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Original article

Synthesis, biological activity, distribution and membrane permeability of novel spiro-thiazines as potent neuroprotectors

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ABSTRACT

New spiro-derivatives of 1,3-thiazine – potential neuroprotectors have been synthesized. It has been determined that the obtained compounds are biologically active and capable of blocking the glutamate-induced calcium ion uptake into synaptosomes of rat brain cortex. The inhibitory activity of the test substances was shown to depend on the chemical nature and structure of the substituents bound with an exocyclic nitrogen atom. Non-polar alkyl and polar radicals with halogen, oxygen and nitrogen atoms were used as substituents. It is typical of the active spiro-thiazines to have alkyl substituents in ortho- and para-position of the benzene ring. Among the investigated spiro-thiazines it is the derivatives with ethyl- and isopropyl-groups in the aril part of the molecules that are the lead-compounds with a high inhibitory ability. We measured the distribution coefficients of the substances in octanol/buffer and hexane/buffer systems and made conclusions about the ability of the investigated drug-like compounds to penetrate the biological membranes. By using the parabolic model we derived a quadratic equation that allowed us to evaluate quantitatively the inhibitory activity of spiro-thiazines with hydrophobic substituents based on lipophilicity data. We also studied the permeability through the phospholipidic membrane and introduced a correlation equation describing the dependence of the investigated spiro-thiazines activity on the descriptors characterizing the donor–acceptor properties.

1. Introduction

In accordance with the contemporary conceptions of the Alzheimer disease pathogenesis the key factor in the development of neurodegenerative processes in the brain is formation of a pathological form of amyloid peptide. In elderly the mechanism of cleavage of this protein changes leading to a reduction in the level of the soluble form and the formation of a peptide β-form. Further aggregation of β-amyloid results in creation of insoluble fibrils, participating in senile plaque formation. Neurodegenerative mechanisms caused by β-form of the peptide involve disturbing the calcium homeostasis, strengthening the oxidative stress, potentiating the toxicity of neurotransmitter amino-acids, as well as initiating apoptose processes. At present a whole series of approaches have been proposed to prevent the cellular destruction and to correct neurodegenerative processes, among them development of potential neuroprotectors with a high biological activity – glutamate-inducing calcium channel-blocking agents [1,2]. While designing new drug compounds it should be taken into account that drugs with neuroprotective action should easily pass from the blood flow to the spinal fluid and brain i.e. to overcome the blood brain barrier (BBB). The main parameter for predicting whether the substance can pass through the BBB is the partition coefficient in the hexane/buffer system [3]. Besides, drug compounds should possess good lipophilicity. It is well known that the distribution coefficient in the octanol/buffer system is a generally accepted parameter of lipophilicity. The distribution coefficients in the above mentioned system are viewed as the most significant physicochemical parameters in all investigations connected with toxicological properties and pharmacokinetic behavior of the substances [4,5]. Another important stage in drug design is investigating bioavailability that indicates whether the molecules are able to

Abbreviations: $K_{43/21}$, Biological Coefficient Describing Ability to Inhibit Glu-Ca-uptake; IC_{50}, concentration of inhibition; $D_{O/B}$, octanol–buffer coefficient; $D_{H/B}$, partition coefficient; $\Delta\log D_{O/H}$, difference between the partition coefficients octanol–buffer and hexane/buffer; P_{app}, apparent permeability; $\Sigma C_{ad}/\alpha$, descriptor – ratio the sum of H-bond donor–acceptor factors in molecule to polarizability.

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penetrate through the semipermeable phospholipid cell membranes in order to interact with intercellular receptors.

Searching for correlations between biological activity and lipophilicity, membrane permeability and other molecular characteristics is the base of the scientific concept named QSAR (Quantitative structure–activity relationship) in modern medicinal chemistry. The methodology of finding the correlations between the structure and biological activity of compounds is developing intensively as the impact of individual substituents on biological activity is being investigated [6]. The above mentioned investigations open opportunities for predicting the physiological activity and rational synthesis of the drug compounds. These investigations make possible to predict the physiological activity of the substances and the efficient synthesis of the drugs for perspective development of the pharmaceutics and clinical chemistry.

By analyzing the accumulated experimental bioscreening results of a huge array of substances, data on their medical applications and structure we managed to single out a number of structures often used in drug substances [7]. Previously the authors [8] had proved the ability of isothiourea derivatives to act as neuroprotectors and cognitive stimulators. We developed a method of synthesizing new 2-aminospirothiazines – cyclic derivatives of isothiourea without substitutions on the bicyclic fragment of the molecule. The present study is aimed at investigating the ability of the synthesized spiro-thiazines to inhibit glutamate-inducing calcium ions uptake and revealing the correlations between the biological activity and the structure, the lipophilicity and the membrane permeability of the substances. The present study is a continuation of our previous investigations into the biological activity, solubility, lipophilicity and membrane permeability of drugs and drug-like compounds [9].

2. Results and discussion

In order to develop drug substances with neuroprotective activity we synthesized a number of 1,3-thiazine spiro-derivatives whose molecular structure is represented in Fig. 1.

We have shown that these compounds can modulate glutamate-dependent uptake of calcium ions in rats' cerebral cortex synaptosomes. The structural formulae of the R substituents are presented in Table 1. The results of the biological activity studies of the investigated compounds are reported in Table 2.

By analyzing the structure–activity correlation we determined biologically active compounds. A histogram of the biological activity values for the synthesized substances is shown in Fig. 2.

As it follows from the above-mentioned data the inhibiting activity depends on the chemical nature and the structure of the substituents bound to the exocyclic nitrogen atom. Spiro-thiazine with an unsubstituted phenyl group (**15**) weakly inhibiting calcium ions uptake was chosen as the fiducial substance to identify the active and inactive substances which allowed us to divide conventionally the synthesized compounds into two groups. The substances (**1**–**14**) with halogen, oxygen and nitrogen atoms in the substituents as well as the compounds in which R is hydrogen or an alkyl group belong to group I – inactive spiro-derivatives. The biological activity investigations have shown that the unsaturated spiro-thiazine (**15**) and its ethyl analog (**9**) inhibit calcium ions uptake poorly, while the methyl derivative (**1**) even potentiates the uptake process. For the aryl derivatives the inhibiting activity depends on the kind of substituents of the ring. It is noteworthy that all the compounds with electron acceptor substituents *n* the benzene ring (**2**, **4**–**8**, **10**–**14**) displayed insignificant activity in this test. Moreover, amino-derivative (**2**) promotes a calcium ion uptake.

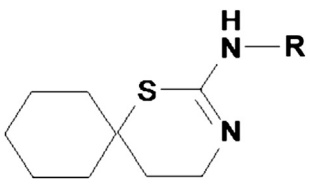


Fig. 1. Molecular structure of spiro-compounds.

Table 1
The structural formulae of the R substituents of compounds studied.

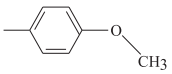
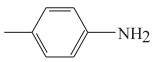
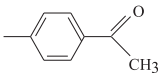
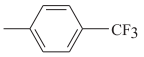
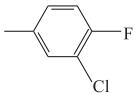
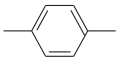
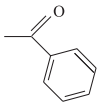
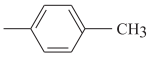
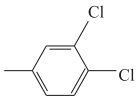
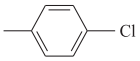
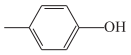
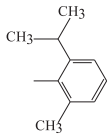
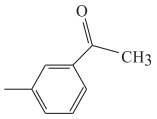
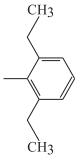
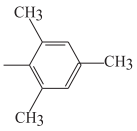
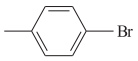
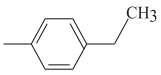
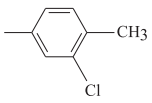
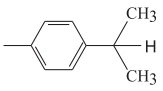
Compound	–R	Compound	–R
1	$-CH_3$	**12**	
2		**13**	
3	–H	**14**	
4		**15**	
5		**16**	
6		**17**	
7		**18**	
8		**19**	
9	$-C_2H_5$	**20**	
10		**21**	
11		**22**	

Table 2
Coefficient of Biological Activity ($K_{43/21}$), Concentration of Inhibition (IC_{50}), Constants of Partition Octanol/Buffer ($D_{O/B}$) and Hexane/Buffer ($D_{H/B}$), Apparent Permeability Coefficient (P_{app}) and Descriptor ($\Sigma C_{ad}/\alpha$) for Spiro-derivatives studied.

