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Журнал «Фармация и фармакология» включен в перечень рецензируемых научных изданий, входящих в международные реферативные базы данных и системы цитирования, и в соответствии с пунктом 5 правил формирования перечня рецензируемых научных изданий, в которых должны быть опубликованы основные научные результаты диссертаций на соискание ученой степени кандидата наук, на соискание ученой степени доктора наук (Перечень ВАК), Scopus, Web of Science (ESCI), РИНЦ, eLibrary, ВИНИТИ, РГБ, Киберленинка, Соционет, EMBASE, Chemical Abstracts (CAS), Directory of Open Access Journals (DOAJ), EBSCO Discovery Service, RNMJ, University of CAMBRIDGE, Ulrich'sWeb, Google Scholar, Biefeld Academic Search Engine (BASE), Directory of Open Access Scholarly Resources (ROAD), Research Bible, Open Archives Initiative, Academic Keys, JournalTOCs, WorldCat, OpenAIRE, University of Oxford, The British Library, Universitait Gent, Université de Montréal, University of Saskatchewan.

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Brain-derived neurotrophic factor as a target for the search of anti-addiction drugs

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The relationship between the influence of the brain-derived neurotrophic factor (BDNF) on the development of alcoholism and possible ways of using this molecule or related compounds (mimetics) as targets of anti-addictive action are discusses in the article.

The aim of the work was to carry out a literature review to identify potential applications of the BDNF signaling pathways to assess the feasibility of developing new drugs.

Materials and Methods. The following abstract databases were used to search for the information materials: PubMed, EMBASE, ResearchGate, elibrary.ru. The key queries for the search included the following ones: 'BDNF', "BDNF TrkB", "BDNF LNGFR", "alcoholism therapy", "anti-addiction drugs", "signaling pathways", "alcoholism", "ethanol", "poisoning". The depth of the search was 40 years (1985–2025). The total number of the sources included in the review is 116.

Results. This study analyzed the molecular mechanisms of the action of BDNF, including its biosynthesis, structural forms (BDNF and pro-BDNF), and functions and features of TrkB and LNGFR receptors. These receptors play a key role in the regulation of the neuronal plasticity, a neuronal survival and apoptotic processes. The performed review of the scientific literature made it possible to establish that at least 9 chemical compounds with a potential anti-addictive activity that affect the receptors and signaling cascades associated with BDNF, have been identified as of 2025. Based on the data obtained, a hypothesis about the prospective use of BDNF and its signaling pathways as potential targets for developing new pharmacological agents aimed at the treatment of alcohol dependence, have been formulated. The established facts can significantly expand the therapeutic opportunities in the fight against the alcoholic dependence and associated neurotoxic conditions.

Conclusion. At least 9 compounds with a potential anti-addictive activity associated with a mimetic effect on the receptors and signaling pathways of the BDNF molecule have been analyzed and found to exist as of 2025.

Keywords: brain-derived neurotrophic factor; BDNF; anti-addiction drugs; addiction syndrome; TrkB receptors; LNGFR receptor

Abbreviations: BDNF — brain-derived neurotrophic factor; LNGFR (p75NTR, NGF) — low-affinity nerve growth factor receptor; TrkB — tropomyosin-related kinase receptor B; NTRK2 — neurotrophic receptor tyrosine kinase 2; PCs (1/2/3) — proprotein convertase (1/2/3); MMPs — matrix metalloproteinases; SHC — Src homology 2 domain-containing)-transforming protein 2; MAPK / ERK — mitogen-activated protein kinase / extracellular signal-regulated kinase; PI3K / AKT — phosphoinositide 3-kinases / serine-threonine-protein kinase; DAG / PKC — diacylglycerol / protein kinase C; IP3 — inositol-trisphosphate 3-kinase; PLC γ — phospholipase C (gamma); SP — signal peptide; LRRNT — leucine-rich N-terminal repeats; LRR — leucine-rich repeats; LRRCT — leucine-rich C-terminal repeats; IGC2-1 and IGC2-2 — immunoglobulin-like domains; CREB — cAMP response element-binding protein; TTIP — truncated TrkB-interacting protein; TACE/ADAM17 — tumor necrosis factor-α converting enzyme; CRD — carbohydrate recognition domain; NT — neurotrophin; NRAGE — neurotrophin-receptor-interacting melanoma antigen-encoding gene homolog; NRIF — neurotrophin receptor interacting factor; aPC — activated Protein C; NFκB — nuclear factor kappa-light-chain-enhancer of activated B cells; TRAF — TNF receptor-associated factor; RIPK2 — receptor-interacting serine / threonine-protein kinase 2; RhoA — Ras Homolog Family Member A; FAP-1 — Fas-Associated Phosphatase-1; GABA — γ-aminobutyric acid; PTSD — posttraumatic stress disorder; ADH — antidiuretic hormone.

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Мозговой нейротрофический фактор как мишень для поиска препаратов, проявляющих антиаддиктивные эффекты

М.С. Халиманов, Е.М. Григоревских, К.А. Завадич, С.И. Сологов, Д.А. Тращенкова, К.А. Татжикова, Е.В. Поликарпов, С.С. Сологова, Д.А. Кудлай, Е.А. Смолярчук

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В данной статье рассматриваются вопросы влияния мозгового нейротрофического фактора (BDNF) на развитие алкоголизма. Рассматриваются возможные пути использования этой молекулы или родственных с ней соединений (миметиков) в качестве мишеней для антиаддиктивного действия.

Цель. Провести анализ литературных источников с целью выявления потенциальных возможностей применения сигнальных путей BDNF в сфере разработки новых лекарственных препаратов.

Материалы и методы. Для поиска информационных материалов использовали реферативные базы данных: PubMed, EMBASE, ResearchGate, elibrary.ru. Ключевые запросы для поиска включали в себя следующие слова и словосочетания: «BDNF», «BDNF TrkB», «BDNF LNGFR», «терапия алкоголизма», «антиаддиктивные препараты», «сигнальные пути», «алкоголизм», «этанол», «отравление». Глубина поиска — 40 лет (1985–2025 гг.). Общее число источников, которые вошли в обзор — 116.

Результаты. В данном исследовании проведён анализ молекулярных механизмов действия BDNF, включая его биосинтез, структурные формы (BDNF и про-BDNF), а также функции и особенности рецепторов TrkB и LNGFR. Эти рецепторы играют ключевую роль в регуляции нейрональной пластичности, выживаемости нейронов и апоптотических процессов. Выполненный обзор научной литературы позволил установить, что по состоянию на 2025 год идентифицировано не менее 9 химических соединений с потенциальной антиаддиктивной активностью, которые воздействуют на рецепторы и сигнальные каскады, связанные с BDNF. На основании полученных данных сформулирована гипотеза о перспективах использования BDNF и его сигнальных путей в качестве потенциальных мишеней для разработки новых фармакологических агентов, направленных на лечение алкогольной зависимости. Установленные факты могут существенно расширить терапевтические возможности в борьбе с алкоголизмом и ассоциированными нейротоксическими состояниями.

Заключение. Было проанализировано и выявлено, что на 2025 год существует не менее 9 веществ с потенциальной антиаддиктивной активностью, связанной с миметическим действием на рецепторы и сигнальные пути молекулы BDNF.

