Biomeditsinskaya Khimiya, 2025 vol. 71, issue 2, pp. 81–94.

REVIEW

SLOW-BINDING INHIBITORS OF ENZYMES: KINETIC CHARACTERISTICS AND PHARMACOLOGICAL INTEREST

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Currently, the search for new slow-binding inhibitors of enzymes (SBI) and their identification primary *in vitro* studies still attracts much attention in the context of their potential role as putative pharmacological agents for the treatment of various diseases. In contrast to their classical reversible analogues, SBI exhibit a slow enzyme binding kinetics, where the equilibrium steady-state is reached not in microseconds, but after longer time intervals. Such compounds could be promising drugs, because regardless of their pharmacokinetics in the bloodstream, they have such advantages as high affinity for the target enzyme, long residence time on the target, and therefore, prolonged action. These pharmacological properties ensure optimized dosage of drugs required to achieve high activity with less side effects. In this review we have considered mechanisms of SBI interaction with enzyme targets, the principles of their recognition at the level of *in vitro* studies and analysis of binding and kinetic parameters.

Keywords: slow-binding inhibitors; enzyme kinetics; inhibition constant; residence time

DOI: 10.18097/PBMCR1536

INTRODUCTION

There is increasing evidence that the pharmacological activity of inhibitor drugs depends not only on affinity to a particular target and duration of its interaction with this target: how quickly the drug binds to the target site and how long it remains there [1, 2]. According to the model proposed by Copeland [3], the effect of a drug lasts as long as it is bound to the target and disappears when the drug-target complex dissociates. Many example are known when pharmacodynamics persists long after most of the drug has been eliminated from the bloodstream [4, 5]. Therefore, in order to search for and develop new drugs, the parameter "the drug-target residence time" has been increasingly used [1] in addition to commonly used thermodynamic, kinetic and traditional pharmacological parameters: LD_{50} (50% lethal dose), IC_{50} (concentration causing 50% inhibition), ED_{50} (half-effective dose), and dissociation constant [1]. Every year, the number of publications, in which the authors propose to include the kinetics of drug binding to the target as one of the stages of drug development, is increasing [6–13]. Incorrect assessment of the ligand affinity to the target [14–16] has serious consequences for drug development, including incorrect predictions of human dosage, confusion in the selection of candidate molecules for their improvement, and discrepancies between assessments of ligand activity *in vivo* and *in vitro*.

Among the large number of drugs, reversible enzyme inhibitors, slow-binding inhibitors (SBI) are of great interest. Usually, in the case of fast reversible inhibitors, as well as for substrates, reversible complexes with the enzyme are formed within a few microseconds (<50 μs). After a short induction period, a steady state of the system occurs. In contrast to classical reversible inhibitors, for SBI the induction period is much longer (>50 μs). The observed induction time of more than 10 s after rapid mixing of the enzyme and reagents at a constant temperature (thermostating) serves as evidence of SBI, which should be confirmed by refined kinetic analysis using analytical equipment for studying the kinetics of fast reactions, for example, the stopped-flow method [17–19]. However, from a practical viewpoint (pharmacological significance), SBIs with a longer retention time (>2 min) are of the greatest interest.

The first works on the kinetics of slow binding of enzyme inhibitors were published in the second half of the last century [20–22]. In 1982, Morrison presented a method for analyzing the kinetic dependencies of enzymatic reactions in the presence of SBI [23]. According to the PubMed search for the keywords "slow-binding", "inhibitors", and "enzyme", more than 500 examples of SBI have been described to date, About 50 of them were published in the last 5 years. Interest in such compounds is due to the fact that, regardless of their pharmacokinetics in the bloodstream, they have a high affinity for the target enzyme and

a long residence time [24, 25]. Therefore, a search for and development of new SBI, potential drugs for the treatment of various diseases, is currently underway [26–29]. Establishing the mechanisms of slow inhibition at the stage of development of *in vitro* drug evaluation allows predicting a longer duration of drug action and simultaneously reducing its dose and side effects [30], thereby predicting the efficacy and safety of the future drug *in vivo* [31, 32].

This review is aimed at considering the mechanisms of SBI binding to enzymes, the principles of SBI recognition at the stage of *in vitro* studies. The review considers examples of tight binding inhibitors with high affinity and functionality in the same concentration range with the enzyme ([E] ~ [I]) [30], which in most cases are irreversible inhibitors. In the first part of the review, we consider the theoretical principles of SBI. The second and third parts describe the methods of SBI recognition, the course of analysis of kinetic dependencies and the determination of the parameters and type of enzymatic reaction mechanisms characterizing SBI. The final part presents examples of SBI found in the literature.

1. THEORETICAL CONSIDERATION OF SLOW-BINDING INHIBITORS

Figure 1 shows a general scheme of the mechanism of interaction of an enzyme (E) and a substrate (S) with formation of a reaction product (P) in the presence of inhibitors (I), which is usually described by the Michaelis-Menten model.

The inhibition constant (K_i), equal to the inhibitor concentration, which inhibits 50% of the enzyme and measured in mol/l, is one of the main parameters of the enzymatic reaction. The lower the K_i value, the smaller the amount of inhibitor is required to achieve the effect, i.e. the lower the dose of the drug, and the fewer side effects are, namely, the pharmacotoxicological effect on secondary targets. Therefore, at present, inhibitors that exhibit an effect in the nano- and picomolar concentration range are of the greatest interest. K_i depends on the rate constants of association (k_{on}) and dissociation (k_{off}) of the enzyme-inhibitor complex (EI) (Equation 1):

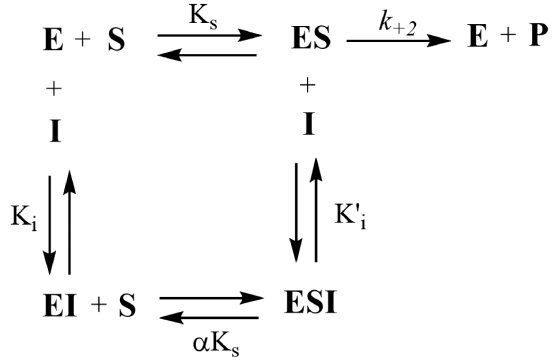


Figure 1. A general scheme of action of classical reversible inhibitors, where K_s is a dissociation constant of the enzyme-substrate complex, K_i and K'_i are inhibition constants (dissociation constants of enzyme-inhibitor complexes), k_{+2} is a rate constant of formation of a reaction product and free enzyme.

$$K_i = \frac{k_{off}}{k_{on}} \quad (1).$$

where k_{on} reflects the energy barrier of the enzyme-inhibitor binding reaction and its value is usually in the range of 10^3–10^8 $M^{-1}s^{-1}$ and k_{off} describes the thermodynamic stability of the EI complex and is measured in reciprocal seconds s^{-1} [33, 34]. The k_{off} value changes depending on the mechanism of action of the enzyme and inhibitor. For example, one recent study has shown that during the computer simulation of the kinetic dependences of enzyme inhibition and subsequent data analysis, a change in k_{off} from 100 s^{-1} to 0.0001 s^{-1}, was accompanied by changes in the type of inhibition from competitive to noncompetitive inhibition [35].

