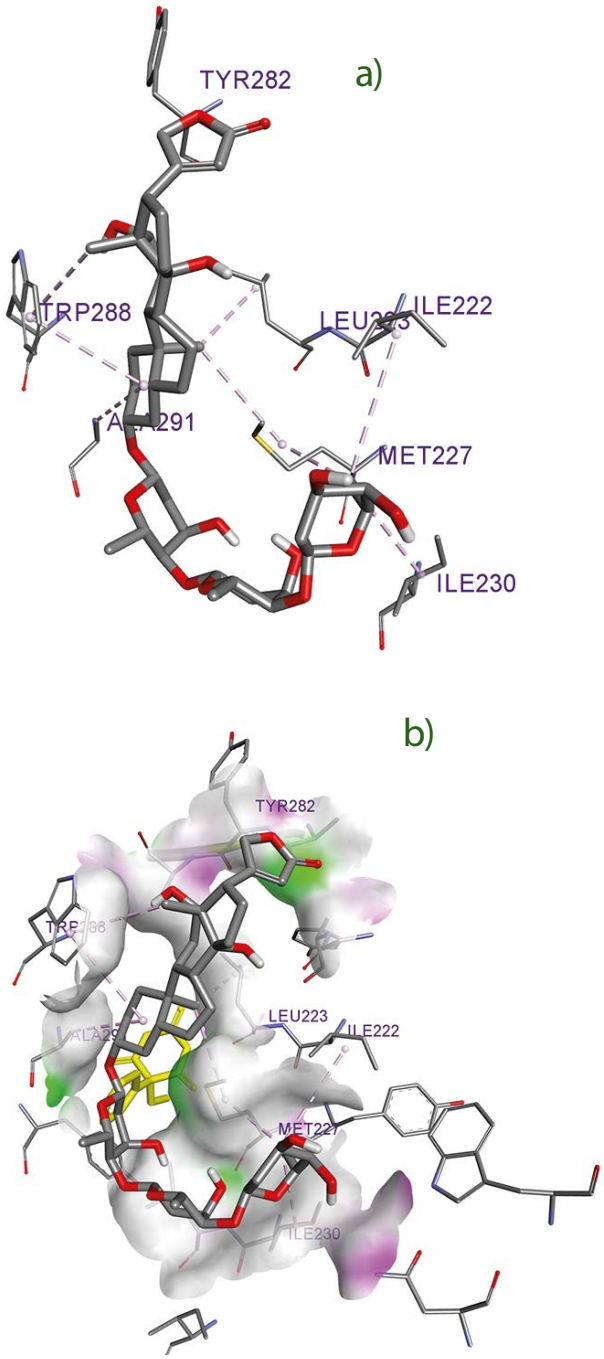
Digoxin's affinity to the site of the positive allosteric modulator (PAM) of the GABAA receptor (PDB ID 6X3W) was significantly inferior to the reference ligand: –5.8 kcal/mol versus –8.5 kcal/mol for phenobarbital (Fig. 1, Table 1). A large molecule of digoxin is not able to sink into a hydrophobic pocket: superficial and weak (due to the small number of hydrogen and hydrophobic bonds) fixation is observed at the entrance to the active site with the participation of amino acid residues that do not form the active site in the experiment (except for alanine Ala291 and leucine Leu223) (Fig. 1b).

Digoxin has a better affinity for the benzodiazepine site of the GABAA receptor (PDB ID 6HUP): –9.0 versus –9.9 kcal/mol for diazepam (Fig. 2, Table 1). The formation of bonds with experimentally determined peptide residues of the active site was recorded, which indicates the possibility of fixation in the active site itself. However, binding to histidine (His102), which is involved in the binding of all benzodiazepines, i.e. is a marker for the manifestation of agonism to the GABAA receptor, is not predicted. Stabilization of the conformation occurs both by the aglycon and by the sugar part, but the latter sinks deeper into the active site, being fixed by hydrophobic bonds in the pocket of benzodiazepines. Taking this into account, the probability of an effect on the GABAA receptor through the benzodiazepine site is unlikely.