Ключевые слова: мозговой нейротрофический фактор; BDNF; антиаддиктивные лекарственные средства; синдром зависимости; TrkB рецепторы; LNGFR рецептор

Список сокращений: BDNF — нейротрофический фактор мозга; LNGFR (p75NTR, NGF) — рецептор фактора роста нервов; TrkB — рецептор семейства тропомиозин-киназ B; NTRK2 — нейротрофический рецептор тирозинкиназы 2; PCs (1/2/3) — пропротеиновая конвертаза (1/2/3); MMPs — матриксные металлопротеиназы; SHC — домен, гомологичный второму домену белка Src; MAPK / ERK — митоген-активируемая протеинкиназа / киназа, регулируемая внеклеточными сигналами; PI3K / AKT — фосфоинозитид-3-киназа / серин-треониновая киназа; DAG / PKC — диацилглицерин / протеинкиназа C; IP3 — инозитол-трисфосфат-3-киназа; PLC γ — фосфолипаза C (гамма); SP — сигнальный пептид; LRRNT — N-концевые повторы, богатые лейцином; LRRR — богатые лейцином повторы; LRRCT — C-концевые повторы, богатые лейцином; IGC2-1 и IGC2-2 — иммуноглобулиноподобные домены; CREB — белок, связывающий цАМФ-чувствительные элементы; TTIP — укороченный белок, взаимодействующий с TrkB; TACE / ADAM17 — фактор некроза опухоли-конвертирующий фермент; CRD — домен распознавания углеводов; NT — нейротрофин; NRAGE — гомолог гена, кодирующего антиген меланомы, взаимодействующий с нейротрофиновым рецептором; NRIF — фактор, взаимодействующий с рецептором нейротрофина; aPC — активированный протеин C; NFκB — ядерный фактор каппа-легкой цепи-усилитель активированных B-клеток; TRAF — фактор, ассоциированный с рецептором TNF; RIPK2 — рецептор серин / треонин-протеинкиназы 2; RhoA — член семейства гомологов Ras A; FAP-1 — Fas-ассоциированная фосфатаза-1; ГАМК — γ-аминомасляная кислота; ПТСР — посттравматическое стрессовое расстройство; АДГ — антидиуретический гормон.

INTRODUCTION

According to the WHO Global Report 2019[1], the global trend in the total per capita alcohol consumption has decreased by 4.5% from 2010 to 2019. At the same time, the highest consumption rates were observed in the WHO European Region (9.2 L/yr).

The problem of chronic alcoholism therapy is extremely important in the modern world. Against the background of alcoholism, exacerbations of diseases

[1] WHO. Global status report on alcohol and health and treatment of substance use disorders. Available from: https://iris.who.int/bitstream/handle/10665/377960/9789240096745-eng.pdf?sequence=1

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associated with the toxic effect of ethanol on the heart, liver, kidneys, lungs, and circulatory nervous system often occur. The nervous system is most susceptible to the toxic effects of ethanol [1].

Ethanol and its metabolites have toxic effects on the brain through several major pathways straight away: a dysregulation of the GABAergic system of the brain [1–3]; a disruption of calcium signaling [4]; a general toxic effect of acetaldehyde, the main metabolite of ethanol [5]; a disruption of the glutamatergic system of the brain [6]; and a formation of neurotoxic conjugates with monoamine neurotransmitters [7–9].

The latter toxic pathway is poorly understood; however, according to some data, these conjugates contribute to the formation of reactive oxygen species and an oxidative stress [10]. In addition, ethanol disrupts neurotropic factors (in particular BNDF). Since this protein is essential not only for the nerve cell proliferation and nerve tissue regeneration, but also for the protection of neurons from adverse effects and maintenance of their viability under normal conditions [11]. This signaling system and its alterations in alcoholism are of great interest for the study of new neuroprotective therapeutic strategies and the development of neuroprotective drugs that may be useful not only in alcoholism, but also in other toxic lesions of the nervous system that affect neurotrophin signaling.

THE AIM of the work was to carry out a literature review to identify functions of the BDNF and its relationship to the course of alcoholism; it was also the search for potential new targets for an anti-addictive action related to the TrkB receptors affected by BDNF.

MATERIALS AND METHODS

Materials for this literature review were searched and systematized in the following abstract Russian and foreign databases: PubMed, Google Academic, EMBASE, ResearchGate scientific information network and a scientific electronic library (elibrary.ru). The key queries for the search included the following ones: “BDNF”, “BDNF TrkB”, “BDNF LNGFR”, “alcohol therapy”, “anti-addiction drugs”, “signaling pathways”, “alcoholism”, “ethanol”, and “poisoning”. The depth of the search was 40 years, as it was from 1985 that the brain-derived neurotrophic factor was first described in publications as a separate protein molecule (publications in most abstract databases from 1985 to 2025). Conducting the publication search, analyzing the sources and correlating with the given target requirements took about 8 months (from January to August 2024).

About 29 899 publications were found by the main keywords: “BDNF” and its receptors. In this process, some articles were excluded due to the nosology mismatch (after specifying the query for “alcoholism”, most studies were on different types of depression (*n*=2 700), effect on the pain response (*n*=1900)), predominance of the description of physiological effects of the neurotrophic factor (*n*=5900), as well as closed access articles. In this literature review, the total number of papers containing the studies of new substances potentially influencing the reduction of the alcohol consumption by the effect on BDNF, was 31. The remaining 85 sources were used to discuss the action of BDNF on its specific receptors and the relationship to the alcohol dependence.

RESULTS AND DISCUSSION

Structure and functions of endogenous BDNF molecule and realization of its effects

The brain-derived neurotropic factor (BNDF) is a hormone of the protein nature discovered in 1982 [12].

It is mainly involved in the development of the nervous system and the process of synaptogenesis. At the molecular level, its functions are to block the triggering of apoptosis through the JAK2-dependent pathway. The BDNF gene is located on a small arm of chromosome 11 (11p14.1). The production of this protein occurs in several stages [13–15].

The initial product of the gene is pre-proBDNF, from which the signal peptide is cleaved by proteases. The product of this reaction is pro-BDNF, which further undergoes hydrolysis from the N-terminus, decomposing into the final product — BDNF proper and pro-BDNF-peptide [12, 16–19]. The studies have shown that each of the products of the BDNF gene has its own functions and the role in the regulation of the neuronal activity and its vital activity. For example, pro-BDNF-peptide has been found in presynaptic terminals and it has been suggested that it may be released by the mechanisms similar to those of classical low molecular weight neurotransmitters [14, 20] and can bind to the receptors for both neurotrophins [15, 21–24] and low molecular weight neurotransmitters [16, 25, 26]. As for the difference between the function and biosynthesis of BDNF and pro-BDNF, the studies have shown a relationship between the neuron activity and the ratio of these two forms – in the active neurons generating more frequent action potentials, proteolysis of pro-BDNF proceeds more rapidly [17, 27–29] due to the increased activity of intracellular convertases PC1, PC5, PACE4, PC7. The extracellular convertases responsible for the conversion of pro-BDNF into BDNF — MMP3, MMP7, MMP9, have also been described. This

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multifactorial regulation of the pro-BDNF / BDNF ratio has been found out to be essential for the synaptic plasticity in the hippocampus and the formation of a long-term potentiation [18] and the development of neuromuscular junctions [19, 30–32].

The main targets of the BDNF molecular action are TrkB (Fig. 1) and LNGFR receptors (Fig. 2).