SBI are characterized by slow binding to the enzyme and then slow dissociation; therefore, it is necessary to introduce a number of kinetic parameters to describe the toxico-pharmacological characteristics of SBI. As such parameters, the values inverse to the dissociation rate constant k_{off} are used: residence time (τ_R) (equation 2) and half-time of dissociation of the enzyme-inhibitor complex ($t_{1/2}$) (equation 3) [1]:

$$\tau_R = \frac{1}{k_{off}} \quad (2);$$

$$t_{1/2} = \frac{\ln 2}{k_{off}} \quad (3).$$

The τ_R parameter can provide a more complete understanding of the dynamic nature of drug-target interactions *in vivo* [36]. In general, τ_R is the "lifetime" of the drug-target complex, i.e. the time during which the ligand and target are in a bound state. The higher this value, the longer the inhibitor is bound to the target enzyme and the longer the effect of the drug will be manifested *in vivo*; this may reduce the dose and prolong the pharmacological effect [37].

The k_{on} also plays an important role in determining the residence time, primarily due to the rebinding effect [7], which consists in the following chain of events. In a limited space (in synapses or in organelles), where the concentration of the inhibitor on the target will be high, after dissociation of the EI complex, the inhibitor can bind again to the same target or to any other target located nearby [4]. This effect contributes to an increase in the duration of action of the drug, since it remains in a bound state longer, despite its fast pharmacokinetics. The rebinding effect will depend not only on the high concentration of the drug on the target and the limited space, but also on k_{on}: the higher the k_{on}, the faster the EI complex is formed [38].

For effective enzyme inhibitors, the K_i values should be as low as possible (in nano/picomolar concentrations), while the τ_R value as high as possible, up to several minutes. However, at high τ_R values, when the dissociation stage of the EI complex is very slow, inhibition can be considered as irreversible. Therefore, kinetic studies of enzyme inhibitors should be supplemented by molecular docking and molecular dynamics to understand the molecular mechanisms of inhibitor binding to the enzyme, which will allow determining the calculated parameters and will give an idea of which compounds need to be studied by experimental methods [39–41].

2. ANALYSIS OF KINETIC DEPENDENCIES AND DETERMINATION OF ENZYME REACTION PARAMETERS FOR SLOW-BINDING INHIBITORS

For classical reversible inhibitors, the steady state in the enzyme-inhibitor-substrate (E-I-S) system is reached in microseconds. For SBI, such a state can be reached in several seconds, minutes, and even hours [2, 42], i.e., the steady state of the system is reached after some time interval.

For classical inhibitors, at the steady state, a linear increase in the concentration of the reaction product [P] is observed over time under conditions of substrate excess (Fig. 2, line 1); it is described by equation (4):

$$[P] = V \times t \quad (4),$$

where $[P]$ is concentration of the reaction product, t is time; V is a steady state reaction rate for a classical inhibitor.

In the case of SBI (Fig. 2, curves 2, 3) the kinetic dependencies are not linear and they follow exponential equation (5) [19, 20]:

$$[P] = V_{ss} \times t + \frac{(V_i - V_{ss}) \times (1 - e^{-k_{obs} \times t})}{k_{obs}} \quad (5),$$

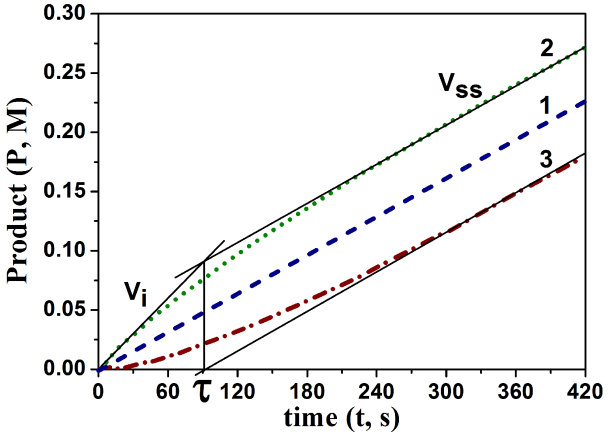


Figure 2. Plots of time-dependent increase of a reaction product concentration in the presence of a classical inhibitor (line 1) and SBI (curves 2 and 3) without pre-incubation (curve 2) and with pre-incubation (curve 3). The plot was built using literature data [23, 30, 48].

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} EI' + P'$$

Figure 3. A scheme of the mechanism of enzyme aging in the presence of inhibitor, where EI′ is an irreversibly modified enzyme-inhibitor complex, P′ is a leaving group.

where $[P]$ is concentration of the reaction product, t is time; V_{ss} is a steady state reaction rate; V_i is initial reaction rate; k_{obs} is a first order reaction rate constant.