№	$K_{43/21}$ (%)	IC_{50} (μM)	$\log D_{O/B}$	$\log D_{H/B}$	$\Delta\log D_{O/H}$	P_{app} (10^6 cm/s)	$\Sigma C_{ad}/\alpha$
1	134.2 ± 13.2	–	0.88	0.91	−0.03	2.70 ± 0.23	0.241
2	114.7 ± 14.5	–	1.88	−0.52	2.40	2.04 ± 0.02	0.347
3	91.4 ± 9.8	–	1.57	1.22	0.35	7.33 ± 0.15	0.254
4	81.5 ± 0.8	–	2.03	0.95	1.08	7.36 ± 0.25	0.266
5	78.1 ± 14.7	–	1.55	1.51	0.04	3.18 ± 0.29	0.231
6	76.9 ± 3.1	–	2.45	1.24[a]	1.21	3.10 ± 0.30	0.251
7	73.5 ± 12.5	–	1.96	–	–	1.72 ± 0.14	0.360
8	67.3 ± 0.4	–	2.37	1.31	1.06	74.73 ± 0.91	0.287
9	65.3 ± 12.1	–	1.38	0.72	0.66	8.78 ± 0.54	0.223
10	56.5 ± 4.7	–	2.53	1.36	1.17	90.31 ± 2.41	0.166
11	45.9 ± 2.3	89.1	2.44	0.91	1.53	23.90 ± 0.31	0.162
12	45.7 ± 1.7	85.1	0.80	1.45	−0.65	1.11 ± 0.05	0.190
13	45.1 ± 6.3	81.3	1.97	1.88	0.09	2.55 ± 0.31	0.198
14	42.1 ± 5.3	77.6	2.18	1.31	0.87	3.06 ± 0.13	0.170
15	33.2 ± 6.3	63.1	1.02	1.69	−0.67	1.81 ± 0.06	0.180
16	20.4 ± 3.9	31.6	1.56	1.48	0.08	5.67 ± 0.20	0.171
17	12.7 ± 7.4	35.5	2.50	1.34	1.15	27.82 ± 1.92	0.170
18	5.4 ± 5.4	20.9	2.36	1.50	0.86	9.04 ± 0.01	0.213
19	3.5 ± 2.7	7.9	1.88	1.28	0.60	21.60 ± 1.54	0.213
20	2.6 ± 2.5	9.3	2.05	1.48	0.57	11.34 ± 0.10	0.225
21	0	11.7	2.31	1.75	0.56	41.02 ± 0.42	0.162
22	0	6.2	1.57	1.45	0.12	15.73 ± 0.98	0.153

[a] compound was not dissolved in hexane.

Introducing a CF_3-group in the para-position of the phenyl ring (**14**) leads to a small decrease in the biological activity when compared to the naked phenyl substituted spiro-thiazine (**15**). The inhibiting ability decreases more significantly when a bromine atom is taken as a substitute (**10**) and mono-substituted chlorine and fluorine derivative is replaced with twice-substituted ones (**4** and **6**). Previously we had determined that spiro-compounds containing halogen atoms in the structure have very low solubility ($\sim 10^{-7}$ mol fraction) in the buffer solution simulating a blood system [10]. Probably, this fact accounts for the decrease in the inhibiting bioaction in a number of halogen-derivatives: CF_3(**14**) > CH_3, Cl (**11**) > Br (**10**) > Cl, Cl (**6**) > F, Cl (**4**). The influence of the oxygen atom in the molecule structure on the biological activity has been studied by the example of the compounds with carbonyl (**5**, **8** and **13**), methoxy (**12**) and hydroxyl (**7**) groups. The obtained results showed that the biological activity is sensitive even to a small change in the position of the substituents. Thus, the structural isomers with acetyl-group in the *para*- and *meta*-positions of the benzene ring (**13** and **8,** respectively) have different inhibiting properties. Another example of how the steric structure of the compounds affects the biological activity is represented by the compound (**5)** in which the acetyl group is nearer to the spiro ring. The effectiveness of the action of the above mentioned compound is lower in comparison with the structurally similar acetyl-substituted substances.

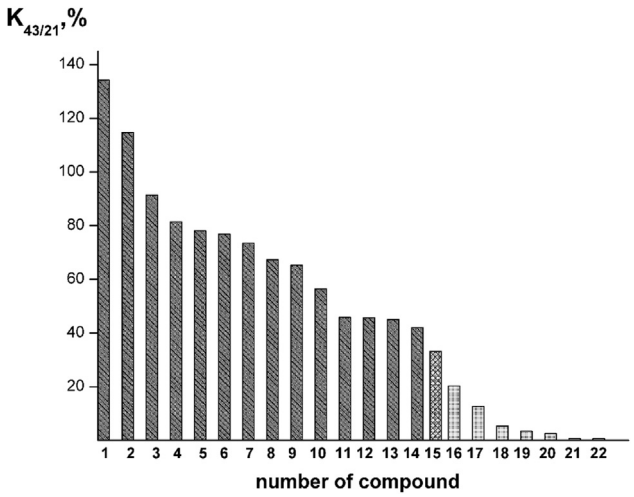


Fig. 2. Biological activity coefficients ($K_{43/21}$) of the investigated compounds.

Group II of the active spiro-thiazines (**16**, **18**–**22**) is characterized by the presence of electron donor alkyl substituents in *ortho*- and *para*-position of the benzene ring. Compound (**17**) with a chlorine atom on the phenyl ring also belongs to this group. The biological activity of these compounds, determined by the $K_{43/21}$ value, changes within the range of 25 to 0%. The elongation of the alkyl chain and the replacement of the methyl-derivative (**16**) with an ethyl-derivative (**21**) has strengthened the inhibiting action and increased the biological activity to the highest value in this test. Branching of the alkyl chain is usually accompanied by a decrease in the biological effect as the volume of the molecule enlarges and the steric hindrance growth impedes the optimal interactions with the bioreceptor active part. But if the substances under investigation are spiro-thiazines, the isomerization of the alkyl substituents is a way to improve their inhibiting properties. It should be noted that the biological activity of the second group of compounds grows when the number of carbon atoms in the structure of the substituents decreases from 4 to 3. As a result along with ethyl-derivative (**21**) the best blocker of glutamate-induced calcium ions uptake is compound (**22**) with an isopropyl group.

As the efficiency of the drug substance action depends on the concentration of the substance near the receptor (i.e. passing the substance through the body membranes), the next part of our study deals with measuring and analyzing the distribution coefficients in organic solvent/buffer systems, modeling the passive drug transport through the biological membranes. The two-phase octanol/buffer system is a model of GI tract membranes and the hexane/buffer one – of BBB membranes. The distribution coefficients of the spiro-thiazines in the mentioned systems are given in Table 2.

The analysis of the obtained data showed that $\log D_{O/B}$ values change within the range of 1.9–2.6 and satisfy the well known requirement [11] that the optimal meanings of the distribution coefficients should be in the range of 1–3. Such drug compounds are characterized by a quite good absorption and diffusion (i.e. they have balanced properties of the hydrophilic–lipophilic characteristics of the molecule), that determine the membrane permeability processes. Poor lipophilicity is typical of non-aryl derivatives (**1**, **3** and **9**), model compound (**15**) and spiro-thiazine with a methoxy-group (**12**). The halogen-derivatives (**6, 10, 11, 14** and **17**) belong to the compounds with high $\log D_{O/B}$ values > 2.18. We have not found any considerable differences in the lipophilicity of the compounds of group I containing polar (hydrophilic) substituents and group II with alkyl (hydrophilic) ones. It should be mentioned that the spiro-derivatives under consideration have a complex molecular structure with hydrophilic amine and hydrophobic phenyl groups (Fig. 1). Therefore, the non-fulfillment of the additivity principle of the impacts of different substituents on the relative hydrophobicity of the investigated spiro-thiazines can be explained on the basis of the concept of considerable influence of the steric arrangement of the substituents in the diphilic compounds [7].