TkrB (tropomyosin-related kinase receptor B) is a brain factor receptor with an autophosphorylation ability. It is encoded by the NTRK2 gene located on the long arm of chromosome 9 (9q21.33). Five different mRNAs have been found to be the product of this gene (NCBI database numbers are: NM_006180.3; NM_001007097.1; NM_001018064.1; NM_001018065.1; NM_001018066.1 — variants A, B, C, D, E, respectively [32]).

Transcripts A and C give a full version of the protein upon translation (the so-called TK+ isoform), all others give a shortened version (TK-). These forms differ both in their domain composition and in their functions [33, 34].

Mechanism of BDNF action on TrkB

The receptor dimerization is necessary to activate the mechanism of action of BDNF on TrkB. After binding to BDNF, the following mechanisms are triggered: mediated by the adaptor protein SHC — the activation of Erk / MAPK and PI3K / Akt signaling pathways triggering the transcription factor CREB; mediated by a PLC γ increase of the DAG level and a subsequent activation of PKC, an increase of the IP3 level resulting in a subsequent increase of the calcium ion level and, as in the case of other signaling pathways, the activation of CREB.

The extracellular part of both TK+ and TK- isoforms (Fig. 3) is BDNF-binding and consists of the following domains: SP, LRRNT, LRR, LRRCT, IGC2-1 and IGC2-2. TK+ forms have also got specific intracellular parts: the SHC1 binding domain, TyrKc, a tyrosine protein kinase domain involved in the phosphorylation process, and the PLCγ binding domain. In TK- forms, the TTIP (truncated TrkB-interacting protein) and Rho GDI1 binding domains are located on the intracellular part (combined into a separate (TK-)-specific exon). In both isoforms, the region between LRRCT and immunogloblin-like domains is responsible for the actual interaction with BDNF.

Other TrkB isoforms resulting from alternative splicing and gene rearrangements have also been described in the scientific literature, but their functions and clinical significance are poorly understood [35, 36].

To bind to BDNF, TrkB must switch to a dimerizated state. At present, the functions of TK+ / TK+ and TK- / TK- homodimers have been described and elucidated [37]; the functions of TK+ / TK- heterodimers are not yet fully clear, but their presence has already been confirmed, as well as the possibility for TK+ and TK- to form heterodimers with other receptors (transactivation), e.g., with the angiotensin receptor AGTR2 [38], TrkC [39] and TrkA [40] (see Fig. 3).

The TK+ homodimer is mainly involved in the processes of synaptic plasticity (the intracellular PLC-binding domain is responsible for this), the differentiation (a Ras-dependent pathway) and a cell survival (a Akt-dependent pathway). Some publications have mentioned the possibility that both homodimers are mutually regulating at the level of intracellular processes [41].

The functions of the TK- homodimer are less studied, but its influence on the regulation of the calcium ion entry into the cell is assumed. In addition, the studies suggest a role of this receptor in the phenomenon of an excitotoxicity — death or a severe damage to neurons from the calcium homeostasis disruption [42]. The influence of this receptor form on the synapto- and morphogenesis of nerve cells has also been found [43]: abnormalities of the hippocampal and amygdala development were detected in TK-deficient mice, and this was reflected in the behavioural phenotype in the form of the increased excitability and anxiety.

Several clinically relevant mutations have been described for this receptor gene, such as a number of single nucleotide polymorphisms (rs1867283, rs10868235, rs1147198, rs11140800, rs1187286, rs2289656, rs1624327, rs1443445, rs3780645, and rs2378672) associated with temporal lobe epilepsy [44] and depressive disorders [45], the rs2289656 polymorphism was also found to be associated with suicidal behavior [46]. Mutant variants of the TrkB receptor were also found in genotyping of lung [47], breast and intestinal tumors [48].

LNGFR

This nerve growth factor receptor has its specific functions, which are to restrict the nerve cell growth and migration [49] by interacting with the signaling proteins NRAGE [50], SC-1 (Schwann cell factor 1) [51], NADE [52] and NRIF [53] (see Fig. 2).

The extracellular part of both TK+ and TK- isoforms is BDNF-binding and consists of the following domains: SP, LRRNT, LRR, LRRCT, IGC2-1 and IGC2-2. TK+ forms also have specific intracellular parts: the SHC1 binding domain, TyrKc, and the PLCγ binding domain. In TK forms, the TTIP and Rho GDI1 binding domains are located on

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the intracellular part (combined into a separate (TK-)-specific exon). In both isoforms, the region between LRRCT and immunogloblin-like domains is responsible for the interaction with BDNF.

It consists of an extracellular part, which contains 4 cysteine-rich domains (CRDs) and a TACE / ADAM17 protease binding site, and an intracellular part, which contains a γ-secretase binding site, and death and Chopper domains (binds to proteins responsible for triggering apoptosis (NADE and NRIF) [54, 55].

However, when dimerized with TrkA [56], caused by the activation of the ephrin-B receptor and a subsequent phosphorylation of the adaptor proteins Kidins220 / ARMS [57], it, on the contrary, stimulates the migration of progenitor cells at the stage of a nervous system development [58]. P75NTR is also able to dimerize with TrkB. This process also occurs with the help of Kidins220, and leads to the formation of a dimeric receptor with an increased sensitivity to BDNF [59]. It is also possible to form a sortilin / TrkB / p75NTR complex, which has increased its sensitivity to pro-BDNF [60].

This receptor gene is located on the large arm of chromosome 17 (17q21.33) and several mutations have been described for it. In animal models with non-functional p75, an impaired axon formation was found, expressed in their excessive growth and reduced branching [61]. It is interesting, that this effect was most strongly expressed in the gustatory cortex of mice: the loss of taste papillae was observed in the experimental animals. In the studies on mice, the role of p75NTR mutations in the development of deafness was also established [62]. The sortilin mutations because of which it could not dimerize with p75NTR, were also described, resulting in an increase in the main, pro-apoptotic function of the latter - clinically it is expressed in the presence of the essential tremor [63].

Alcoholism and BDNF

The relationship of the of BDNF influence on the development of the alcohol dependence will be considered.

The study conducted by D. Silva-Peña et al. [64] showed a direct link between an alcohol consumption, cognitive deficits and reduced BDNF levels. In this experiment, the alcohol dependence was modeled in experimental animals for several weeks - periods of an uncontrolled access to alcohol were interspersed with taking away and returning alcohol drinks, i.e. the withdrawal component of dependence was also modeled. In addition, as part of their publication, Daniel Silva-Peña et al. also performed both a serum analysis and a statistical data collection from the alcoholic patients. Using ELISA, the levels of BDNF, NT-3, IGF-1 and IGF-2 in the serum of mice and humans were measured and their correlation with the level of cognitive and mnestic deficits (measured by FAB and MFE tests) was calculated. In the control group of patients, BDNF was at the level of 0.75–0.83 ng/mL, in patients with alcoholism (without a pronounced cognitive or mnestic deficit) —0.45–0.55 ng/mL, in patients with alcoholism aggravated by at least one of the forms of cognitive or mnestic deficit — 0.3–0.4 ng/mL. In the humans, the difference in NT-3 and IGF-2 levels was present but less pronounced. In the mice, however, the difference between the levels of BDNF and NT-3 in the blood of the control and experimental groups was much more pronounced — 650-700 mg/mL in the control group and 250–300 mg/mL in the experimental group for BDNF; 0.22–0.27 ng/mL in the control group and 0.05–0.07 ng/mL in the experimental group for NT-3. In addition, the relative mRNA levels of BDNF, NT-3, TrkB, TrkC and p75NTR in the hippocampus were measured in the mice; a decrease in the expression of BDNF and NT-3 was found in the experimental group compared to the control group, the levels of TrkB and TrkC remained almost unchanged, and the mRNA level of p75NTR increased more than one and a half times in the experimental group compared to the control. All the above data give a picture of the inhibition of neurogenesis and the work of natural neuroregenerative mechanisms [65] during the alcohol consumption (a decrease in BDNF and NT-3), and an increase in the number of apoptosis events (an increase in p75NTR [66]).