In the course of complication of the reaction mechanism, for example, during irreversible chemical modification of the EI complex, when $V_{ss} = 0$ (Fig. 3), equation (5) is simplified to equation (6):

$$[P] = \frac{V_i \times (1 - e^{-k_{obs} \times t})}{k_{obs}} \quad (6).$$

As can be seen from Figure 2, typical dependences of the SBI without preincubation with the enzyme (when the substrate is in excess), look as follows: 1) a sharp increase in the initial velocity V_i; 2) a decrease in the reaction rate to V_{ss} due to reaching a steady state of the system; 3) the induction time τ (lag time) [43, 44], i.e. the moment of time at which the concentration of the product at the initial velocity and at the steady state velocity would be the same. The reciprocal value of τ is equal to k_{obs} (equation 7) [28]:

$$\tau = \frac{1}{k_{obs}} \quad (7).$$

In some cases, enzyme-inhibitor binding takes a long time [42], so the enzymatic reaction is carried out with preliminary incubation of the enzyme with SBI for some time, required for the stable EI complex formation. During incubation, equilibrium is established between the enzyme and the inhibitor. As a result of the slow stage of dissociation of the EI complex, a small slope is observed in the initial section of the kinetic dependence 3 (Fig. 2), and the growth of the reaction product at a steady state occurs only after some time. In this case, the initial velocity V_i will be lower than the steady state velocity [30]. Some cases are also known in the literature, when after incubation of the enzyme with the inhibitor, the initial reaction velocity remained unchanged compared to the reaction carried out without incubation, since inhibition occurred only in the presence of the substrate [45].

The plots of V_i, V_{ss}, and k_{obs} versus inhibitor concentration may be used to determine the type and mechanism of SBI action. As a rule, the type of any inhibitor is determined graphically by plotting the $1/V_{ss}$ versus inhibitor concentration (Dixon plot) and $[S]/V_{ss}$ versus inhibitor concentration (Cornish-Bowden plot) [46].

The type of inhibition can also be determined plotting k_{obs} versus substrate concentration. Tian and Tsou showed a relationship between

the k_{obs} value and substrate concentration for competitive, non-competitive, and uncompetitive types of inhibition [30, 47]. Thus, for a non-competitive inhibitor, the k_{obs} value does not depend on substrate concentration (Fig. 4, curve 1). For an uncompetitive inhibitor, the k_{obs} values will increase with increasing substrate concentration (Fig. 4, curve 2). For a competitive SBI, a decrease in k_{obs} is observed with increasing substrate concentration (Fig. 4, curve 3).

3. MECHANISMS OF ENZYMATIC REACTION IN THE PRESENCE OF SLOW-BINDING INHIBITORS

Three mechanisms of enzymatic reaction occurring in the presence of slow-binding inhibitors have been proposed (Fig. 5) [23, 30, 48].

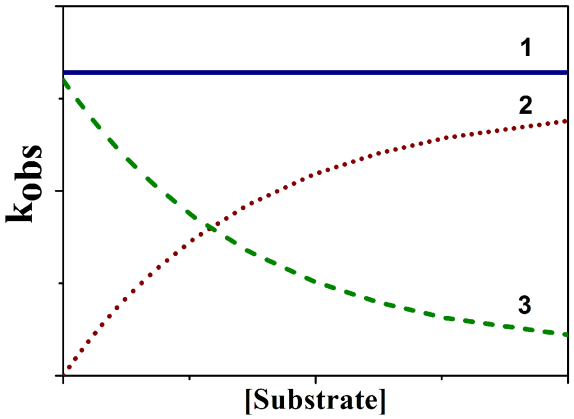


Figure 4. Plots of k_{obs} dependence on substrate concentration for noncompetitive (1), uncompetitive (2) and competitive (3) types of inhibition [30, 47].

3.1. Mechanism A

Figure 5A shows the scheme of the enzymatic reaction in the presence of SBI, occurring by mechanism A. As can be seen, this is a single-step process, which is characterized by a slow stage of inhibitor binding to the enzyme, the EI complex formation and then a slow stage of dissociation of this complex. For mechanism A, $k_{off} = k_{-3}$ and $k_{on} = k_{+3}$. The ratio of association (k_{+3}) and dissociation (k_{-3}) rate constants, as for classical inhibitors, is equal to the inhibition constant (K_i). The association/ dissociation mechanism of the EI complex is described by the above equation (1). For mechanism A, the dependence of k_{obs} on the inhibitor concentration will be linear and described by equation (8), and the initial reaction rate V_i does not depend on the inhibitor concentration (Fig. 6A):

$$k_{obs} = k_{-3} + \frac{k_{+3}[I]}{1 + [S]/K_m} \qquad (8).$$

The dissociation constant of the enzyme-inhibitor complex (k_{-3}) will be defined as the intercept on the ordinate axis (Fig. 6A).

3.2. Mechanism B

In the case when the reaction mechanism follows mechanism B (Fig. 5B), the inhibitor "classically" binds to the enzyme to form the EI complex, accompanied by its slow isomerization into another conformation (E*I). This isomerization may also be related to the slow transfer of the inhibitor from the initial binding site to the final one. Thus, the first inhibition constant (interaction between enzyme E