The distribution coefficient in hexane/buffer system is the ratio of the substance concentrations in the lipophilic phase (hexane) to its concentration in the hydrophilic medium (phosphate buffer). The $\log D_{H/B}$ values do not exceed 1.9 (Table 2). The obtained data testify that all the compounds except **2** prefer lipophilic delivery pathways to penetrate BBB. For all the investigated compounds, except the following three (**1, 12** and **15**) with low lipophilicity, the

inequality $\log D_{O/B} > \log D_{H/B}$ is satisfied. In order to predict the BBB permeability we have calculated $\Delta\log D_{O/H}$ – parameter of the spiro-derivatives given in Table 2. An increase in $\Delta\log D_{O/H}$ is caused, as a rule, by a decrease in the permeability and brain distribution [3]. The values of this parameter for the investigated spiro-derivatives satisfy the well known requirement that the optimal $\Delta\log D_{O/H}$ for brain penetration should be less than two units.

The distribution coefficient logarithm in the octanol/buffer system is the most often used descriptor for estimating quantitative correlations structure–activity. Fig. 3 shows the dependences of the biological activity on the molecular lipophilic properties characterized by $\log D_{O/B}$. As it follows from the represented data for group I of the compounds with polar substituents, no correlation was revealed between the activity and distribution coefficients. In order to explain this fact we used the correlation Equation (1) of Hansch [6], applied to compounds of a certain kind of activity and analogous chemical structure:

$$\log(1/k) = a_0 + a_1 \log D_{O/B} - a_2\left(\log D_{O/B}\right)^2 + a_3\sigma + a_4 E_s, \qquad (1)$$

where k is any experimental value characterizing the biological activity, $\log D_{O/B}$ is the distribution coefficient of the substance between octanol and water phases, parameters σ and E_s are electron and steric influences of the substituents, a_i is the constants obtained when the experimental data are processed by the least-squares method. Since the spiro-derivatives of group I poorly interact with glutamate receptors, it is probably necessary to take into consideration the steric impacts of the substituents as well as the hydrophobic properties in order to estimate the correlations between the compounds structure and the biological activity.

The dependence of $\log(1/K_{43/21})$ of $(\log D_{O/B})$ for the compounds of group II with alkyl substituents is also represented in Fig. 3. As an exception this group of biologically active spiro-thiazines also includes compound (**17**) containing a chlorine atom. It should be refined that the most active substances (**21** and **22**) with $K_{43/21} = 0$ are not indicated in Fig. 3. We used the parabolic model to describe the non-linear correlations lipophilicity–activity. According to this model, in order to connect with the target molecules the drug compounds should have an opportunity, on the one hand, to circulate in the blood stream (i.e. to be dissoluble in water), and on the other hand, to penetrate through the cell membranes (i.e. to be soluble in lipids). In this case the activity of the substances with very small and very large distribution coefficients, i.e. highly hydrophilic (**16**) or highly lipophilic ones (**17** and **18**), will be not high

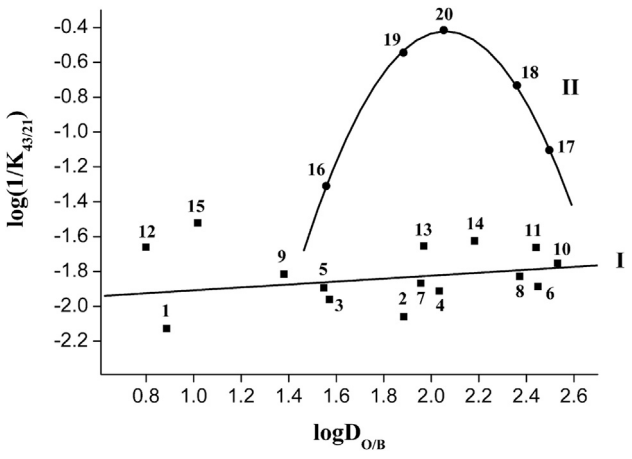


Fig. 3. Correlation between the biological activity coefficient $(\log(1/K_{43/21}))$ and the distribution coefficients $(\log D_{O/B})$ of spiro-compounds studied.

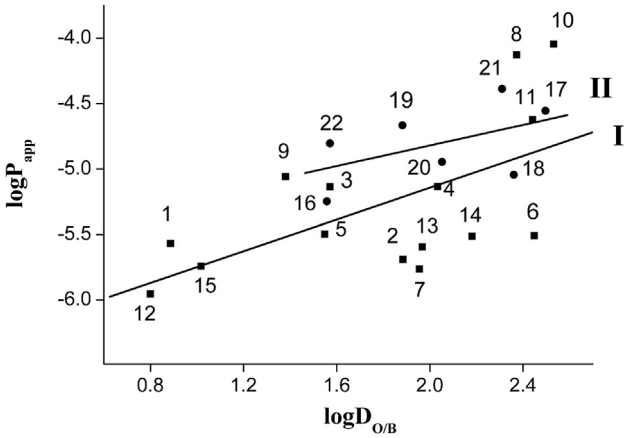


Fig. 4. Correlation between the membrane permeability coefficients $(\log(P_{app}))$ and the distribution coefficients $(\log D_{O/B})$ of spiro-compounds studied.

as in the first case the compounds reveal a poor absorption in the lipophilic phase, whereas in the second one, they don't penetrate the membranes due to a strong interaction with lipid matrix displayed in a poor diffusion. As a result, the dependence of the activity of the spiro-thiazines under consideration with hydrophobic substituents on the distribution coefficient is a parabolic curve with a maximum. Substances (**19**–**22**) belong to the group of leader-compounds balanced by their lipophilicity/hydrophilicity. The correlation equation, in which the biological activity acts as a dependent variable and the distribution coefficient acts as an independent one, is presented below:

$$\log\left(1/K_{43/21}\right) = -15.43 + 14.58 \log D_{O/B} - 3.54\left(\log D_{O/B}\right)^2 \quad (R = 0.9994) \qquad (2)$$

The statistical criteria of Equation (2) testify that it is possible to quantitatively assess the inhibiting ability of spiro-thiazines with hydrophobic substituents on the basis of lipophilic data. The obtained correlations can be used to predict the structure of the leader compounds with a high biological activity in a series of the studied spiro-derivatives. The permeability of the compounds through different membranes determines their bioavailability which is of principal importance in drug development. It is well known that most orally administered drugs overcome the intestine epithelium by passive transport which to a great extent depends on the drug physicochemical properties. In our study we used Phospholipid vesicle Permeability assay [12] to imitate the intestine epithelium of the human body. The results of the investigation of the spiro-derivative membrane permeability are given in Table 2. As it follows from the permeability coefficient results, all the compounds under investigation have $P_{app} > 0.9 \cdot 10^{-6}$ cm/s, therefore, they can be classified as substances with a high ability to permeate through phospholipid membranes. The authors [13] suggested the hypotheses that cell permeability depends, in the first place, on the lipophilicity, determined by the distribution coefficient $\log D_{O/B}$ (pH 7.4), as well as on the polar surface area and molecular weight of a compound. Fig. 4 presents the correlation between the logarithms of the permeability coefficients and the logarithms of the distribution coefficients in buffer/octanol system. As the experiment results show, in spite of the dependence of the membrane permeability on the lipophilicity, modeling permeability is a complicated task. It was estimated that for the compounds of groups I and II the permeability increases as the lipophilicity grows. This is coordinated with literature data: when

$\log D_{O/B}$ values are lower than 3.5, the transepithelial permeation coefficients increase as the lipophilicity rises [14]. Meanwhile, the low correlation coefficients ($R < 0.6$) only reveal a qualitative character of the obtained correlations. The analysis of the results represented in Fig. 4 shows that the permeability of the studied spiro-compounds depends on the chemical nature of the functional groups in the substance molecules. In spite of their small molecular volume the non-aryl derivative (**1**), the compound with the unsubstituted benzene ring (**15**) and the methoxy-group substituted one (**12**) penetrate through the membranes rather poorly due to their low lipophilicity ($\log D_{O/B} < 1.4$). The maximal permeability is observed for acetyl- and bromine-substituted aryl derivatives (**8** and **10**), which have a high lipophilicity ($\log D_{O/B} > 1.9$). It should be mentioned that the correlation line «permeability-lipophilicity» for group II of the compounds with a high biological activity is located above the analogous line for group I of the non-active derivatives. This fact indicates that among the studied substances the biologically active spiro-thiazines can penetrate through the phospholipid membranes better than the others.