Due to their ability to modulate neurotransmission through interaction with GABAA receptors, endogenous neurosteroids are able to influence the clinical symptoms of epileptic disorders, providing anticonvulsant and antiepileptic effects (15). The neurosteroid binding site is located between the helices lining the pore of the GABAA receptor ion channel and modulates the conformation of the desensitization gate. It has been demonstrated that modulation through this site is responsible for physiological heteromeric potentiation of the GABAA receptor (14). Since digoxin also has a steroid structure, its affinity for the neurosteroid site located between the β3–α5 helices of the extracellular domain of the GABAA receptor was evaluated.

The affinity of digoxin to the neurosteroid binding site was somewhat inferior to the affinity of the native reference ligand: –7.5 kcal/mol in digoxin versus –9.5 kcal/mol in pregnenolone. The detailing of relationships with amino acid residues indicates the placement of the digoxigenin aglycon in the neurosteroid fixation cavity: hydrophobic interaction between cyclopentaneperhydrophenanthrene cycle and the indole cycle of tryptophan (Trp249), the pyrrole cycle of proline (Pro403), alanine (Ala303), the interaction between the angular methyl group (10 position) and isoleucine (Ile242). It should be noted the formation of an identical to reference ligand hydrogen bond with the hydroxyl group of threonine (Thr309), which fixes the first monomer of the glycone (Fig. 3a).

Fig. 1. *3D visualization of the interaction of digoxin (gray molecule) with the amino acid residues of the GABAA receptor active site (a) and the compatible conformation with phenobarbital (b)*



The compatible conformation of the native ligand and digoxin also confirms the fixation by the steroid cycle itself and their similar spatial arrangement (Fig. 3b). Such molecular docking results may suggest the possibility of digoxin stimulation of the GABAA receptor through the neurosteroid site.

Taking into account the previously established effectiveness of digoxin under the conditions of strychnine seizures, the mechanism of which consists in blocking glycinergic neurotransmission 16), to predict the influence of digoxin on glycinergic properties, docking was carried out in the active site of the glycine receptor (GlyR), crystallized in the conformation with its agonist glycine and AM-3607 – a positive allosteric modulator (PDB ID 5TIN).

Fig. 2. *3D visualization of the interaction of digoxin with the amino acid residues of the active site of diazepam GABAA receptor (a) and the compatible conformation of digoxin with respect to diazepam (b)*