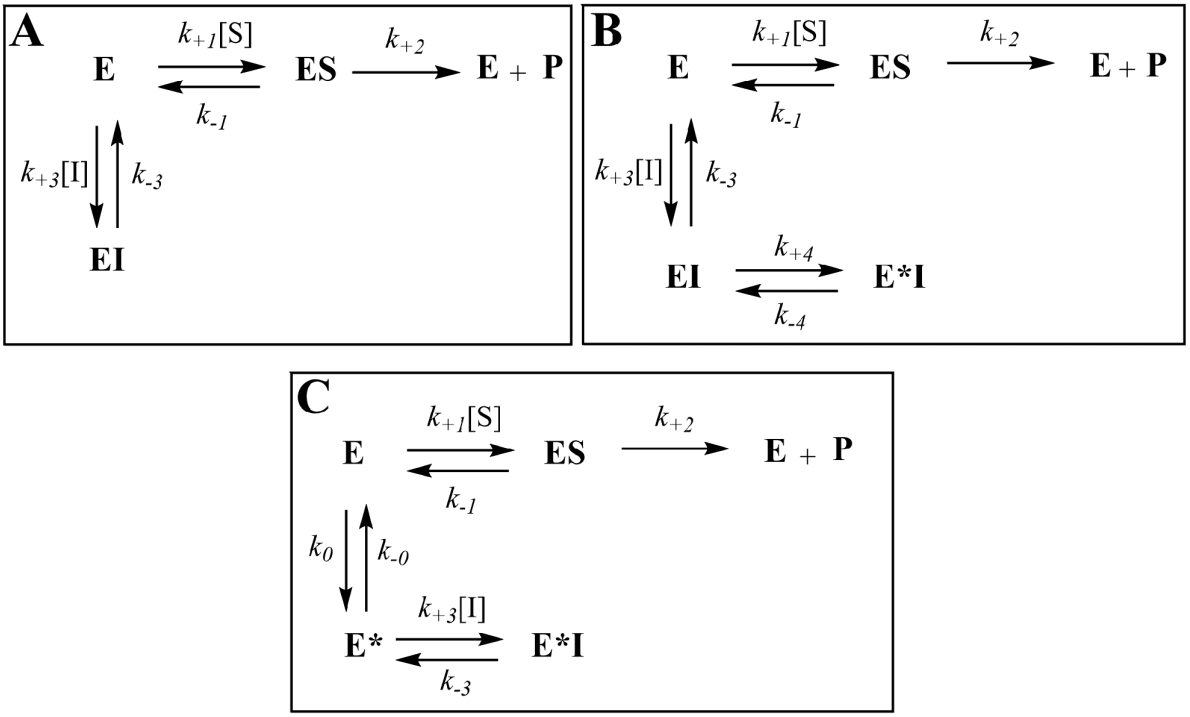


Figure 5. The mechanisms A, B, and C of enzymatic reaction in the presence of SBI.

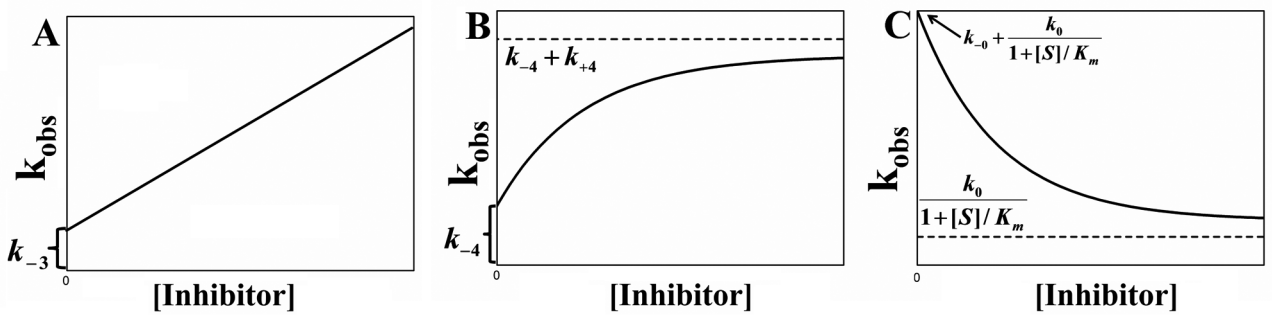


Figure 6. Plots of k_{obs} dependence on inhibitor concentration for mechanisms A, B, C of enzymatic reaction in the presence of SBI.

and inhibitor I) is described by equation (1), but the association/dissociation is rapid, as for classical inhibitors. The second inhibition constant (K^*_i), related to the isomerization of the E*I complex, is described by equation (9) provided that the inhibitor is in excess (I>>E). This mechanism is often referred to as the induced fit model:

$$K^*_i = \frac{[E][I]}{[EI]+[EI^*]} = \frac{K_i k_{-4}}{k_{+4}+k_{-4}} \quad (9).$$

In such a reaction mechanism, both the isomerization of EI into E*I (rate constant k_{+4}) and the reverse process of transformation of E*I into EI (rate constant k_{-4}) can be slow stages. In some cases, when $k_{-4} << k_{+4}$ (i.e., when the reverse isomerization of the enzyme into the initial conformation is much slower than the direct process) k_{-4} is considered negligible and the process itself is irreversible [49]. For mechanism B, the k_{off} parameter is described by equation (10):

$$k_{off} = \frac{k_{-3}\, k_{-4}}{k_{-3}+k_{+4}+k_{-4}} \quad (10).$$

For mechanism B, the dependence of k_{obs} on the inhibitor concentration is convex-hyperbolic (Fig. 6B) and is approximated by the nonlinear equation (11). In this case, the intersection of the dependence with the ordinate axis gives the k_{-4} value (Fig. 6B). The initial reaction rate V_i will also depend hyperbolically on the inhibitor concentration:

$$k_{obs} = k_{-4} + \frac{k_{+4}[I]}{K_i(1+[S]/K_m)+[I]} \quad (11).$$

3.3. Mechanism C

In the case of mechanism C (Fig. 5C), two forms of the enzyme (E and E*) are in slow equilibrium with each other (hysteresis behavior of the enzyme), and only one form of the enzyme binds rapidly to the inhibitor (however, a more complex model in which inhibitor binding is slow to both conformations cannot be ruled out), thus causing isomerization of the enzyme into a conformation capable of binding to the inhibitor (E<=>E*). This mechanism is often referred to as the conformational selection model [23].

For mechanism C $k_{off} = k_{-3}$. The dependence of k_{obs} on inhibitor concentration, shown in Figure 6C, is concave-hyperbolic and is described by equation (12):

$$k_{obs} = \frac{k_{-0}}{1+[I]/K_i} + \frac{k_0}{1+[S]/K_m} \quad (12).$$

3.4. Irreversible and Quasi-Irreversible SBI

In most cases, slow inhibition is reversible. However, when the mechanism becomes more complex, irreversible steps leading to enzyme inactivation are also possible (Fig. 3). Conformational transformations of the EI complex can cause changes in the stability of the enzyme, for example, reducing its conformational plasticity with the formation of a more rigid structure [50, 51].

For example, after reversible slow inhibition, a covalent step is possible; it results in covalent bond formation between the inhibitor and the active center of the enzyme. This is most often observed for inhibitors that are analogs of the transition state. Such type of inhibition was shown by Mucha et al. for metalloaminopeptidases [52]. The study of the mechanism of slow covalent inhibition of aminopeptidases by similar inhibitors (amastatin and bestatin derivatives) [53, 54] has shown that it corresponds to the mechanism B SBI (Fig. 5B). The slow isomerization of the EI complex into E*I is followed by a step that results in the formation of a covalent bond between E* and I. Other examples of transition state inhibitors that cause covalent slow inhibition have been considered in [17]. For example, trifluoroketone (TFK; 1-(3-*tert*-butylphenyl)-2,2,2-trifluoroethanone), an SBI of human acetylcholinesterase type B with τ_R = 20 min, forms a hemiketal bond with the active center serine residue upon binding to the enzyme. Deacylation of the active center serine residue by water is slow, and the enzyme activity is restored after 40 min [55]. Irreversible inhibition of cholinesterases by phosphorylation of the active center serine may be preceded by slow binding

stages. For example, cresyl saligenin phosphate, the active metabolite of tri-*ortho*-cresyl phosphate, is an irreversible type C SBI of cholinesterases [56, 57].