Another work contains data on the study of changes in the relative mRNA levels of individual exons of the BDNF gene, and a group of researchers studied not only the effect of ethanol *per se*, but that of the promising phytoestrogen resveratrol on the expression of this gene [67]. As a result, it was found out that the amount of mRNA belonging to the 9th exon of the BDNF gene decreased most of all (starting from the minimum dose of 0.25 g/kg of the animal body weight). Exon 9 of the BDNF gene is important from the point of view of molecular pathology and modeling of various pathological conditions, since in rodents it is the exon that encodes the protein structure directly [68], while the other exons are regulatory.

Another very important study conducted on mice, which is worthy of highlighting, found an association between an uncontrolled alcohol consumption at an early age (25-day-old rats were used) and an increased

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likelihood of developing a depression [69]. This study used the BrdU tagging method to assess the nerve cell proliferation, which resulted in a quantitative measurement of the neuronal proliferation inhibition in the dentate gyrus in the mice compared to the control. It was reduced by 30–50% in alcohol-drinking mice.

The study of the neurotropic factors' dynamics in connection with a withdrawal syndrome in alcoholism is also of great interest. It has been found that during the alcohol withdrawal, the level of GDNF (glial cell line-derived neurotrophic factor) in blood decreases [70], while the level of BDNF is inversely related to the severity of the withdrawal syndrome. This may be due to the hyperactivity of compensatory mechanisms at the early stages of the withdrawal syndrome, when the BDNF level increases too sharply [71]. The evidence that elevated BDNF is a by-product of the compensatory mechanisms' activation is also supported by the fact that levels of IL-10, an anti-inflammatory cytokine, are simultaneously increased [72]. It is also of interest that the above-mentioned increase in BDNF levels is also observed in the addiction induced by other pharmacological agents, for example, is morphine [73], cocaine [74] and nicotine [75].

It was found out that the BDNF level also vary in different brain regions, both in norm and pathology. The authors distinguish: a) cell-dependent and b) activity-dependent types of BDNF expression [76]. In the first case, a constitutive expression determined by the cell type and differentiation per se is being discussed, while in the other case any other factors influencing the expression of this protein — learning, physical activity, pathological conditions and intake of various chemicals, should be understood. Besides, if this protein localization is being discussed, the levels of anatomical and cellular structures can be also conditionally distinguished. On the cellular scale, BDNF is usually found in large amounts in glutamatergic neurons, closer to the synaptic terminal [77]. If anatomical structures of the CNS are considered, the studies on rats have shown that the largest amount of BDNF (by the mRNA level and immunohistochemical staining) is in the hippocampus [78] (detected both in the neuron body and in axons with dendrites, with a peak intensity in the CA4 region). Their large amounts are also in the cortex, with a peak intensity in layer VI and the areas adjacent to the corpus callosum. It is of interest that layer I (a molecular) of the cortex had the lowest intensity of immunohistochemical staining and mRNA levels. When considered by sections, in the cortex, the highest relative intensity was observed in temporal and parietal ones. In the amygdala, the BDNF levels are extremely low except in the central nucleus, where it is found mainly in neuronal processes.

In pathological conditions, the level of BDNF can also change. For example, in a major depressive disorder, it has been shown that its relative amount in dendrites can increase sevenfold compared to the neuron body [79]. This is thought to be due to the length of the non-coding fragment that is the substrate of the DNA / RNA-binding protein translin. It has also been shown that the increased amounts of pro-BDNF decrease the length of hippocampal dendritic spines [80]. In depressive spectrum disorders, a decrease in the relative amount of BDNF in the perirhinal and entorhinal cortex is also observed. It is assumed that this is associated with an impaired long-term potentiation [81].

The studies have also shown the effects of alcohol on the gene expression in different parts of the brain. For example, the study by Finnish scientists [82], conducted on two lines of the mice alcohol-avoiding (ANA) and alcohol-preferring (AA) — showed that a chronic ethanol consumption decreases the BDNF expression. Moreover, a dose-dependent decrease in mRNA was found in the hippocampus and nucleus accumbens (NA), and conversely, an increase was found in the ventral tegmentum area (VTA). In the frontal lobes, an interesting effect was found out: in low doses, alcohol decreased the expression compared to the intact initial state, while high doses, conversely, increased the expression. In contrast, in the amygdala, a dose-dependent increase in mRNA was found with increasing doses of alcohol. Other studies have shown an increase in BDNF mRNA in pVTA during a direct administration of nicotine and ethanol [83].

Prospective approaches to the treatment of alcoholism aimed at correcting the effectiveness of BDNF

It is difficult to underestimate an important role of the brain-derived neurotrophic factor in the development of alcoholism (especially in the presence of withdrawal) and its severity. This signaling system is currently an object of interest for research and development of anti-addiction pharmacological agents.

Resveratrol is one of such agents, which in studies has shown a clear ability to restore the mRNA level of exon 9 of the BDNF gene. Although the effect is partial and, at the same time, this agent increases mRNA levels of regulatory exons 1 (4-fold), 3 (3-fold) and 4 (6-fold) in the control animals, the research is currently ongoing, as the exact consequences of such dysregulation of the expression are currently unknown.

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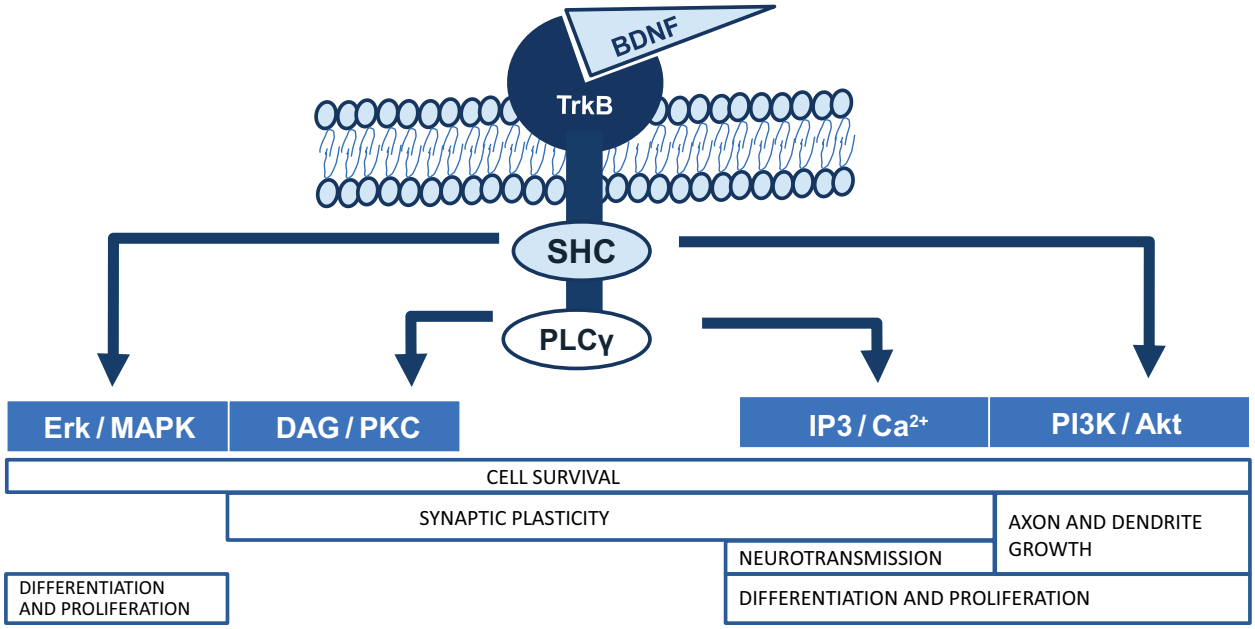