The dependences of $\log(1/IC_{50})$ and $\log(1/K_{43/21})$ of the spiro-derivatives on their permeability is presented in Fig. 5. For the biologically active compounds (group II) the dependences have a parabolic type. It should be noted that compounds **21** and **22** with $K_{43/21} = 0$ are not represented in Fig. 5b, due to mathematical limitations when using Hansch Equation (1). The obtained results showed that the poor inhibiting action of the compounds of group I is not determined by the membrane permeability. For the compounds of group II we revealed a parabolic dependence of activity on the permeability which can be described by the equation:

$$\log\left(1/K_{43/21}\right) = -153.88 - 63.10 \log P_{app} - 6.49(\log P_{app})^2 \quad (R = 0.9347) \qquad (3)$$

As it follows from the presented data, the maximal biological activity is typical of compounds with a medium permeability. We can make a conclusion that the high inhibiting ability of the leader compounds (**19**–**22**) is provided by the optimal combination of the lipophilic and hydrophilic characteristics of the molecules, permeability through the membranes and geometrical/topological structure that leads to a better interaction with the receptors.

Lipophilicity of chemical compounds is used as an important physicochemical parameter practically in all investigations associated with biological activity of drug substances. It is well known that the distribution of substances in the two-phase octanol/water system, characteristic of lipophilicity, is determined by the contributions of van-der-Waals forces and tendencies to hydrogen bond formation. These different effects can be separated and, when searching for correlations between the structure and activity, considered to be additive [15]. The most pronounced basic center of the investigated spiro-derivatives is the nitrogen atom of the secondary amino-group which can form a hydrogen bond with the hydrogen atom of the octanol hydroxyl group. This fact makes it necessary to take into account not only non-specific interactions with the organic solvent but also the interactions caused by hydrogen bonding when analyzing the activity of the spiro-thiazines under investigation. That is why we chose the $\Sigma C_{ad}/\alpha$-parameter to assess the donor–acceptor hydrogen bonding ability of the molecules, which was calculated by the HYBOT program package [16]. The $\Sigma C_{ad}/\alpha$ descriptor is the ratio of the sum of H-bond donor–acceptor factors in the molecule to polarizability. The statistical criteria of the equations, where $\Sigma C_{ad}/\alpha$ act as independent variables, were the best among the descriptors. Fig. 6 presents the dependences of the logarithm of the aryl compound activity on the $\Sigma C_{ad}/\alpha$ –value (non-aryl derivatives **1**, **3** and **9** are excluded from consideration). As we can see from Fig. 6, the biological activity of the substances depends on their chemical nature. The activity of spiro-derivatives of group I with polar substituents is decreasing as the descriptor grows. The opposite tendency is observed for compounds of group II with non-polar substituents: the activity rises if the descriptor increases. It should be mentioned that the model compound (**15**) with unsubstituted benzene ring is appropriately placed between the estimated correlation lines of substance groups I and II. In our opinion, the reason for the revealed correlations is changing the basicity of the nitrogen atom of the spiro-thiazine amino-group when polar and non-polar radicals are introduced into the benzene ring. The electron-acceptor substituents of the spiro-derivatives of group I (carbonyl, alkoxy, hydroxy and Hal) reduces the basicity of the nitrogen in the amino-group. The electron-donor substituents (alkyl) act in the opposite way – they increase the electron density on the nitrogen atom and raise its basicity. As a result, the electron pair of the nitrogen atom is held less firmly and interacts more easily with a proton forming a stronger hydrogen bond. Thus, the obtained results indicate that the ability of molecules to take part in specific interactions has an important influence on the biological activity of the investigated spiro-thiazines. Moreover, it should be taken into consideration that realizing these interactions in a living organism is determined by complicated processes of bioavailability and receptor binding activity.

The resulting equations, where the activity acts as the dependent variable and $\Sigma C_{ad}/\alpha$ – as the independent one are presented below. For the compounds of group I with electron-acceptor substituents:

$$\log\left(K_{43/21}\right) = 1.4156 + 1.6073 \sum C_{ad/\alpha} \quad (R = 0.8130) \qquad (4)$$

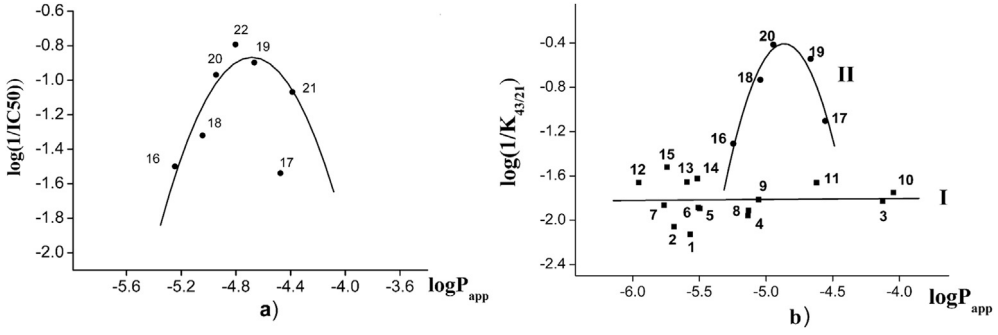


Fig. 5. Correlation between the biological activity coefficients and the membrane permeability coefficients ($\log P_{app}$) of spiro-compounds studied: a) – $\log(1/IC_{50})$; b) – $\log(1/K_{43/21})$.

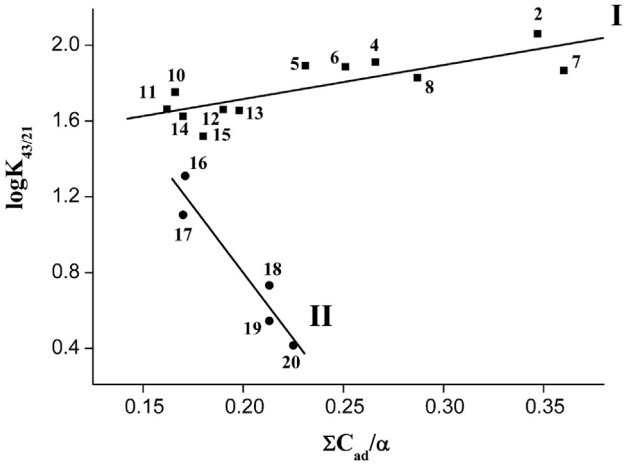


Fig. 6. Correlation of the biological activity ($\log(K_{43/21})$) and $\Sigma C_{ad}/\alpha$ descriptor of spiro-compounds.

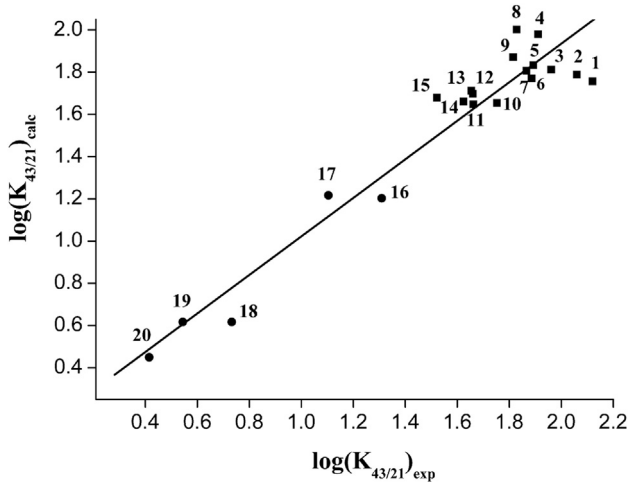


Fig. 7. Comparative analysis of the experimental $\log(K_{43/21})_{exp}$ and predicted $\log(K_{43/21})_{cal}$ values for the test set.

For the compounds of group II with electron-donor substituents:

$$\log\left(K_{43/21}\right) = 3.5898 - 13.9556\sum C_{ad/\alpha} \quad (R = 0.9605) \qquad (5)$$

The calculations carried out by Equations (4) and (5) have led us to the following correlation of the experimental and calculated activity values:

$$\log\left(K_{(43/21)calc}\right) = 0.6369 + 0.6414\log\left(K_{(43/21)exp}\right) \quad (R = 0.8014) \qquad (6)$$

Comparing the experimental and calculated activity values (Fig. 7) has revealed a rather high prognostic ability of the introduced approach for quantitative description of the structure–activity correlation of the studied spiro-thiazines.