3.5. Allosteric Enzymes and SBI

The mechanisms described above are characteristic of enzymes following Michaelis-Menten kinetics. However, there are more complex mechanisms. For example, non-Michaelis-Menten kinetics can appear due to activation or inhibition of the enzyme by substrate excess and non-cooperative conformational changes [58], or due to allosteric effects in oligomeric enzymes resulting from cooperativity between subunits. In the first case, to avoid complicated kinetics at high substrate concentrations [59], it is recommended to study reversible inhibition at low substrate concentrations, that is, under conditions in which the enzyme follows Michaelis-Menten kinetics. In the second case, for allosteric enzymes that follow the classical Monod-Wyman-Changeux concerted model or the sequential Koshland-Nemethy-Filmer model, allosteric effector binding at binding sites other than the active center causes competitive or noncompetitive inhibition [60].

In this case, graphical kinetic analysis of slow binding inhibition can be difficult due to interactions between spatial and kinetic cooperativity. Various cases and formal methods for kinetic analysis have been described by Segel [61]. Theoretical kinetic analysis of allosteric enzyme SBI has been performed using type C SBI and the Monod-Wyman-Changeux model [62]. This approach shows the complexity of analyzing such cases and the need for very accurate experimental data. In any case, kinetic analysis of allosteric enzyme SBI must be accompanied by molecular modeling (molecular docking and molecular mechanics) and biophysical methods to study conformational changes. Therefore, a combination of different approaches combining kinetic and structural analysis of enzymes is needed [63–65].

4. EXAMPLES OF SBI WITH DIFFERENT TYPES OF MECHANISMS

There are many examples of SBI for different target enzymes in the literature (Table 1).

4.1. Natural SBI

A natural polypeptide neurotoxin, fasciculin, isolated from the venom of *Dendroaspis angusticeps* (green mamba), is one of the best known examples of type A SBI, exhibiting high affinity and selectivity for acetylcholinesterase (AChE). The K_i value for inhibition of AChE from the eel *Electrophorus electricus* is 0.33 nM with a residence time of about 14.5 min [66]. Its affinity for mammalian AChE is much higher (K_i of 0.002 nM for mouse AChE inhibition) and residence time is much longer [67], thus explaining why the green mamba venom is very dangerous. Such potent inhibition is due toxin-induced blockade of the entrance to the active center channel of the enzyme. Butyrylcholinesterase (BChE)

Table 1. Examples of SBI: constants, mechanisms, residence time

Target enzyme	Mechanism	Inhibitor	K_i, nM	τ_R, min	Reference
AChE	A	Fasciculin-2	0.33	14.5	[66]
		Fasciculin-2	0.0023	227	[67]
		α-Tocopherol	4900 (K_i) / 1600 (K_i')	2	[57]
		Huperzine A	40.5	50.5	[70]
	B	C-547	0.022	20	[78]
BChE	C	Cresyl saligenin phosphate	—	—	[62]
Tyrosinase	A	2-Phloroeckol	8200	12.8	[72]
	B	2-O-(2,4,6-trihydroxyphenyl)-6,6′-bieckol	5800	—	
	A	Puerol A	107.5	333	[73]
Neuraminidase	A	Chromenone derivatives	310–460	20–24	[74]
PTP1B	A	Theaflavanoside IV	0.2	8.3	[76]
	A	Ugonin J	123.4	14	[77]
LpxC	A	Pyridone methylsulfone derivatives	0.010–0.274	5–124	[83]
20S proteasome	A	Ixazomib	4.9	7.2	[85]
	A	Bortezomib	1.6	43.8	
	A	Delanzomib	1.4	138	
Plasmin	A	Macrocycles with a C-terminal benzylamine group	1.83–16.80	4.4–13.7	[86]
Urease	A	Cinnamate-based phosphonic acids	509–6040	6.8–24.4	[87]

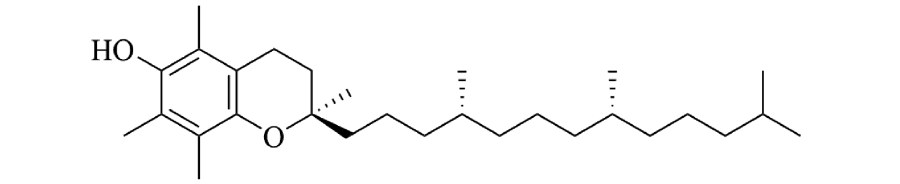
has a lower affinity for fasciculin (by eight orders of magnitude, K_i = 0.21 mM). The replacement of three amino acid residues in the AChE structure Tyr72Asn, Tyr124Gln, Trp286Arg, located at the peripheral binding site (initial ligand binding site), with the corresponding amino acid residues, as in the BChE structure, also resulted in a lower affinity of fasciculin for the enzyme (K_i = 0.23 mM).

α-Tocopherol (vitamin E) is another example of a reversible type A SBI of the human AChE enzyme (Fig. 7). Steady-state kinetics analysis performed by the Ellman method using acetylthiocholine as substrate showed that α-tocopherol acted as a mixed type inhibitor of AChE with inhibition constants K_i = 0.49 μM and K'_i = 1.6 μM; the residence time for α-tocopherol was 2 min. Molecular dynamics simulations confirmed that α-tocopherol formed multiple nonspecific interactions with the AChE surface near the gorge entrance, binding to the peripheral side and then slowly sliding down the gorge to the catalytic active center without causing significant conformational changes in the enzyme (Fig. 8) [57]. The results obtained are consistent with the AChE inhibition constant of the electric eel (*Electrophorus electricus*) by α-tocopherol hemisuccinate [68].