Figure 1 – Effects of BDNF when stimulated by TrkB.

Note: SHC — Src homology 2 domain containing; Erk / MAPK — mitogen-activated protein kinase / extracellular signal-regulated kinase; DAG / PKC — diacylglycerol / protein kinase C; IP3 — inositol-trisphosphate 3-kinase; PI3K/Akt — phosphoinositide 3-kinases / serine-threonine-protein kinase.

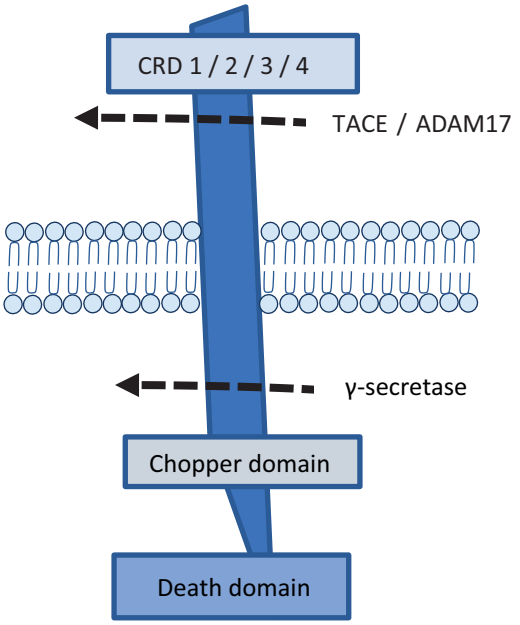

Figure 2 – Structure of LNGFR receptor.

Notes: TACE / ADAM17 — tumor necrosis factor-α converting enzyme; CRD — carbohydrate recognition domain.

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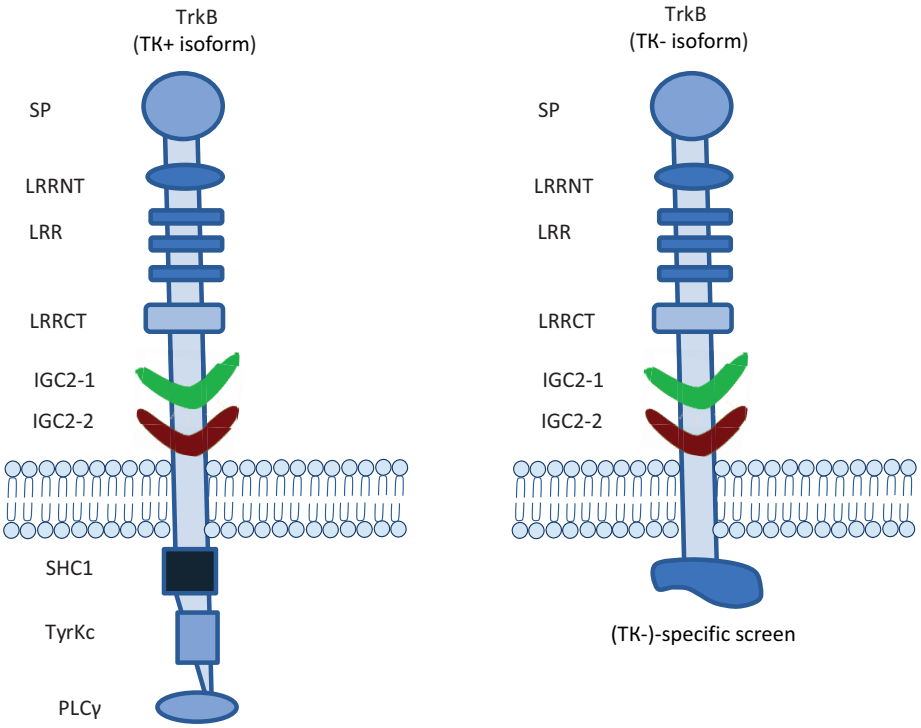

Figure 3 – Structure of TK+ and TK- isoforms of TrkB.

Notes. SP — signal peptide; PLC γ — phospholipase C (gamma); SHC1 — Src homology 2 domain containing; LRRNT — leucine-rich N-terminal repeats; LRR — leucine-rich repeats; LRRCT — leucine-rich C-terminal repeats; IGC2-1 and IGC2-2 — immunoglobulin-like domains.

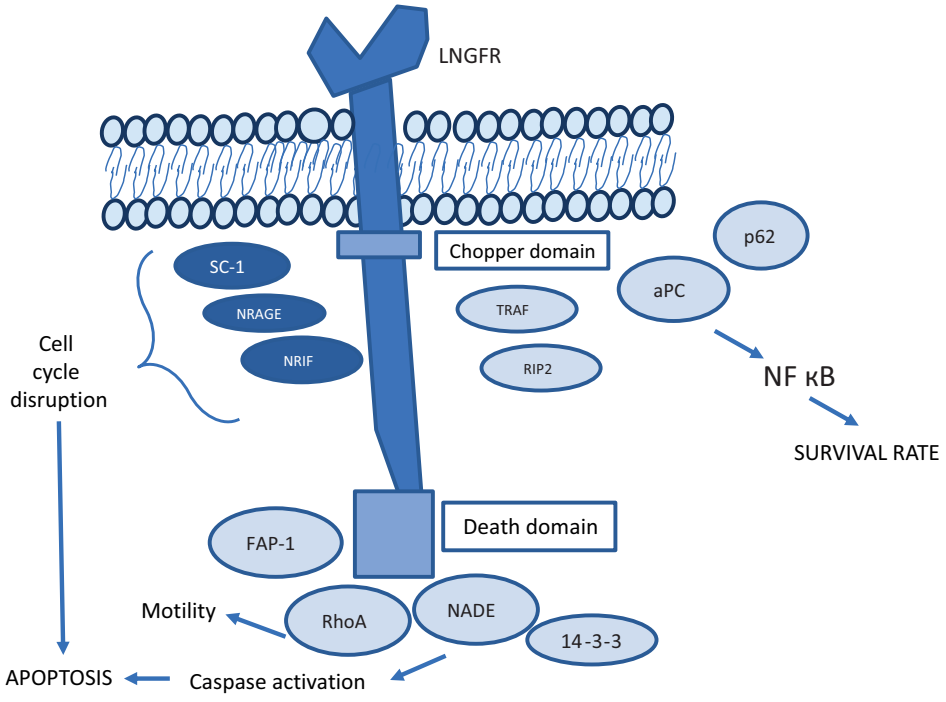

Figure 4 – Biochemical signaling pathways of LNGFR receptor.