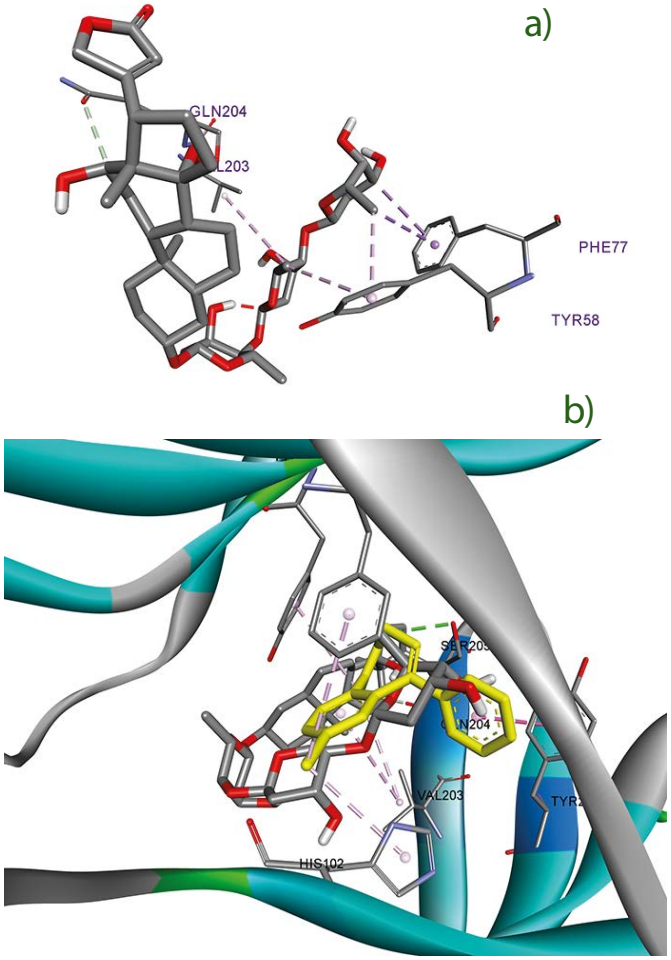
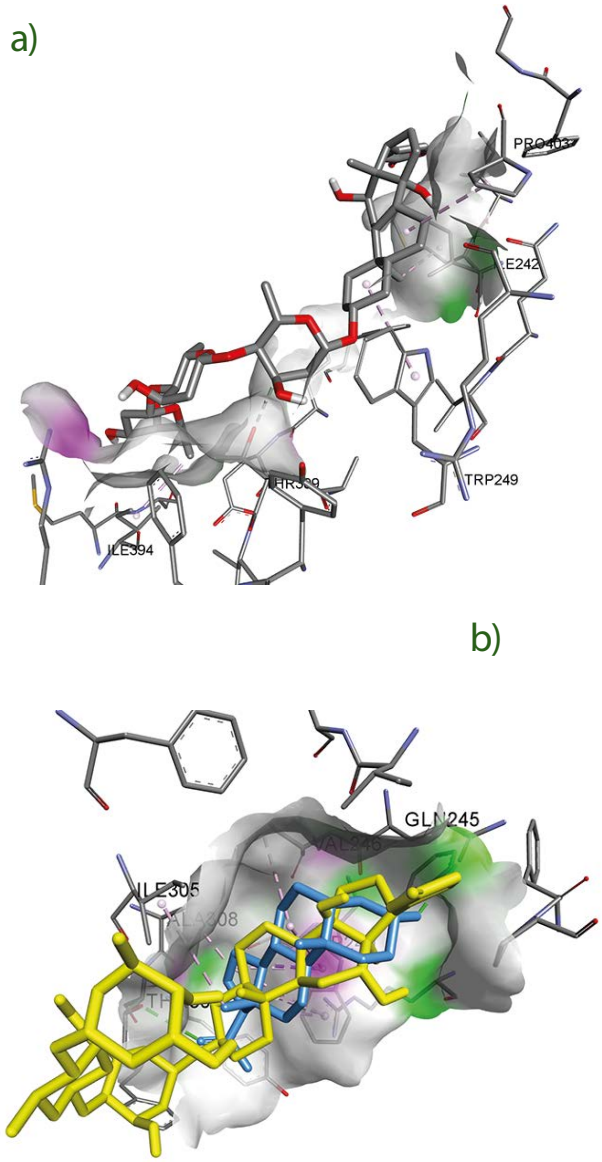


Fig. 3. *3D visualization of the conformation of digoxin in the neurosteroid active site of the GABAA receptor: a) interaction with amino acid residues; b) compatible conformation of digoxin (yellow molecule) and pregnenolone (yellow molecule)*



The value of the digoxin scoring function (28 kcal/mol versus –11.4 kcal/mol) and 11 "unfavorable bumps" in detailing the interaction with amino acid residues prove the impossibility of the existence of such a conformation and, accordingly, the modulation of the glycine receptor by digoxin (Fig. 4a, b).

Despite the verified in vivo ability of digoxin to prevent the development of thiosemicarbazide convulsions and therefore counteract the excessive effects of glutamate (7), a low degree of affinity and an unsatisfactory profile of the cardiac glycoside to all targets of the glutamatergic mechanism were established. Docking was performed and the lack of affinity was determined for such biotargets as:

- branched chain amino acid aminotransferase (BCAT) inhibitor site – gabapentin (PDB ID 2COI);
- metabotropic receptors – mGlu5 group I active site – MPEP, topiramate (PDB ID 6FFI); the active site of the mGlu8 group III orthosteric agonist with anticonvulsant properties –3,4-dicarboxyphenylglycine, DCPG (PDB ID 6E5V);
- ionotropic receptors – site of an inhibitor of AMPA receptors – perampanel (PDB ID 5L1F);
- NMDA receptor glycine antagonist site – TK-40 and felbamate (PDB ID 4KFQ).

Modulation of neuronal voltage-gated potassium channels KCNQ2 is another recently established mechanism of influence on epileptogenesis, which has great potential in the development of new strategies for the treatment of refractory forms of the disease (16). Therefore, it was interesting to investigate the possible affinity of digoxin to this biological target.