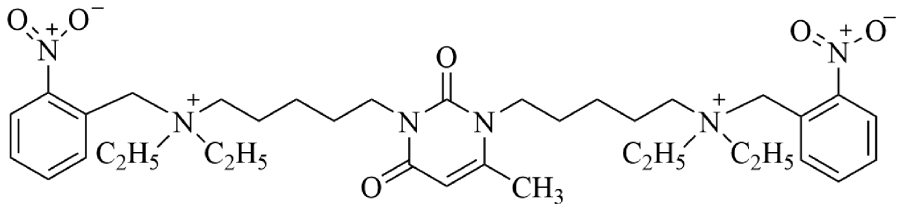
In 1998, Sepčić et al. found that the first 20 min of AChE inhibition by a polymer containing N-butyl(3-butylpyridinium) monomeric links from the marine sponge *Reniera sarai* were characterized by nonlinear time-dependent kinetics. The authors suggested that the polymer rapidly formed a reversible AChE-polymer complex, and then there was a slow binding of the polymer pyridinium residues at other AChE binding sites [69].

The alkaloid huperzine is another example of a type A SBI with respect to human AChE and BChE. The inhibition constant is K_i = 40.5 nM with half-dissociation time $t_{1/2}$ = 35 min and, respectively, residence time of about 50.5 min and K_i = 42 μM for human BChE [70].

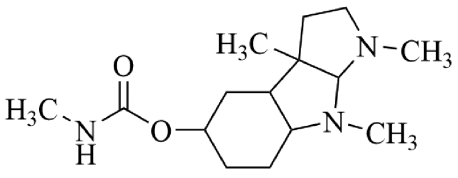
The alkaloid physostigmine (eserine) is a reversible type B SBI of BChE (Fig. 7). At the initial stage of the enzyme-inhibitor interaction there was a rapid but relatively weak binding of eserine to the active center of the enzyme with further formation of the conformational complex EI*. After that, carbamylation of serine of the enzyme active center was observed, followed by the slowest stage, deacylation [71].



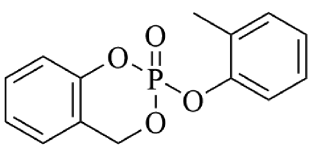
α–Tocopherol



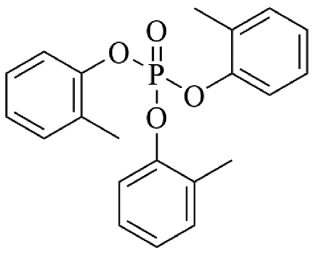
C-547



Physostigmine



Cresyl saligenin phosphate (CSP)



Tri-o-cresyl phosphate (TOCP)

Figure 7. Structures of SBI acting on AChE and BChE.

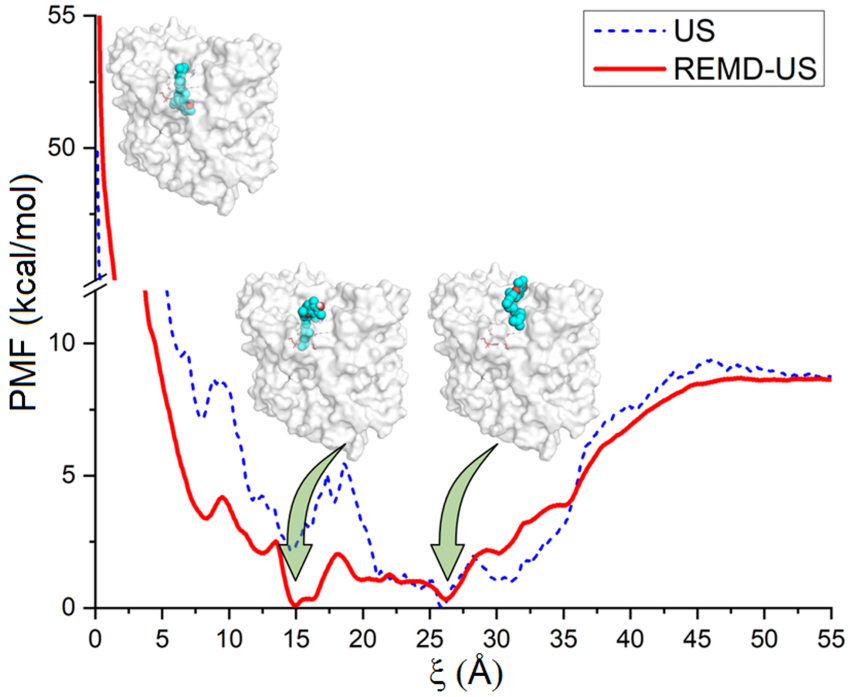


Figure 8. Free energy profile of α-tocopherol binding to human AChE. US (Umbrella sampling) and REMD-US (Replica exchange molecular dynamics) are molecular dynamics methods. PMF is Potential mean force. ξ is a distance between the chromanol ring of α-tocopherol and the active center of AChE enzyme [57] (with permission from Elsevier).

A group from Korea [72] investigated several florotannins isolated from the marine brown algae *Ecklonia cava* (family Laminariaceae) for their inhibitory activity against tyrosinase (EC 1.14.18.1), a key metalloenzyme responsible for the processes associated with the production of melanin to protect the skin from ultraviolet radiation and reactive oxygen species, and neuromelanin associated with the processes of neurodegeneration and Parkinson's disease. The authors found that two compounds with IC_{50} values below 10 μM belonged to type A and B SBI with the following inhibition constant for 2-floroecol (K_i = 8.2±1.1 μM, residence time of 12.8 min) and 2-O-(2,4,6-trihydroxyphenyl)-6,6′-biecol (K_i = 5.8±0.8 μM). It was confirmed by molecular modeling method that these molecules bound to His85 and Asn260 of the tyrosinase active center. The study of phenolic compounds isolated from methanol extracts of *Amorpha fruticosa* roots by the same group showed that one of the components, puerol A, acted as a reversible, competitive SBI with a dissociation rate constant of 0.003 min^{-1} and, accordingly, a residence time of 333 min [73].

Chromenone derivatives isolated from methanol extracts of the root part of *Flemingia philippinensis*, including two novel compounds philipin D and philipin E, exhibited properties of reversible competitive type A SBI of bacterial neuraminidase. This enzyme plays a key role in the pathogenesis of several microbial diseases, inflammatory cytokine release, sepsis, and biofilm development. The inhibition constants for philipin D and philipin E were 0.46±0.07 μM and 0.31±0.02 μM, and the residence times were 24 min and 20 min, respectively [74]. The data were validated by molecular modeling. Another group performed in-depth analysis including molecular docking and molecular dynamics, indicating that neuraminidase inhibitors, xanthones containing geranyl and prenyl groups isolated from the root bark of *Cratoxylum cochinchinense*, had high affinity and low inhibition constants (IC_{50} ranged from 0.38–38.9 μM). The residence time of the leader compound was 49 min [75].