3. Conclusions

We have synthesized new spiro-derivatives of 1,3-thiazine as potential neuroprotectors. It was estimated that the obtained compounds are biologically active and can block glutamate-induced calcium ion uptake into rats' brain cortex synaptosomes. It was shown that the inhibiting activity of the tested compounds depends on the chemical nature and the structure of the substituents bound with an exocyclic nitrogen atom. The inactive spiro-thiazine group includes the compounds with halogen, oxygen and nitrogen atoms in the substituents as well as the non-aryl derivatives. The group of active spiro-thiazines contains alkyl substituents in the *ortho*- and *para*-position of the phenyl ring. Among the investigated spiro-thiazines the derivatives with a high inhibiting activity have ethyl- and isopropyl-radicals on the aryl part of the molecule.

The ability of drug-like compounds to pass through the barriers of the body was investigated using the distribution coefficients of the compounds in octanol/buffer and hexane/buffer systems. The analysis of the results has shown that $\log D_{O/B}$ values for spiro-derivatives are within the range of 1–3, which allows us to conclude that all the investigated spiro-thiazines have balanced characteristics of solubility and permeability by passive diffusion and, as a consequence, are easily absorbed. The $\log D_{H/B}$ values were estimated to be less than 1.9. The data obtained testify that all spiro-compounds prefer lipid paths of delivery to overcome BBB.

The parabolic model was applied to description of non-linear correlations "lipophilicity–activity". We experimentally confirmed the commonly accepted views that leader compounds should have balanced lipophilicity/hydrophilicity parameters. We have obtained a second-degree equation allowing quantitative assessment of the inhibiting activity of spiro-thiazines with hydrophobic substituents based on the lipophilicity data.

We have investigated the compounds permeability through the phospholipidic membranes and made a conclusion that the high inhibiting ability of the leader compounds is provided by the optimal combination of lipophilicity, membrane permeability and interaction with the receptors. It was estimated that the ability of the investigated spiro-derivatives to take part in specific interactions has a considerable effect on their biological activity. We have suggested a correlation equation describing the dependence of such activity of the investigated spiro-thiazines on the descriptors that characterize donor–acceptor properties.

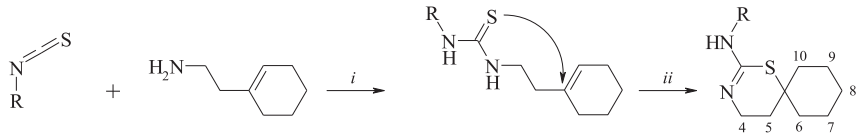
4. Experimental section

4.1. Materials and physical measurements

All chemicals used were purchased from Aldrich (USA). All reagents used in current study were of analytical grade. ^{1}H NMR spectra were recorded on Bruker CXP-200 instrument (Germany) in $CDCl_3$. Chemical shifts δ are given in the d scale relative to Me_4Si. The solvents were removed using rotary evaporator under water pump vacuum. Elemental analysis of the compounds obtained was performed on a CHN analyzer (Carlo Erba). Melting points were determined by microscope «Leitz Laborlux 12 Pol» with hot stage «Mettler FP 82». The pH values were measured by the use of a Toledo MP 220 pH meter (Mettler, USA) standardized with pH 1.68 and 9.22 solutions. Spectrophotometer Spectramax 190 (Molecular Devices Corporation, California, USA) was used for measuring the optical density of solutions.

4.2. General procedure for the synthesis of compounds 1–22

Synthetic approaches to novel 22 spiro-derivates were carried out according to Scheme:



To a stirred solution of 2-(1-cyclohexenyl)ethylamine II (1.25 g, 0.01 mmol) in diethyl ether (20 mL), a solution of appropriate isothiocyanate I in diethyl ether (0.01 mmol) was added dropwise. Then the reaction mixture was stirred for 2–5 h at ambient temperature until formation of precipitate. The precipitate of thiourea III was filtered off, dried, suspended in 48% aqueous HBr (10 mL), and refluxed for 5 h; the precipitate dissolved during the reaction. After the completion of the reaction, the mixture was cooled to ambient temperature, diluted with water (20 mL) and dichloromethane (50 mL), and then saturated aqueous $NaHCO_3$ was carefully added until the solution was basic. The organic layer was separated and dried with Na_2SO_4. The drying agent was filtered off and the solvent was removed. The residue was recrystallized from propane-2-ol to give N-substituted 1-thia-3-aza-spiro[5.5]undec-2-en-2-ylamine (IV) [17].

*4.2.1. Methyl-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**1**)*

Methyl-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (1) was obtained from methyl-isothiocyanate in a similar way in 72% yield; mp 72–73 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.30 (m, 1H, CH), 1.52 (m, 1H, CH), 1.59 (m, 6H, 6CH), 1.71 (t, 2H, J = 5.8 Hz, $NCH_2\underline{CH_2}$), 1.88 (m, 2H, 2CH), 3.66 (C, 3H, CH_3), 3.66 (t, 2H, J = 5.8 Hz, $N\underline{CH_2}CH_2$). Anal. Calcd for $C_{15}H_{20}N_2S$: C, 60.54; H, 9.17; N, 14.12. Found: C, 60.52; H, 9.18; N, 14.11; Calcd. Mass: 198.3862; Found: 198.3860.

*4.2.2. 4-Aminophenyl(1-thia-3-azaspiro[5.5]undec-2-en-2-yl) amine (**2**)*

The overall yield for the two steps was 67%, mp 161 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.31 (1H, m, C(8)H$\underline{H}$); 1.59 (7H, m, C(6)H$\underline{H}$, C(7)H_2, C(8)$\underline{H}$H, C(9)H_2, C(10)H$\underline{H}$); 1.89 (4H, m, C(5)H_2, C(6)$\underline{H}$H, C(10)$\underline{H}$H); 3.59 (2H, m, C(4)H_2); 4.94 (2H, br.s, NH_2); 6.20 (1H, br.s, NH); 6.64 (2H, d, ArH, J = 8.6 Hz); 6.99 (2H, d, ArH, J = 8.6 Hz). Anal. Calcd for $C_{15}H_{21}N_3S$: C, 65.35; H, 7.62; N, 15.25. Found: C, 65.24; H, 7.67; N, 15.31; Calcd. Mass: 275.4191; Found: 275.4193.

*4.2.3. 1-Thia-3-aza-spiro[5.5]undec-2-en-2-ylamine (**3**)*

Yield 67%; mp 104–106 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.27 (m, 1H, CH), 1.56 (m, 7H, 7CH), 1.67 (t, 2H, J = 5.9 Hz, $NCH_2\underline{CH_2}$), 1.85 (m, 2H, 2CH), 3.59 (t, 2H, J = 5.9 Hz, $N\underline{CH_2}CH_2$), 3.66 (C, 2H, NH_2). Anal. ($C_9H_{16}N_2S$) C,H,N. Anal. Calcd for C_9H_2S: C, 58.66; H, 8.74; N, 15.20. Found: C, 58.63; H, 8.73; N, 15.24; Calcd. Mass: 184.2913; Found: 184.3051.

*4.2.4. (3-Chloro-4-fluoro-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**4**)*

Yield 71%, mp 154.6 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.23 (1H, m, C(8)H$\underline{H}$), 1.40 (7H, m, C(6)H$\underline{H}$, C(7)H_2, C(8)HH, C(9)H_2, C(10)H$\underline{H}$), 1.83 (4H, m, C(5)H_2, C(6)$\underline{H}$H, C(10)$\underline{H}$H), 3.45 (2H, m, C(4)H_2), 6.82 (1H, m, ArH), 6.94 (1H, m, ArH), 7.10 (1H, m, ArH). Anal. Calcd for $C_{15}H_{18}ClFN_2S$: C, 57.59; H, 5.79; N, 8.95. Found: C, 57.55; H, 5.80; N, 8.98; Calcd. Mass: 312.8421; Found: 312.8419.