Docking into voltage-gated potassium channels was performed in the crystal structure of the KCNQ2 protein – PDB ID 7CR2. The reference ligand is a retigabine modulator that stabilizes the open form of the potassium channel. For digoxin, the value of the scoring function at the level of the comparison drug was calculated: –7.8 kcal/mol. Detailing of amino acid interactions demonstrates the participation of peptide residues that form the experimentally determined active site, including tryptophan (TRP236), a marker for retigabine fixation: a tetrahedral net of bonds between the benzene ring and a bidentate bond with the pyrrole of tryptophan and the steroid cycle of digoxin. Fixation of the

Fig. 4. *3D and 2D visualization of the conformation of digoxin in the site of the Gly receptor*

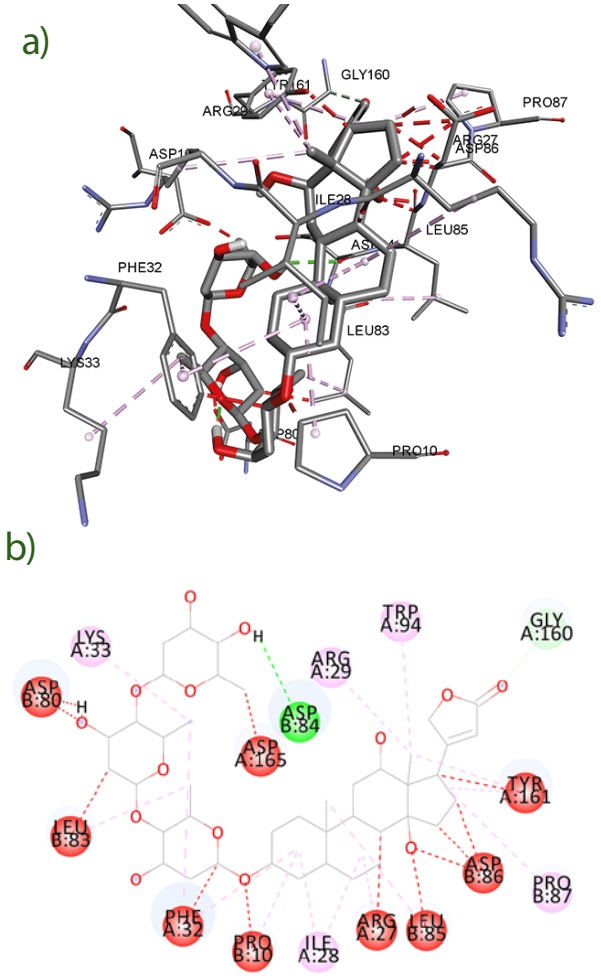
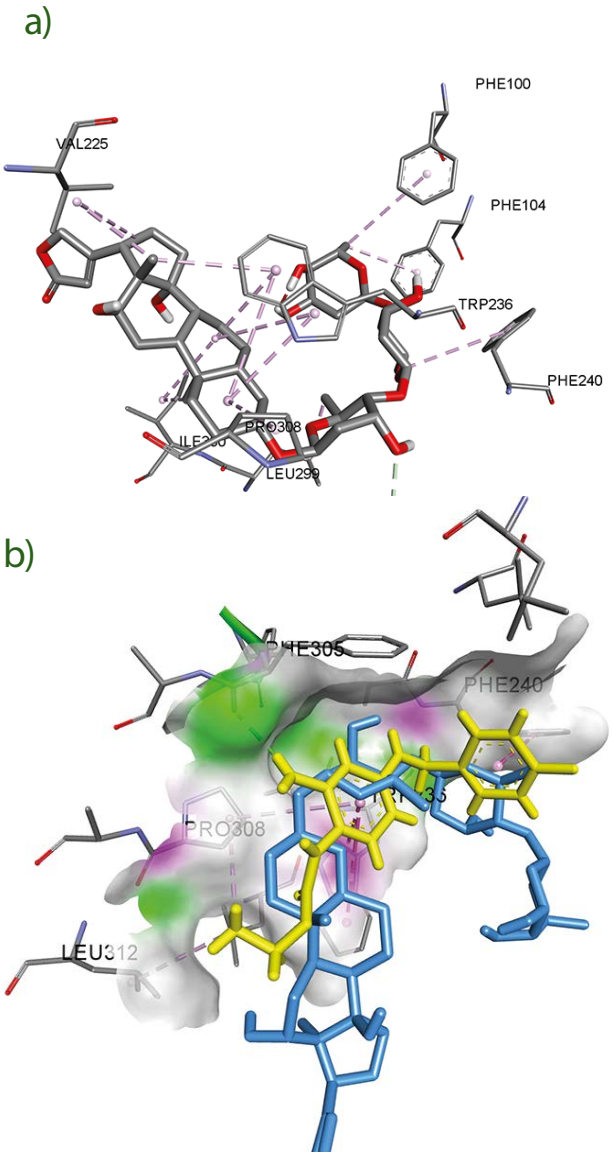