Note: LNGFR (NGFR) — low-affinity nerve growth factor receptor; NGF — nerve growth factor; NT — neurotrophin; NRAGE — Neurotrophin-receptor-interacting melanoma antigen-encoding gene homolog; NRIF — NGF receptor-interacting factor; aPC — activated protein C; NFκB — nuclear factor kappa-light-chain-enhancer of activated B cells; TRAF — TNF receptor-associated factor; RIPK2 — receptor-interacting serine / threonine-protein kinase 2; RhoA — Ras homolog family member A; FAP-1 — Fas-associated phosphatase-1. When LNGFR interacts with neurotrophin molecules (NGF, NT-3, BDNF, and NT-4/5), mechanisms of the apoptosis initiation are triggered by the activation of NRAGE, SC-1, NADE, and NRIF signaling proteins by the receptor. In some cases, receptor-ligand interactions trigger mechanisms that support a nerve cell survival by the activation of NFkB. The interaction of the receptor with the signaling protein RhoA leads to an increase in the cell motility, as one of the main functions of this protein is the regulation of actin protein functions.

The next promising agent is 7,8-DHF (7,8-dihydroxyflavone) — a TrkB agonist or "BDNF-mimetic" [84], which has been found to have neuroprotective effects [85], like the basic molecule BDNF [86–88] plus antidepressant effects [89, 90]. In other animal studies [69] this effect was also shown, only already in relation to alcoholism — in rodents treated with 7,8-DHF, when further counting proliferating cells of dentate gyrus (by the BrdU inclusion level), the proliferation level was almost equal to the control group. Similar results were obtained in behavioural tests such as "Sucrose preference test" and "Open field test". The experimental group of animals that received both 7,8-DHF and the alcohol-containing solution showed similar results to the control group of mice that did not receive alcohol. 7,8-DHF also showed the ability to return to the normal levels of circulating BDNF in the blood and the levels of phosphorylated TrkB (pTrkB) on cell membranes. At the moment, not only 7,8-DHF itself is being investigated, but also the compounds close to it, such as the prodrug R13, which has shown a therapeutic effect in models of neurodegenerative diseases [91], and another compound R7, which is an independent TrkB agonist and has better pharmacokinetic parameters than 7,8-DHF [92]. Although the pharmacodynamics of 7,8-DHF on humans is under-researched, the molecule is still considered a highly promising compound [93].

LM22A-4 is a BDNF-mimetic different in structure, but similar in essence. In in vitro experiments, it showed neuroprotective effects [94], and in animal models, it reduced an alcohol consumption [95, 96].

A well-known compound that has an affinity for the same receptor as BDNF is amitriptyline, an antidepressant and adjuvant analgesic [97]. The studies that included the use of this drug in alcoholism were aimed at treating comorbid depressive disorders, not the addictive effects of alcohol itself since its use in the alcohol dependence syndrome is limited due to severe side effects and a low tolerability in patients with this disease [98].

An important substance, which, by its characteristics, can be used as a neuroprotective agent in patients with alcoholism, is a synthetic steroidal drug BNN-20 (an affinity to several receptors at once: TrkA, TrkB, and p75NTR). There are published results of its testing on various animal models of neurodegenerative diseases [99, 100], but not on the model of the alcohol dependence, although, based on its mechanism of action, this substance could potentially be a promising candidate for a further testing.

Deoxygedunin (deoxygedunin) can be promising in the light of the alcohol dependence treatment and the reduction of alcohol neurotoxic effects. Its neuroprotective effect has been proved in relation to nigral neurons, in model lesions by a selective MPTP toxin [101] and a mechanical damage of the nerve fibres [102].

Currently, one of the most common means of treating alcoholism is disulfiram, an outdated ADH blocker. It stops the ethyl alcohol metabolism at the stage of the toxic acetaldehyde formation, which is manifested by hyperaemia, tachycardia, vomiting and anxiety in the patient [103].

Therefore, alcoholism, as a socially significant disease, should be studied as deeply as possible. It is necessary to pay attention not only to the behavioural abnormalities [104] caused by its use [105–107], but also to know the molecular basis of this disease [108] — its preconditions (including genetic ones [109–111]), biochemical changes occurring in cells [112], their consequences and correction potential.

Low molecular weight BDNF mimetics are being developed in the Russian Federation. Currently, there is evidence of studies of the anxiolytic activity on rodents in the "elevated cruciform maze" test of the GTS-201 molecule [113, 114], which is important because the development of alcoholism is often associated with anxiety disorders. Separate studies have been conducted on an ethanol consumption, they have also shown a potential reduction in an alcohol-motivated behaviour [115]. Pharmacokinetic and metabolomic studies have shown that the mimetic GTS-201 has both an effect on increasing serotonin, dopamine concentrations in the CNS and lowering serum cortisol [116].

CONCLUSION

The study of the brain neurotrophin system holds promise for the development of innovative and safe therapeutic strategies in the treatment of the alcohol dependence. A review of the sources from the last 40 years helped to establish that at least 9 chemical compounds with potential anti-addictive activity that target BDNF-related receptors and signaling cascades, have been identified as of 2025.

Based on these findings, it can be concluded that BDNF and its signaling pathways may become promising targets for the development of new drugs for the alcohol dependence treatment. This may significantly improve the methods of alcoholism therapy and related neurotoxic conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Mikhail S. Khalimanov — search and analysis of literature sources; Ekaterina M. Grigorevskikh — search and analysis of literature sources, systematization of information, manuscript writing; Sergey I. Sologov — search and analysis of literature sources, systematization of information, manuscript writing; Ksenia A. Zavadich — search and analysis of literature sources; Daria A. Trashchenkova — search and analysis of literature sources, systematization of information, manuscript writing; Kristina A. Tatzhikova — search and analysis of literature sources, systematization of information, manuscript writing; Evgeny V. Polikarpov — search and analysis of literature sources, systematization of information, manuscript writing; Susanna S. Sologova — systematization of information, manuscript editing, Dmitry A. Kudlai — systematization of information, manuscript editing; Elena A. Smolyarchuk — systematisation of information, manuscript editing.

All the authors confirm that their authorship meets the ICMJE international criteria (all the authors contributed substantially to the conceptualization, research and preparation of the article, read and approved the final version before the publication).

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A rational approach to dose reduction of CDK4/6 inhibitors in the treatment of patients with advanced breast cancer: a Narrative Review

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The use of CDK4/6 inhibitors in the treatment of $HR^+/HER2^-$ breast cancer (BC) has become increasingly widespread in recent years. When assessing the safety of CDK4/6 inhibitors, it was found that during therapy, a significant number of patients require a reduction in the initial dose of the drug due to adverse events (dose reduction), but publications summarizing such data are absent. At the same time, the time to dose reduction and its stages can significantly affect the organization of drug supply for patients with drugs of this group, having an economic and administrative effect on the healthcare system. In this regard, a review of the results of the use of CDK4/6 inhibitors presented in the literature, describing the features of dose reduction, is timely and relevant.