*4.2.5. N-(1-Thia-3-aza-spiro[5.5]undec-2-en-2-yl)-benzamide (**5**)*

Yield 79%, mp 130.4 °C. ^{1}H NMR [200 MHz, DMSO-d_6] δ: 1.36 (1H, m, C(8)H$\underline{H}$), 1.68 (7H, m, C(6)H$\underline{H}$, C(7)H_2, C(8)$\underline{H}$H, C(9)H_2, C(10)H$\underline{H}$), 2.00 (4H, m, C(5)H_2, C(6)$\underline{H}$H, C(10)$\underline{H}$H), 3.64 (2H, m, C(4)H_2), 7.40 (3H, m, ArH), 8.08 (2H, m, ArH), 11.52 (1H, br s, NH). Anal. Calcd for $C_{16}H_{20}N_2OS$: C, 66.63; H, 6.99; N, 9.71. Found: C, 66.55; H, 6.85; N, 9.70; Calcd. Mass: 288.4146; Found: 288.4146.

*4.2.6. (3,4-Dichloro-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**6**)*

Yield 75%, mp 151.5 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.20 (1H, m, C(8)H$\underline{H}$), 1.48 (7H, m, C(6)H$\underline{H}$, C(7)H_2, C(8)$\underline{H}$H, C(9)H_2, C(10)H$\underline{H}$), 1.81 (4H, m, C(5)H_2, C(6)$\underline{H}$H, C(10)$\underline{H}$H), 3.50 (2H, t, C(4)H_2, J = 5.8 Hz), 6.60 (2H, m, ArH), 7.25 (1H, m, ArH). Anal. Calcd for $C_{15}H_{18}Cl_2N_2S$: C, 54.71; H, 5.50; N, 8.51. Found: C, 54.66; H, 5.51; N, 8.56; Calcd. Mass: 329.2961; Found: 329.2959.

*4.2.7. (4-Hydroxy-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**7**)*

Yield 63%, mp 192.9 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.44 (8H, m, C(6)H$\underline{H}$, C(7)H_2, C(8)H_2, C(9)H_2, C(10)H$\underline{H}$), 1.80 (2H, m, C(6)$\underline{H}$H, C(10)$\underline{H}$H), 1.99 (2H, t, J = 5.6 Hz, C(5)H_2), 3.60 (2H, t, J = 5.6 Hz, C(4)H_2), 6.78 (2H, d, J = 8.8 Hz, ArH), 6.96 (2H, d, J = 8.8 Hz, ArH), 10.60 (1H, br.s, OH). Anal. Calcd for $C_{15}H_{20}N_2OS$: C, 65.12; H, 7.24; N, 10.13; Found: C, 65.17; H, 7.21; N, 10.11; Calcd. Mass: 276.4030; Found: 276.4034.

*4.2.8. 3-Acetylphenyl(1-thia-3-azaspiro[5.5]undec-2-en-2-yl) amine (**8**)*

Yield 63%, mp 138.2 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.29 (1H, m, C(8)H$\underline{H}$); 1.56 (7H, m, C(6)H$\underline{H}$, C(7)H2, C(8)$\underline{H}$H, C(9)H_2, C(10)H$\underline{H}$); 1.85 (2H, t, C(5)H_2, J = 5.9 Hz); 1.88 (2H, m, C(6)$\underline{H}$H, C(10)$\underline{H}$H); 3.61 (2H, t, C(4)H_2, J = 5.9 Hz); 2.51 (3H, s, C(O)CH_3); 6.28 (1H, br.s, NH); 7.46 (1H, m, ArH); 7.56 (1H, m, ArH), 7.79 (1H, m, ArH); 8.60 (1H, s, ArH). Anal. Calcd for $C_{17}H_{22}N_2OS$: C, 67.51; H, 7.33; N, 9.26. Found: C, 67.40; H, 7.45; N, 9.28; Calcd. Mass: 302.4417; Found: 302.4422.

*4.2.9. Ethyl-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**9**)*

Yield 62%, mp 107 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.16 (t, 3H, J = 7.2 Hz, CH_3), 1.32 (m, 1H, CH), 1.52 (m, 1H, CH), 1.59 (m, 6H, 6CH), 1.72 (t, 2H, J = 5.8 Hz, $NCH_2\underline{CH_2}$), 1.88 (m, 2H, 2CH), 3.26 (q, 2H, J = 7.2 Hz, $N\underline{CH_2}CH_3$), 3.64 (t, 2H, J = 5.8 Hz, $N\underline{CH_2}CH_2$). Anal. Calcd for $C_{11}H_{20}N_2S$: C, 62.22; H, 9.48; N, 13.19. Found: C, 62.27; H, 9.46; N, 13.21; Calcd. Mass: 212.3413; Found: 212.3416.

*4.2.10. (4-Bromo-phenyl)-1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**10**)*

Yield 72%, mp 175.3 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.32 (1H, m, C(8)H$\underline{H}$), 1.61 (7H, m, C(6)H$\underline{H}$, C(7)H_2, C(8)$\underline{H}$H, C(9)H_2, C(10)H$\underline{H}$), 1.91 (4H, m, C(5)H_2, C(6)$\underline{H}$H, C(10)$\underline{H}$H), 3.59 (2H, m, C(4)H_2), 7.08 (2H, d, J = 8.6 Hz, ArH), 7.39 (2H, d, J = 8.6 Hz, ArH). Anal. Calcd for $C_{15}H_{19}BrN_2S$: C, 53.10; H, 5.64; N, 8.26. Found: C, 53.30; H, 5.65; N, 8.15; Calcd. Mass: 339.3001; Found: 339.3000.

*4.2.11. (3-Chloro-4-methyl-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**11**)*

Yield 74%, mp 149.3°C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.28 (1H, m, C(8)HH), 1.55 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.87 (4H, m, $C(5)H_2$, C(6)HH, C(10)HH), 2.29 (3H, s, CH_3), 3.54 (2H, m, $C(4)H_2$), 6.88 (1H, dd, J = 1.1 8.1 Hz, ArH), 7.08 (1H, d, J = 8.1 Hz, ArH), 7.18 (1H, d, J = 1.1 Hz, ArH). Anal. Calcd for $C_{16}H_{21}ClN_2S$: C, 62.22; H, 6.85; N, 9.07. Found: C, 62.30; H, 6.85; N, 9.05; Calcd. Mass: 308.8762; Found: 308.8766.

*4.2.12. (4-Methoxy-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**12**)*

Yield 65%, 115.5 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.33 (1H, m, C(8)HH), 1.60 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.88 (4H, m, $C(5)H_2$, C(6)HH, C(10)HH), 3.61 (2H, m, $C(4)H_2$), 3.81 (3H, s, OCH_3), 4.50 (1H, br s, NH), 6.84 (2H, d, J = 8.8 Hz, ArH), 7.13 (2H, d, J = 8.8 Hz, ArH). Anal. Calcd for $C_{16}H_{22}N_2OS$: C, 66.17; H, 7.64; N, 9.65. Found: C, 66.22; H, 7.66; N, 9.69; Calcd. Mass: 290.4305; Found: 290.4310.

*4.2.13. 1-[4-(1-Thia-3-aza-spiro[5.5]undec-2-en-2-ylamino)-phenyl]-ethanone (**13**)*

Yield 69%, mp 163.6 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.29 (1H, m, C(8)HH), 1.56 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.85 (2H, t, J = 5.9 Hz, $C(5)H_2$), 1.88 (2H, m, C(6)HH, C(10)HH), 2.55 (3H, s, $C(O)CH_3$), 3.61 (2H, t, J = 5.9 Hz, $C(4)H_2$), 6.25 (1H, br s, NH), 7.24 (2H, d, J = 8.6 Hz, ArH), 7.87 (2H, d, J = 8.6 Hz, ArH).

Anal. Calcd for $C_{17}H_{22}N_2OS$: C, 67.52; H, 7.31; N, 9.27. Found: C, 67.46; H, 7.40; N, 9.28; Calcd. Mass: 302.4417; Found: 302.4422.