Fig. 5. *Visualization of the location of digoxin in the active site of voltage-gated potassium channels: a) interaction with amino acid residues and b) compatible conformation of digoxin (blue molecule) and retigabine (yellow molecule)*



conformation also occurs due to hydrophobic bonds with angular methyl groups and methyl groups of the glycone. The ability to dive into the active site of potassium channels to fix its open form was also demonstrated with the compatible conformation of the reference and the studied ligand (Fig. 5): digoxin is able to completely occupy the position of retigabine, although the "tail" of the glycone slightly exceeds the limits – fixation by phenylalanine residues (Phe100, 104). In summary, we can assume the ability of digoxin to allosteric modulation of potassium channels.

To assess the possibility of realizing the anticonvulsant effect of digoxin by affecting neuroinflammation, docking was carried out in the active sites of inhibitors of cyclooxygenase-2 (PDB ID – 3N8Y) and cyclooxygenase-1 (PDB ID – 3LN1), reference drugs celecoxib and diclofenac, respectively. The degree of affinity was unsatisfactory: –1.5 kcal/mol versus –12.5 for celecoxib; –2.5 kcal/mol versus –8.5 for diclofenac. Detailing of amino acid interactions also confirmed the impossibility of existence of such conformations and the inability to sink into selected active sites. The obtained results confirm the previously made assumption that changes in the levels of cerebral COX-1 and COX-2 in mice under the influence of digoxin are a consequence of the nuclear effect of cardiac glycoside, which has a steroid structure, on the expression of these enzymes in the brain (9).

In silico results have been verified in vivo. Thus, digoxin both per se and in combination with sodium valproate at ½ ED_{50} exerted the maximum protective effect on the model of acute bicuculline-induced seizures (Tab. 2), completely protecting animals from the development of convulsions. At the same time, digoxin and its combination with low-dose AED were significantly more effective than monotherapy with sodium valproate at ½ ED50, and for some indicators (in particular, the number of animals with clonic and tonic convulsions, the severity of seizures and the duration of the period of seizures), they statistically significantly exceeded even the effect of sodium valproate at ED_{50}.

Under the conditions of simulated kindling, digoxin per se showed only a rather vague anticonvulsant effect – it did not reduce the total number of days with paroxysms in the group compared to the control, but it statistically significantly reduced the % of animals with clonic-tonic paroxysms from the 13th to the 16th day of the experiment (Fig. 6, 7). At the same time, however, digoxin revealed itself as a powerful enhancer of

Tab. 1. *Results of docking of digoxin and reference ligands into active sites of anticonvulsant biotargets*

Target	Scoring function, kcal/mol	Interaction with amino acids	Reference ligand, kcal/mol
GABA PAM site	–5.8	a) Ala291, Ile222*, Met227(2)*, Ile230*, Leu223, Trp288* b) Tyr282*	–7.3 phenobarbital
$GABA_A$ benzodiazepine site	–9.0	a) Phe77(2), Val203*, Tyr58 b) Gln204(2)	–9.9 diazepam
$GABA_A$ neurosteroid site	–7.5	a) Pro403, Ile394, Ile242, Trp249, ALa303 b) Thr309	–9.0 pregnenolone
KCNQ2 agonist site	–7.8	a) Pro308*, Leu299, Ile300(2), Val225(2), Trp236(5), Phe240, Phe100*, Phe104* b) Phe305	–7.8 retigabine

*a) Hydrophobic interaction; b) Hydrogen bonds; ( ) number of connections; * amino acid residues that do not form the active site in the experiment*

sodium valproate: together in the combination, it provided the maximum protective properties of a subeffective dose of classical AED, at the same time surpassing the effectiveness of sodium valproate monotherapy not only in ½ ED_{50}, but even in ED_{50} ($p < 0.05$) – during all 16 days of administration of pentylenetetrazole against the background of a combination of digoxin and valproate, no spontaneous convulsions were observed in animals.