The aim. To conduct a literature review in order to summarize and systematize the results of the use of CDK4/6 inhibitors, describing the features of dose reduction.

Materials and methods. The literature search was carried out in the MedLine (PubMed) and Google Scholar databases from January 2016 to January 2024. The literature search was carried out using the following search queries: "ribociclib OR palbociclib OR abemaciclib" AND "breast cancer and randomized clinical trial", "CDK4/6 inhibitors OR cyclin-dependent kinase 4/6 inhibitors" AND "metastatic breast cancer" AND "real-world" AND "dose Intensity OR dose reduction". As a result of the search, 384 publications were found, and 15 publications were included in the final analysis. Data on dose reduction were systematized according to the following criteria: the proportion of patients who underwent the first and, if available, the second reduction, the time to dose reduction, and the intensity of dosing.

Results. Analysis of data from randomized clinical trials showed that a dose reduction was required in 31.8–57.4% of patients using CDK4/6 inhibitors. At the same time, the second dose reduction was carried out in 17.4–40% of patients. The median time to the first stage of reduction ranged from 1.2 to 3.2 months. The median relative dose intensity ranged from 66.3 to 93.0%. According to the results of the analysis of real clinical practice data, dose reduction was carried out in 28.1–59.1% of patients. At the same time, the first stage of reduction was carried out at 1–3 months of therapy, and the second at 4–17 months from the start of treatment.

Conclusion. A literature review was conducted to systematize the results of the use of CDK4/6 inhibitors, describing the features of dose reduction. Approximately up to 60% of patients need a dose reduction, regardless of the selected CDK4/6 inhibitor. Data on the frequency and time to dose reduction vary; therefore, the need for reduction in an individual patient may arise at any time, which may complicate the process of planning the provision of anti-tumor therapy drugs.

Keywords: $HR^+/HER2^-$; breast cancer; CDK4/6 inhibitor; abemaciclib; palbociclib; ribociclib; dose reduction

Abbreviations: BC — breast cancer; HR — hormone receptor; HER2 — human epidermal growth factor receptor 2; EGFR — epidermal growth factor receptor; RCT — randomized clinical trial; CDK — D-cyclin-dependent kinase; Rb — retinoblastoma.

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Рациональный подход к редукции дозы ингибиторов CDK4/6 при лечении пациентов с распространённым раком молочной железы: описательный обзор

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Применение ингибиторов CDK4/6 при лечении $HR^+/HER2^-$ рака молочной железы (РМЖ) в последние годы получает все большее распространение. При оценке безопасности ингибиторов CDK4/6 было установлено, что в процессе терапии значительному числу пациентов требуется снижение первоначальной дозы препарата в связи с нежелательными явлениями (редукция дозы). Однако публикации, обобщающие такие данные отсутствуют. При этом время до редукции дозы, ее этапность, могут значительно влиять на процесс организации лекарственного обеспечения пациентов препаратами этой группы, оказывая экономический и административный эффект на систему здравоохранения. В связи с этим, проведение обзора представленных в литературе результатов применения ингибиторов CDK4/6, описывающих особенности снижения дозы, своевременно и актуально.

Цель. Провести обзор литературы с целью обобщения и систематизации результатов применения ингибиторов CDK4/6, описывающих особенности редукции дозы.

Материалы и методы. Поиск литературы проводился в базах данных MedLine (PubMed) и Google Scholar с января 2016 по январь 2024 года. Литературный поиск был проведён по следующим поисковым запросам: «ribociclib OR palbociclib OR abemaciclib» AND «breast cancer and randomized clinical trial», «CDK4/6 inhibitors OR cyclin-dependent kinase 4/6 inhibitors» AND «metastatic breast cancer» AND «real-world» AND «dose Intensity OR dose reduction». В результате поиска было найдено 384 публикации, в финальный анализ попали 15 публикаций. Систематизацию данных о редукции доз проводили по следующим критериям: доля пациентов, которым выполнена первая и, при наличии, вторая редукция, время до редукции дозы, интенсивность дозирования.

Результаты. Анализ данных рандомизированных клинических исследований показал, что снижение дозы потребовалось 31,8–57,4% пациентам при применении ингибиторов CDK4/6. При этом 17,4–40% пациентам было проведено второе снижение дозы. Медианное время до первого этапа редукции составило от 1,2 до 3,2 мес. Медиана относительной эффективности дозы находилась в интервале от 66,3 до 93,0%. По результатам анализа данных реальной клинической практики — 28,1–59,1% пациентам была проведена редукция дозы. При этом первый этап редукции осуществлялся на 1–3 мес. терапии, а второй — на 4–17 мес. с момента начала лечения.

Заключение. Проведён обзор литературы для систематизации результатов применения ингибиторов CDK4/6, описывающих особенности редукции дозы. Примерно до 60% пациентов нуждаются в проведении редукции дозы вне зависимости от выбранного ингибитора CDK4/6. Данные о частоте и времени до снижения дозы разнятся, следовательно, необходимость редукции у отдельного пациента может возникнуть в любой момент, что может затруднять процесс планирования обеспечения препаратами противоопухолевой терапии.

Ключевые слова: $HR^+/HER2^-$; рак молочной железы; ингибитор CDK4/6; абемациклиб; палбоциклиб; рибоциклиб; редукция дозы

Список сокращений: РМЖ — рак молочной железы; HR — рецептор гормона; HER2 — рецептор эпидермального фактора роста, тип 2; EGFR — рецептор эпидермального фактора роста; РКИ — рандомизированное клиническое исследование; CDK — D-циклин-зависимая киназа; Rb — ретинобластома.

INTRODUCTION

Most cases (approximately 70%) of breast cancer (BC) worldwide are positive for hormonal receptor and/or progesterone receptor (HR) expression and do not express human epidermal growth factor receptor 2 (HER2), i.e., have the $HR^+/HER2^-$ phenotype[1] [1]. Although blocking signaling from epidermal growth factor receptors (EGFR) is a base of $HR^+/HER2^-$ BC treatment, many patients develop resistance to endocrine therapy. This resistance is difficult to overcome and is associated with a negative prognosis [2–4]. A number of mechanisms are involved in the development of resistance to endocrine therapy, including changes in cell cycle checkpoints. For example, regulation of cyclin

[1] National Cancer Institute. SEER. Cancer Stat Facts: Female Breast Cancer Subtypes. Available from: https://seer.cancer.gov/statfacts/html/breast-subtypes.html.

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D-cyclin-dependent kinase (CDK) 4/6 with INK4 proteins and the retinoblastoma (Rb) pathway (CDK4/6-INK4-Rb), which affects cell proliferation, is often impaired in HR^+ BC and other types of cancer [5–7]. Constant expression of cyclin D1 and phosphorylation of Rb support the use of CDK4/6 inhibitors in HR^+ BC [8]. Inhibition of the CDK4/6-INK4-Rb pathway along with endocrine therapy may be effective in patients with $HR^+/HER2^-$ BC compared with endocrine therapy alone. Currently, three selective CDK4/6 inhibitors are registered in the Russian Federation: ribociclib, palbociclib, and abemaciclib [9]. Differences between these drugs include, above all, dosing regimens (ribociclib and palbociclib are used once daily for 3 weeks with a 1-week break, abemaciclib twice daily) and different selectivity for CDK4 compared to CDK6, which has been shown in some preclinical studies [10–13].