*4.2.14. (1-Thia-3-aza-spiro[5.5]undec-2-en-2-yl)-(4-trifluoromethyl-phenyl)-amine (**14**)*

Yield 77%, mp 149.8 °C, ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.29 (1H, m, C(8)HH), 1.58 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.85 (4H, t, J = 5.9 Hz, C(6)HH, C(10)HH, $C(5)H_2$), 3.60 (2H, t, J = 5.9 Hz, $C(4)H_2$), 6.11 (1H, br s, NH), 7.25 (2H, d, J = 8.4 Hz, ArH), 7.49 (2H, d, J = 8.4 Hz, ArH). Anal. Calcd for $C_{16}H_{19}F_3N_2S$: C, 58.52; H, 5.83; N, 8.53. Found: C, 58.33; H, 5.85; N, 8.65; Calcd. Mass: 328.4024; Found: 328.4028.

*4.2.15. Phenyl-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**15**)*

Yield 79%, mp 113.5 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.22 (1H, m, C(8)HH), 1.50 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.78 (4H, m, $C(5)H_2$, C(6)HH, C(10)HH), 3.55 (2H, m, $C(4)H_2$), 6.20 (1H, br s, NH), 6.90 (1H, m, ArH), 7.19 (4H, m, ArH). Anal. Calcd for $C_{15}H_{20}N_2S$: C, 69.19; H, 7.74; N, 10.76. Found: C, 69.30; H, 7.85; N, 10.85; Calcd. Mass: 260.4041; Found: 260.4040.

*4.2.16. 4-Methyl-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**16**)*

Yield 72%, mp 108.3 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.30 (1H, m, C(8)HH), 1.58 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.71 (2H, t, J = 5.8 Hz, $C(5)H_2$), 1.88 (2H, m, C(6)HH, C(10)HH), 2.81 (3H, s, CH_3), 3.66 (2H, t, J = 5.8 Hz, $C(4)H_2$), 3.88 (1H, br s, NH). Anal. Calcd for $C_{10}H_{18}N_2S$: C, 60.56; H, 9.15; N, 14.12. Found: C, 60.59; H, 9.33; N, 14.05; Calcd. Mass: 198.3324; Found: 198.3327.

*4.2.17. (4-Chloro-phenyl)-1-thia-3-aza-piro[5.5]undec-2-en-2-yl)-amine (**17**)*

Yield 75%, mp 162.7 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.29 (1H, m, C(8)HH), 1.59 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.89 (4H, m, $C(5)H_2$, C(6)HH, C(10)HH), 3.57 (2H, m, $C(4)H_2$), 7.06 (2H, d, *m*, ArH), 7.35 (2H, m, ArH).

Anal. Calcd for $C_{15}H_{19}ClN_2S$: C, 61.10; H, 6.50; N, 9.50. Found: C, 61.22; H, 6.53; N, 9.45; Calcd. Mass: 294.8491; Found: 294.8495.

*4.2.18. (2-Methyl-6-Isopropyl- phenyl)-(1-thia-3-aza-spiro[5.5] undec-2-en-2-yl)-amine (**18**)*

Yield 75%, mp 98 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.22 (6H, t, J = 6.8 Hz, $CH(CH_3)_2$), 1.31 (1H, m, C(8)HH), 1.56 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.85 (2H, m, C(6)HH, C(10)HH), 2.01 (2H, t, J = 5.7 Hz, $C(5)H_2$), 2.23 (3H, c, CH_3), 3.11 (1H, m, $CH(CH_3)_2$), 3.54 (2H, m, $C(4)H_2$), 6.25 (1H, br s, NH), 7.11 (3H, m, ArH).

Anal. Calcd for $C_{19}H_{28}N_2S$: C, 72.11; H, 8.91; N, 8.85. Found: C, 72.13; H, 8.90; N, 8.81; Calcd. Mass: 316.4850; Found: 316.4851.

*4.2.19. (2,6-Diethyl-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**19**)*

Yield 69%, mp 119.6 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.11 (3H, t, J = 7.5 Hz, CH_3), 1.28 (1H, m, C(8)HH), 1.42 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.71 (2H, m, C(6)HH, C(10)HH), 1.84 (2H, t, J = 5.6 Hz, $C(5)H_2$), 2.44 (4H, q, J = 7.5 Hz, CH_2), 3.40 (2H, t, J = 5.6 Hz, $C(4)H_2$), 6.96 (3H, m, ArH). Anal. Calcd for $C_{19}H_{28}N_2S$: C, 72.11; H, 8.91; N, 8.85. Found: C, 72.09; H, 8.93; N, 8.86; Calcd. Mass: 316.4850; Found: 316.4848.

*4.2.20. (2,4,6-Trimethyl-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**20**)*

Yield 76%, mp 148.1 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.33 (1H, m, C(8)HH), 1.55 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.83 (2H, m, C(6)HH, C(10)HH), 2.17 (6H, c, $2CH_3$), 1.93 (2H, t, J = 5.7 Hz, $C(5)H_2$), 3.52 (2H, t, J = 5.7 Hz, $C(4)H_2$), 2.29 (3H, c, CH_3); 6.21 (1H, br s, NH). 6.86 (2H, c, ArH). Anal. Calcd for $C_{18}H_{26}N_2S$: C, 71.53; H, 8.66; N, 9.27. Found: C, 71.59; H, 8.44; N, 9.22; Calcd. Mass: 302.2479; Found: 302.2480.

*4.2.21. 4-Ethyl-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**21**)*

Yield 62%, mp 139.5 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.16 (3H, t, J = 7.2 Hz, CH_3), 1.32 (1H, m, C(8)HH), 1.58 (7H, m, C(6)HH, $C(7)H_2$, C(8)HH, $C(9)H_2$, C(10)HH), 1.72 (2H, t, J = 5.8 Hz, $C(5)H_2$), 1.88 (2H, m, C(6)HH, C(10)HH), 3.28 (2H, q, J = 7.2 Hz, $NHCH_2$), 3.61 (1H, br s, NH), 3.64 (2H, t, J = 5.8 Hz, $C(4)H_2$)0. Anal. Calcd for $C_{11}H_{20}N_2S$: C, 62.22; H, 9.49; N, 13.19. Found: C, 62.33; H, 9.43; N, 13.05; Calcd. Mass: 212.3595; Found: 212.3598.

*4.2.22. (4-Isopropyl-phenyl)-(1-thia-3-aza-spiro[5.5]undec-2-en-2-yl)-amine (**22**)*

Yield 73%, mp 139.7 °C. ^{1}H NMR [200 MHz, $CDCl_3$] δ: 1.21 (6H, d, J = 6.7 Hz, $C(CH_3)_2$), 1.30 (1H, m, C(8)HH), 1.56 (7H, m, C(6)HH, C(7)H_2, C(8)HH, $C(9)H_2$, C(10)HH), 1.84 (2H, t, J = 5.9 Hz, $C(5)H_2$), 1.88 (2H, m, C(6)HH, C(10)HH), 2.84 (1H, m, $CH(CH_3)_2$), 3.65 (2H, t, J = 5.9 Hz, $C(4)H_2$), 7.10 (2H, m, ArH). Anal. Calcd for $C_{18}H_{26}N_2S$: C, 71.47; H, 8.66; N, 9.26. Found: C, 71.40; H, 8.85; N, 9.25; Calcd. Mass: 302.4853; Found: 302.4852.

Purity of the spiro-derivatives was 0.98 (mass fraction).

4.3. Permeation studies

Egg phosphatidyl choline, Lipoid E-80 was obtained from Lipoid (Germany). Culture inserts (Transwell-Clear, d = 6.5 mm) and plates were purchased from Corning Inc. Corning, USA, filters in existence were removed, and mixed cellulose ester filters (0.65 μm pore size) from Millipore, Billerica, USA were fused on. Phospholipid vesicle-based barriers were prepared according to the procedure described earlier [12].

The method of the liposome preparation involves film hydration/filter extrusion. Egg phosphatidyl choline (1.8 g) was dissolved in a mixture of chloroform and methanol (2:1) (6 mL) in a round-bottomed flask. The organic solvent was removed under vacuum at 55 °C. The deposited lipid film was exposed to vacuum of 55 hPa at room temperature for an additional period of 3 h to remove traces of solvent, before hydration with phosphate buffer, containing 10% (v/v) ethanol to obtain a 6% (w/v) liposomal dispersion. The liposome dispersion was then filter-extruded through 0.8 and 0.4 μm polycarbonate membrane filters (Millipore) at room temperature. The extrusion was done by hand with a syringe through the filters in a 25 mm Swinnex Filter Holder from Millipore. To obtain liposomes of two different sizes, one portion was extruded five times through the 0.8 μm polycarbonate membrane filter while another portion was extruded five additional passages through filters with 0.4 μm pore size.