Overexpression of one of the key enzymes, protein tyrosine phosphatase (1BPTP1B), leads to insulin resistance and ultimately to hyperglycemia and metabolic disorders, which are important pathogenic causes of diabetes mellitus and obesity. Theaflavanoside IV (IC_{50} = 8.7±1.1 μM) and caffeine (IC_{50} = 37.9±3.5 μM), isolated from the methanol extract of *Camellia sinensis* seed shells, inhibited PTP1B, as did ursolic acid (IC_{50} = 22.4±1.9 μM), a competitive inhibitor of this enzyme that exhibited mixed type inhibitory effect. The dissociation rate constant and residence time of theaflavanoside IV were 0.002 min^{-1} and 8.3 min, respectively [76]. The flavonoid ugonin J, isolated from the ethyl acetate extract of *Helminthostachys zeylanica* roots, had the highest affinity for tyrosine phosphatase 1B and exhibited the properties of a competitive SBI with a longer residence time (τ_R = 14 min, k_{off} = 0.0705 min^{-1}); in addition, this flavonoid also effectively inhibited α-glucosidase [77].

4.2. Synthetic SBI

The alkylammonium derivative of 6-methyluracil, working title C-547 (Fig. 7), being developed as a pharmacological agent drug for the treatment of myasthenia, is an example of an SBI that interacts with human AChE via mechanism B. The authors showed [78] that the formation of the initial EI complex was characterized by an inhibition constant of K_i = 0.14 nM, and then, during the induced fit stage, the final complex was formed with K^*_i = 0.022 nM. Molecular modeling confirmed that, due to conformational changes in the enzyme compound C-547 could cross the narrow "bottleneck" of the AChE active site channel with subsequent formation of the final complex (Fig. 9). The calculated k_{off} value was 0.05 min^{-1}, and τ_R was 20 min. However, with respect to human BChE, C-547 acted as a classical reversible mixed-type inhibitor with K_i = 1.77 μM and K_i' = 3.17 μM. Further studies showed that at a dose of 0.01 mg/kg C-547 protected mice poisoned with paraoxon ($2\times LD_{50}$) for 96 h [79].

In order to reduce the dose of drugs and expand the therapeutic safety window of existing drugs a group of Chinese researchers is developing new-generation AChE inhibitors for the treatment of Alzheimer's disease. For this purpose they synthesized 44 new donepezil derivatives containing a fluorine atom in the structure. In the case of the most active compounds the dissociation constants of the enzyme-inhibitor complex varied within 0.004768–0.01764 s^{-1}, which corresponded to a residence time of about 1–3.5 min. Preclinical studies of fluoropezil (DC20) have shown that this compound can be used as a new generation AChE inhibitor with an established mechanism, high selectivity for the pharmacological target and a wide therapeutic safety window [80]. Clinical trials of fluoropezil are currently underway [81].

In order to expand the therapeutic window of the developed inhibitors of the metalloenzyme UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC), new pyridone sulfonamide based compounds were synthesized and the structure-kinetic relationship was studied [82]. These compounds demonstrated inhibitory activity against LpxC with different residence times on the target [83]. LpxC is known to be involved in the biosynthesis of lipid A, an important component of the cell wall of gram-negative bacteria. At present, none of the LpxC inhibitors have passed clinical trials. The development of a new compound with a residence time of up to 124 min and post-antibiotic effect values of 4 h opened prospects for the creation of new inhibitors with an increased residence time on the target, leading to an extension of the post-antibiotic effect after the elimination phase, i.e. a better therapeutic window. Conformational changes in the enzyme upon inhibitor binding represent the slow stage.

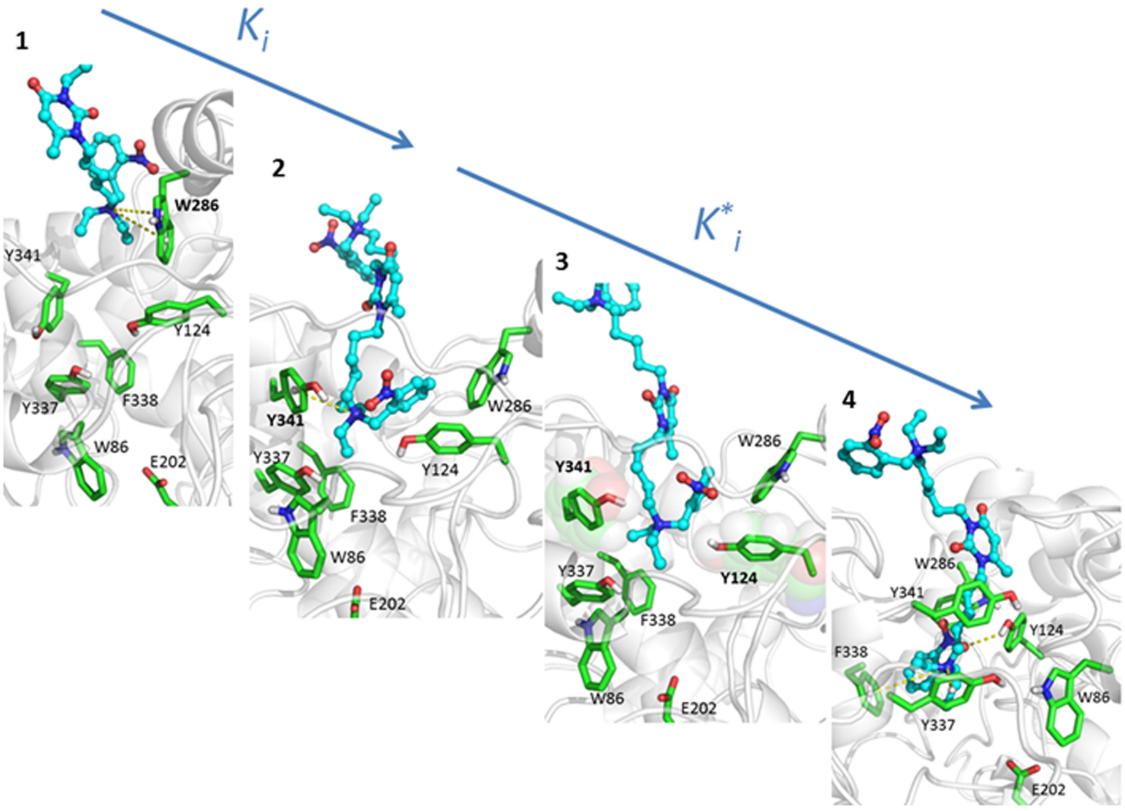


Figure 9. Molecular modeling of AChE inhibition in the presence of compound C-547 [2] (with permission from Elsevier).