The obtained *in vivo* results of the anticonvulsant effect of digoxin study fully confirm the GABAergic properties of the cardiac glycoside established by molecular docking.

Thus, it can be considered proven that the anticonvulsant properties of digoxin are associated with a stimulating effect on GABAergic inhibition in the central nervous system. Digoxin is obviously a pharmacodynamic enhancer of GABA (like benzodiazepines), which was established in *in silico* experiments by the high affinity of the molecule to neurosteroid site of GABAA receptor, as well as KCNQ – voltage-gated potassium channels that regulate GABA release (17), in the absence of affinity for glycine and glutamate biotargets.

The similarity of the chemical structure of cardiac glycosides to neurosteroids is obviously decisive for the realization of central properties, in particular, anticonvulsant action. At the same time, the difference in the anticonvulsant potential that we established for various cardiac glycosides (digoxin, lanatoside C, strophantin G and corglycon) is determined by the lipophilicity of the molecule and, as a result, its cerebral bioavailability (18). As mentioned above, the results of molecular docking comparing digoxin and pregnenolone testify in favor of the possibility of a stimulating effect of this cardiac glycoside on GABAA receptors through the site of neurosteroids.

The steroid structure of cardiac glycosides may also be associated with their anti-inflammatory properties, in particular, the effect on neuroinflammation as a component of epileptogenesis (19). Although the lack of affinity of digoxin to the active sites of COX-1 and COX-2 has been established *in silico*, it is possible to suggest the ability of cardiac glycoside to penetrate the nucleus of cells (glial cells – in the CNS (20)) and affect the gene expression of these enzymes, thereby regulating their synthesis.

In silico GABAergic properties of digoxin have been confirmed in vivo: the cardiac glycoside proved to be the most effective anticonvulsant agent in an acute model of bicuculline-induced seizures associated with blocking GABAergic neurotransmission. Although at the same time, under the conditions of chronic epileptogenesis, digoxin showed fairly moderate protective properties, it significantly increased the anticonvulsant potential of the classic AED sodium valproate, which mechanism of action is to increase GABAergic inhibition through various indirect pathways. The moderate effect of digoxin *per se* on the model of chronic epileptogenesis may be due to pronounced changes in the balance of neurotransmitters occurring in the brain of mice under pentylenetetrazole kindling. For example, we previously established that in addition to GABA deficiency, kindling animals have profound disturbances in the metabolism of other cerebral neuroactive amino acids, in particular, glutamate and aspartate accumulate as well as the glycine pool is depleted (8).

The results of the study allow pharmacists not only to achieve a deep understanding of the mechanisms of the central (in particular, anticonvulsant) action of digoxin, but also confirm the benefits of the combined use of digoxin with classic widely used antiepileptic drugs. Thus, from the pharmacist's point of view, the combination of digoxin and sodium valproate at ½ ED_{50} is beneficial, which is valuable from the standpoint of pharmaceutical care of patients with epilepsy.

Conclusion

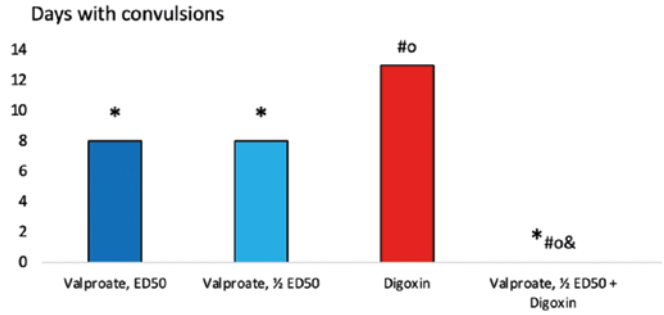
The study of potential molecular targets of digoxin has been carried out in silico, and the proposed mechanisms of the anticonvulsant action of the cardiac glycoside conditions were verified *in vivo*.