Stock solutions of the compounds under study ($2.5\cdot10^{-5}$ to $2.5\cdot10^{-3}$ mol L^{-1}) were prepared by dissolving the drug in phosphate buffer solution pH 7.4. Phosphate buffer solution (pH 7.4) was prepared by mixing the solutions of appropriate phosphoric acid sodium and potassium salts. Containing KH_2PO_4, $Na_2HPO_4\cdot12H_2O$, NaCl, NaN_3, and water, the solution was adjusted to pH 7.4 with HCl/NaOH if necessary. Concentrations of different drug solutions were chosen on the assumption of analytical considerations as well as for obtaining the sink conditions during permeation studies. The concentrations of different drug solutions had to be high enough for the obtainment of a definite amount of a drug in an acceptor chamber during permeation studies to be quantified by means of UV-absorbance and still be below the solubility limit.

Permeation studies were performed after loading the inserts (the facility for bearing the 0.65 μm pore size mixed cellulose ester filter) with drug solution (100 μL) and placing them into separate acceptor compartments containing phosphate buffer solution (600 μL) (pH 7.4). The permeation experiment was carried out at room temperature without agitation, and inserts were moved to wells containing equal quantities of fresh buffer solution. Taking into account the low solubility of the compounds under investigation, the operational range of 12–24 h was optimal. In conclusion of the permeation experiment, samples (200 μL) from each acceptor compartment were transferred into 96-well UV 96-well black plates (Costar) and drug concentrations were measured spectrophotometrically at the most appropriate wavelength for each drug. A blank well was filled with pure phosphate buffer pH 7.4. The electrical resistance of the lipid barriers was measured (Millicell-ERS, Millipore, USA) immediately after completion of permeation studies. To find out the resistance of the lipid barrier itself, a value of 119 Ω resulting from the filter characteristics was subtracted from the observed resistance meaning. Such a difference was then multiplied by the surface area (0.33 cm^2) to be normalized for the dimensions of the insert. The experiments were performed at least in triplicate with three inserts in each parallel for every compound. The mean values and standard deviations are reported.

The standard time dependences of the cumulative amount of permeated drug consisted of 6–9 points, and every point represented the mean value of three parallels. The r2-meanings were always higher than 0.99. Repeatability corresponded to 3.9–20.1%, and an intermediate precision of 2.7–29.4% was observed for different drugs using such a novel method. The linear part of the slope of the curve of the standard time dependence of the cumulative amount represented the steady-state flux rate. If a lag time was observed before the steady-state conditions were attained, and the saturation conditions took place at the end of the experiments, only the middle points of the cumulative plot were used for the calculation procedure, based on the steady-state flux. The obtained flux rates were used to calculate the apparent permeability coefficient (P_{app}, cm s^{-1}) by means of the following equation:

$$P_{app} = J/(A\cdot(C_d - C_a)) \quad (7)$$

where J is the observed flux rate at steady-state (nmol s^{-1}), A is the surface area of the insert (cm^2), and C_d and C_a are the concentrations of the solutions in the donor and acceptor chambers (nmol/mL), respectively. The experiments were performed under the sink conditions; that is, the drug concentration in the acceptor chamber did not exceed 10% of the drug concentration in the donor chamber at any time [18].

Table 3
Effect of spiro-derivatives studied on glutamate-induced $^{45}Ca^{2+}$ uptake into synaptosomes of rat cortex.

Compound	Concentration, μM	Percentage $^{45}Ca^{2+}$ of control (control − 100%)	Inhibition, %
MK-**801**	100	26.9	73.1
	50	35.0	65.0
	25	46.5	53.5
	10	65.2	34.8
	5	76.1	23.9
	1	97.5	2.5
	0.5	98.4	1.6
11	100	45.9	54.1
	50	69.1	30.9
	25	92.9	7.1
12	100	45.7	54.3
	50	68.3	31.7
	25	97.0	3.0
13	100	45.1	54.9
	50	63.7	36.3
	25	82.3	17.7
14	100	42.1	57.9
	50	63.2	36.8
	25	98.8	1.2
15	100	33.2	66.8
	50	54.7	45.3
	10	80.4	19.6
16	100	20.4	79.6
	50	31.3	68.7
	25	60.4	39.6
17	100	12.7	87.3
	50	33.1	66.9
	25	65.7	34.3
	10	93.3	6.7
18	100	5.4	94.6
	50	14.8	85.2
	25	41.5	58.5
	10	72.3	27.7
19	100	3.5	96.5
	50	25.1	74.9
	25	28.2	71.8
	10	45.7	54.3
	5	55.7	44.3
20	100	2.6	97.4
	50	14.2	85.8
	25	27.6	72.4
	10	50.1	49.9
	5	61.9	38.1
21	100	0.0	100.0
	50	20.8	79.2
	25	36.1	63.9
	10	52.2	47.8
	5	69.9	30.1
22	100	0.0	100.0
	50	0.1	99.9
	25	5.0	95.0
	10	38.2	61.8
	5	55.3	44.7

4.4. Determination of partition coefficients

The isothermal saturation method was used to determine the partition coefficients in n-octanol/buffer (pH 7.4), $D_{O/B}$, and n-hexane/buffer (pH 7.4), $D_{H/B}$, systems. All the experiments were carried out at 25 °C. The substance concentrations were measured spectrophotometrically using calibration curves. The partition coefficient was calculated using the following equation:

$$D_{O/B} = C_{O/B}/C_{B/O} \quad (8)$$

where $C_{O/B}$ and $C_{B/O}$ are the molar concentrations of solute in the mutually saturated phases of octanol and water. The accuracy of the D value was verified by checking the mass balance of the starting amount of compound i compared to the total amount of the compound partitioned between the two phases:

$$m_i = m_{O/B} + m_{B/O} \quad (9)$$

where $m_i = C_i V_i$ is the starting mass (in moles) of the compound, $m_{O/B} = C_{O/B} V_{O/B}$ is the mass of the substance dissolved in the water-saturated octanol phase, and $m_{B/O} = C_{B/O} V_{B/O}$ is the mass of the substance dissolved in the octanol-saturated water phase.

4.5. Calcium-blocking property experiments

Interaction between the compounds and the glutamate-dependent calcium uptake system was studied on newborn (8–11 days old) rat brain synaptosomal P2-fraction isolated according to the following method: synaptosomes were suspended in incubation buffer A (132 mM NaCl, 5 mM KCl, 5 mM HEPES) pH 7.4 stored at 0 °C during the experimental stage. The aliquots of synaptosomes (50 μL) were transposed to buffer A containing test compound and 45Ca-samples. Calcium ion uptake was stimulated by introducing the glutamate solution (200 μM). After 5 min incubation at 37 °C, the process was terminated by the filtration on GF/B-filters. The sample was washed three times with cold buffer B (145 mM KCl, 10 mM Tris, 5 mM Trilon B) followed by radioactivity measuring with scintillator counter SL-4000 Intertechnic. In preliminary experiments, all the compounds were tested for the ability to inhibit glutamate stimulated Ca uptake at the concentration of 100 μM. If the inhibition of Glu-Ca-uptake was 50% and more, then further studies were carried $K_{43/21}$ out to determine the concentration dependence of inhibition and the corresponding value of was measured according to the following equation:

$$K_{43/21} = 100 \cdot [(Ca_4 - Ca_3)/(Ca_2 - Ca_1)] \quad (10)$$

where Ca_1 is the Ca^{2+} influx in the blank experiment (without glutamate and test compounds); Ca_2 is the Ca^{2+} influx in the presence of glutamate only (Glu-Ca-uptake); Ca_3 is the Ca^{2+} influx in the presence of test compound (without glutamate); and Ca_4 is the Ca^{2+} influx in the presence of both glutamate and test compound.

The concentrations of the compounds and characteristics of inhibition are presented in Table 3. NMDA-antagonist MK-801 have been used for positive control. Dizocilpine (MK-801) with purity 98% was purchased from Sigma–Aldrich (Saint Louis, MO, USA).

4.6. Calculation procedure

Physicochemical descriptors were calculated with the program package HYBOT-PLUS (version of 2003) in Windows [16].

Acknowledgments

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