The development of histone deacetylase (HDAC) inhibitors has led to the creation of several drugs for the cancer treatment [19]. However, their therapeutic efficacy is limited due to poor selectivity and side effects on physiological functions. In addition, HDAC has been shown to be a target in the regulation of the immune response, in the development of neuropathies and Alzheimer's disease. Recently, it has been shown that 1,3,4-oxydiazoles form HDAC-inhibitor complexes with a long residence time [84] and exhibit high selectivity for this target [51]. The resulting SBI are substrate analogs and form a tightly bound complex with the enzyme.

In 2018, Hasinoff and Patel analyzed the kinetic parameters of known anticancer drugs for the treatment of multiple myeloma, 20S proteasome inhibitors, based on boronic acids and epoxy ketones and studied myocyte damage and side effects associated with cardiotoxicity. It was found that Ixazomib (K_i = 4.9 nM), Bortezomib (K_i = 1.6 nM), and Delanzomib (K_i = 1.4 nM) acted as 20S proteasome SBI with the residence time of 7.2 min, 43.8 min, and more than 138 min, respectively [85].

Increased activity of plasmin, a trypsin-like serine protease, which plays a key role in fibrinolysis, can cause blood clotting disorders. In 2023, Wiedemeyer et al. synthesized a series of highly active and selective macrocyclic plasmin inhibitors with inhibition constants in the range of 1.83–16.80 nM and residence times of 4.4–13.7 min. The binding mechanisms of the macrocycles are explained by their high selectivity as plasmin inhibitors [86].

Inhibitors containing phosphonate/phosphinate groups have affinity for metallohydrolases, including metalloproteases, and other hydrolases, such as urease. The development of urease inhibitors is driven by their use in the treatment of various pathogens associated with infections caused by urease-dependent microorganisms, such as ulcers induced by *Helicobacter pylori* or chronic urinary tract infections induced by *Proteus* species. The search for inhibitors with high affinity for bacterial ureases is focused on the synthesis of several groups of active compounds based on phosphonates/phosphinates. Cinnamic acid-based phosphonic acids are *Sporosarcina pasteurii* urease SBI with inhibition constants of 0.509–6.040 μM and residence times of 6.8–24.4 min [87]. The most active urease SBI were catechol-based phosphonates or (2-carboxyethyl)phosphinates [88], with following inhibition parameters of the studied inhibitors: K_i^* = 0.13 μM and τ_R = 205 min.

Thus, from the examples considered above, it is clear that interest in SBI is due to the fact that they exhibit long residence times. Over the past few years, this parameter has become an indispensable indicator of compound effectiveness at the early stage of drug development and discovery [4, 37, 89].

CONCLUSIONS

The concept proposed by Copeland and presented in this review confirms the importance of the monitoring and optimization of drug-target kinetics *in vitro* for drug discovery. Parameters such as residence time, inhibition constant, association and dissociation rate constants determine the efficacy of the drug application *in vivo*. Drugs with fast pharmacokinetics but long residence time will have a prolonged effect, allowing dosing distribution and achieving high efficacy with fewer side effects. Therefore, SBI are of great pharmacologic interest. However, we should not forget Paracelsus' aphorism “Everything is poison, everything is medicine; both are determined by the dose”. Therapeutic interest in such compounds is always a compromise between binding affinity and ligand-receptor binding kinetics. Ligands with high affinity and tightly bound inhibitors with very low K_i (K_i~[E]) but very long residence times can be characterized by high toxicity. In particular, the high toxicity may be related to the location of the target a limited space (e.g. confined to synapses, in mitochondria) where, after dissociation of the ligand-target complex, their re-binding contributes to a much longer residence time of the ligand on the target.

FUNDING

This work was performed within the frame of the Strategic Academic Leadership Program “Priority 2030” of the Kazan Federal University of the Government of the Russian Federation.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Received: 19. 11. 2024.
Revised: 14. 03. 2025.
Accepted: 24. 03. 2025.

ИНГИБИТОРЫ МЕДЛЕННОГО СВЯЗЫВАНИЯ ФЕРМЕНТОВ: КИНЕТИЧЕСКИЕ ХАРАКТЕРИСТИКИ И ФАРМАКОЛОГИЧЕСКИЙ ИНТЕРЕС

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В настоящее время актуален поиск новых ингибиторов медленного связывания ферментов (ИМС) — потенциальных лекарственных веществ для лечения различных заболеваний — и их распознавание на этапе *in vitro* исследований. ИМС, в отличие от их классических обратимых аналогов, проявляют кинетику медленного связывания с ферментом, в ходе которой стационарное состояние в системе достигается не за микросекунды, а в течение более продолжительного времени. Подобные соединения могут быть перспективными лекарствами, так как вне зависимости от их фармакокинетики в кровотоке они обладают такими преимуществами, как высокая аффинность к мишени-ферменту, длительное время удержания, пролонгированное действие. Такие фармакологические свойства обеспечивают правильную дозировку лекарств для достижения их высокой активности с меньшими побочными эффектами. Цель настоящего обзора заключается в рассмотрении механизмов взаимодействия ИМС с ферментами, принципов распознавания ИМС на этапе *in vitro* кинетических исследований и фармакологической значимости.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: ингибиторы медленного связывания; ферментативная кинетика; константа ингибирования; время удержания

Финансирование. Работа выполнена за счёт средств субсидии, выделенной в рамках государственной поддержки Казанского (Приволжского) федерального университета в целях повышения его конкурентоспособности среди ведущих мировых научно-образовательных центров (Приоритет 2030).

Поступила в редакцию: 19.11.2024; после доработки: 14.03.2025; принята к печати: 24.03.2025.