Tab. 2. *Results of the study of the effectiveness of sodium valproate, digoxin and their combination on the course of acute bicuculline-induced seizures in mice (M ± m)*

Group of animals (n = 6)	Latency, min	Number of clonic-tonic seizures in 1 mouse	% of mice with convulsions		Severity of seizures, points	Period of seizures, min	Time to death, min	Lethality, %
			clonic	tonic				
Control – bicuculline (untreated seizures)	5.0 ± 1.6	1.8 ± 0.4	100	100	5.7 ± 0.3	2.7 ± 1.1	6.5±0.7	83
Valproate, ED50	42.0 ± 11.4**	0.7 ± 0.4 *	33 **	33 **	1.7 ± 1.1 *	1.0 ± 0.9	10.6	17 **
Valproate, ½ ED50	9.5 ± 1.3 ##	1.7 ± 0.3 #	100 ##	100 ##	5.0 ± 0.5 ##	3.9 ± 1.6	12.2 ± 2.3	50
Digoxin	60.0 ± 0.0 **°°	0.0 ± 0.0 **°	0 **#°°	0 **#°°	0.0 ± 0.0 **#°°	0.0 ± 0.0 **#°°	–	0 **°°
Valproate, ½ ED50 + Digoxin	60.0 ± 0.0 **°°	0.0 ± 0.0 **°	0 **#°°	0 **#°°	0.0 ± 0.0 **#°°	0.0 ± 0.0 **#°°	–	0 **°°

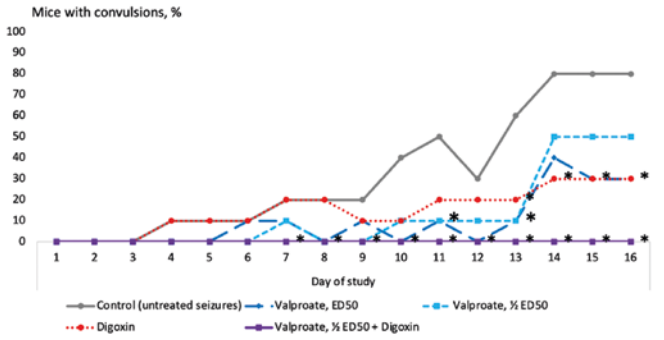
*n – number of animals in each group; * – $p < 0.05$ when compared with control, ** – $p < 0.01$ when compared with control; # – $p < 0.05$ when compared with sodium valproate at ED50, ## – $p < 0.01$ when compared with sodium valproate at ED50; ° – $p < 0.05$ when compared with sodium valproate at ½ ED50, °° – $p < 0.01$ when compared with sodium valproate at ½ ED_{50}*

Fig. 6. *Results of a study of the effectiveness of sodium valproate, digoxin and their combination on the number of days with convulsions in mice under pentyleneterazole kindling*



** – p < 0.05 when compared with control; # – p < 0.05 when compared with sodium valproate at ED50; ° – p < 0.05 when compared with sodium valproate at ½ ED_{50}; & – p < 0.05 when compared with digoxin*

Fig. 7. *The results of the study of the effect of sodium valproate, digoxin and their combination on the % of mice with convulsions in the dynamics of pentyleneterazole kindling*



** – p < 0.05 when compared with control*

According to the results of *in silico* docking, it was established that digoxin shows high affinity to GABAergic biotargets, in particular, the $GABA_A$ site of neurosteroids, as well as identical to retigabine affinity to voltage--gated potassium channels KCNQ2, in the absence of affinity to glycine and glutamate biotargets, as well as active centers of cyclooxygenases.

Under the conditions of GABA-negative seizures *in vivo*, powerful anticonvulsant properties of digoxin were established in the model of acute primary-generalized bicuculline-induced seizures and a moderate anticonvulsant effect under pentylenetetrazole kindling. At the same time, the ability of digoxin to maximally enhance the anticonvulsant potential of sodium valproate has been proven.

Thus, it has been proven that the anticonvulsant properties of digoxin are most likely related to the ability to enhance the inhibitory properties of GABA and GABAergic agents.

Authorship contribution statement

Vadym Tsyvunin: design of the study, acquisition, analysis and interpretation of data, drafting the article. **Hanna Severina**: design of the study, acquisition, analysis and interpretation of data, drafting the article. **Sergii Shtrygol'**: conceptualization, revising the article. Victoriya **Georgiyants**: conceptualization, revising the article. **Diana Shtrygol'**: conceptualization, revising the article. All authors finally approved the version to be submitted.

Conflicts of interest:

The authors have no conflict of interest to declare.

Acknowledgments :

The authors express their gratitude to all defenders of Ukraine who made this research possible. This work was supported by the Ministry of Health of Ukraine and carried out at the expense of the State Budget of Ukraine in 2020/2022 (Grant No. 0120U102460).

REFERENCES

1. Perucca E, Perucca P, White HS, et al. Drug resistance in epilepsy. Lancet Neurol. 2023;22:723-734.
2. Łukawski K, Czuczwar SJ. Emerging therapeutic targets for epilepsy: preclinical insights. Expert Opin Ther Targets. 2022;26:193-206.
3. Dhir A. An update of cyclooxygenase (COX)-inhibitors in epilepsy disorders. Expert Opin Investig Drugs. 2019;28:191-205.
4. Tsyvunin V, Shtrygol S, Havrylov I, et al. SGLT-2 inhibitors as potential anticonvulsants: empagliflozin, but not dapagliflozin, renders a pronounced effect and potentiates the sodium valproate activity in pentylenetetrazole-induced seizures. Sci Pharm Sci. 2022;39:83-90.
5. Freitas ML, Oliveira CV, Mello FK, et al. Na+, K+-ATPase Activating Antibody Displays in vitro and in vivo Beneficial Effects in the Pilocarpine Model of Epilepsy. Neurosci. 2018;377:98-104.
6. Pawlik MJ, Miziak B, Walczak A, et al. Selected Molecular Targets for Antiepileptogenesis. Int J Mol Sci. 2021;22:9737.
7. Tsyvunin V, Shtrygol S, Mishchenko M, et al. Digoxin at sub-cardiotonic dose modulates the anticonvulsive potential of valproate, levetiracetam and topiramate in experimental primary generalized seizures. Ceska Slov Farm. 2022;71:78-88.
8. Tsyvunin VV, Shtrygol SY, Gorbach TV. Effect of digoxin, sodium valproate, their combination and celecoxib on neuroactive amino acids content and cerebral Na+, K+-ATPase activity in pentylenetetrazole-kindled mice. Pharmacol Drug Toxicol. 2023;17:227-239.
9. Tsyvunin V, Shtrygol S, Mishchenko M, et al. Effect of digoxin, sodium valproate, and celecoxib on the cerebral cyclooxygenase pathway and neuron-specific enolase under the pentylenetetrazole-induced kindling in mice. Ceska Slov Farm. 2023;72:172-183.
10. Baber JC, Thompson DC, Cross JB, et al. GARD: a generally applicable replacement for RMSD. J Chem Inf Model. 2009;49:1889-1900.
11. Krasovska N, Berest G, Belenichev I, et al. 5+1-Heterocyclization as preparative approach for carboxy-containing triazolo[1,5-c]quinazolines with anti-inflammatory activity. Eur J Med Chem. 2024;266:116137.
12. Johnston GA. Advantages of an antagonist: bicuculline and other GABA antagonists. Br J Pharmacol. 2013;169:328-336.
13. Reddy DS, Vadassery A, Ramakrishnan S, et al. Kindling Models of Epileptogenesis for Developing Disease-Modifying Drugs for Epilepsy. Curr Protoc. 2024;4:e70020.
14. Miller P, Scott S, Masiulis S, et al. Structural basis for GABAA receptor potentiation by neurosteroids. Nat Struct Mol Biol. 2017;24:986-992.
15. Miziak B, Chrościńska-Krawczyk M, Czuczwar SJ. Neurosteroids and Seizure Activity. Front Endocrinol (Lausanne). 2020;11:541802.
16. Gao K, Lin Z, Wen S, et al. Potassium channels and epilepsy. Acta Neurol Scand. 2022;146:699-707.
17. Graziano B, Wang L, White OR, et al. Glial KCNQ K+ channels control neuronal output by regulating GABA release from glia in C. elegans. Neuron. 2024;112:1832-1847.
18. Tsyvunin VV, Shtrygol SY, Shtrygol DV, et al. Anticonvulsive potential of cardiac glycosides under conditions of pentilentetrazole-induced seizures in mice: comparative study. Acta Med Leopol. 2021;27:63-69.
19. Sanz P, Rubio T, Garcia-Gimeno MA. Neuroinflammation and Epilepsy: From Pathophysiology to Therapies Based on Repurposing Drugs. Int J Mol Sci. 2024;25:4161.
20. Yu C, Deng XJ, Xu D. Microglia in epilepsy. Neurobiol Dis. 2023;185:106249.