When assessing the safety of CDK4/6 inhibitors, it was found that during therapy, a significant number of patients require a reduction in the initial dose of the drug (reduction, dose modification) due to the occurrence of various adverse events [14, 15]. Unlike reducing the frequency of the required dose, reduction allows managing drug toxicity while maintaining the effectiveness of therapy and adherence to treatment [14, 16]. At the same time, the time to dose reduction and its stages can significantly affect the organization of drug supply for patients with drugs of this class, having an economic and administrative impact on the healthcare system. In this regard, an actual question is the analysis of the data available in the literature on the features of dose reduction when using drugs of this group.

THE AIM. To conduct a literature review to systematize the results of the use of CDK4/6 inhibitors, describing the features of dose reduction of drugs.

MATERIALS AND METHODS

A literature search was conducted in the MedLine (PubMed) and Google Scholar databases from 2016 to January 2024. The literature search was carried out using the following search queries:

For **PubMed**, the following filters were used: “since 2016”, “English language”, “preprints excluded”.

- (ribociclib OR palbociclib OR abemaciclib) AND breast cancer AND randomized clinical trial;
- (CDK4/6 inhibitors OR cyclin-dependent kinase 4/6 inhibitors) AND (metastatic breast cancer) AND real-world AND (dose Intensity OR dose reduction);

For **Google Scholar**, the filter “from 2016” was used.

- (CDK4/6 inhibitors OR cyclin-dependent kinase 4/6 inhibitors, metastatic breast cancer, real-world data, (dose intensity OR dose reduction).

A search for real-world clinical practice studies was additionally carried out in the Google Scholar system due to the frequent lack of their indexing in PubMed.

The literature search and preparation of the review were carried out in accordance with the PRISMA methodology[2].

It is worth noting that randomized clinical trials (RCTs) are currently the “gold standard” for assessing the clinical efficacy and safety of drugs [17, 18]. Despite the limitations due to the design of RCTs, such as strict inclusion and exclusion criteria for patients in the study population, the inability to assess the long-term consequences of the therapy under study, and systematic errors, the homogeneity of the data obtained in them is significantly higher than that of real-world clinical practice data (electronic medical records, registers, prescription data and reports of adverse events, data obtained from patients, data from mobile applications and wearable devices, etc.). In this regard, preference was given to RCTs in the selection of publications. However, RCTs that allow simultaneous comparison of all three CDK4/6 inhibitors in terms of frequency, duration, and proportion of patients requiring dose reduction are not available. Therefore, real-world clinical practice data cannot be neglected, because, of course, they should be taken into account as a supplement to RCTs [19].

Article and data selection was carried out independently by two researchers. Disagreements were resolved through discussion. Studies that met the following criteria were excluded:

- Therapy for another nosology against the background of CDK4/6 inhibitors;
- Line of therapy after discontinuation of CDK4/6 inhibitors;
- Use of CDK4/6 inhibitors as part of neoadjuvant therapy;
- Use of CDK4/6 inhibitors in combination with immunobiological drugs;
- Works that do not contain quantitative information about dose reduction (intensity, time to reduction, proportion).

Inclusion criteria were:

- Clinical trials and real-world clinical practice studies evaluating the use of CDK4/6 inhibitors in the treatment of patients with metastatic $HR^+/HER2^-$ advanced breast cancer;
- Clinical trials and real-world clinical practice studies evaluating dose reduction or dose intensity of CDK4/6 inhibitors used;

[2] The PRISMA2020 statement: An updated guideline for reporting systematic reviews. Available from: https://www.equator-network.org/reporting-guidelines/prisma/

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- Real-world clinical practice studies including all three CDK4/6 inhibitors (ribociclib, palbociclib, and abemaciclib).

Suitable data extraction, met the inclusion criteria, was carried out independently by two authors. The following information was extracted: name, surname and initials of the first author, year of publication, journal, type of study, patient condition, age and number of patients, treatment regimen, response to therapy, study design, as well as data on dose reduction (proportion of patients who underwent the first and, if available, second reduction, time to dose reduction, dosing intensity). The data obtained were combined without quantitative synthesis of the results of individual homogeneous studies using meta-analysis) [22].

Quantitative data on dose reductions were extracted together with confidence intervals (CI), minimum and maximum data values where this information is presented, however, CIs were not applicable to the proportions of patients who underwent reduction because the proportion of patients is not an average value.

Figure 1 shows a diagram reflecting the publication search strategy.

Publications systematizing data on dose reduction for all three CDK4/6 inhibitors were not found. In this regard, a summary of the data currently available in the literature on this issue is presented below, and potential problems related to adherence to therapy are also discussed.

RESULTS

Data on dose modification presented in RCTs are summarized in Table 1.

In extensive large-scale studies PALOMA-2 and PALOMA-3, the dose reduction of palbociclib is initially carried out from 125 to 100 mg, at the second stage — from 100 mg to 75 mg. In postmenopausal patients, according to PALOMA-2, the median relative dose intensity was 93% in the palbociclib group, and a reduction was required for 39.4% of patients. At the same time, the median time to the first dose reduction was 3.2 months. (1–28 months). A second reduction was necessary for 36% of patients [13, 20, 21]. A detailed analysis of the PALOMA-3 study [21, 23] showed that the median relative dose intensity was comparable to the data in PALOMA-2 and amounted to 89.8%, for placebo — 100%. In the palbociclib group, 42.3% in pre- and 31.8% of postmenopausal patients required a dose reduction. Among patients who had only one dose reduction, the median time to reduction was 57.0 days (from 125 to 100 mg) and 36.0 days (from 125 to 75 mg). In patients who underwent two dose reductions, the average time to the first reduction was 33.5 days (from 125 to 100 mg), and the average time to the second was 119.5 days (from 100 to 75 mg). Among patients in the palbociclib group who had at least one dose reduction, only 31% received treatment at a dose of 100 mg and 9% — 75 mg [22]. Thus, according to the results of clinical studies of palbociclib, dose reduction was required in an average of 39.4–42.3% of patients. According to the combined analysis of J. Ettl et al. [21], which included the results of palbociclib studies PALOMA-1, -2, -3, 413 patients out of 875 (47.2%) required a dose reduction. At the same time, a second dose reduction was required for 105 out of 413 patients (24.4%). The median time to the first dose reduction in the combined analysis was 70 days (interval 15.0–1269.0 days). The median time to the second dose reduction was 106.0 days (interval 29.0–699.0 days). [21] The wide range of time to reduction may indicate that the occurrence of such a need depends on the individual characteristics of patients and cannot be predicted in advance.

Abemaciclib dose reduction is described in the MONARCH-2 and MONARCH-3 studies and was required in 42.9 and 43.6% of patients, respectively. The first reduction was carried out from 150 to 100 mg, and the second from 100 to 50 mg [24, 25]. The median relative dose intensity was 86% for abemaciclib and 98% for the placebo group [25]. With a median follow-up of 17.8 months, the median number of cycles of therapy received in the abemaciclib group was 16, in the placebo group — 15 cycles [25]. Data on the time to the onset of the first and second dose modification, as well as the distribution of patients between them — were absent. According to the combined analysis of M.P. Goetz et al. [26], which includes the results of abemaciclib studies MONARCH 2 and -3, 42.7% of patients (under 65 years of age), 55.4% of patients (from 65 to 75 years of age) and 55.4% of patients (over 75 years of age) required dose reductions.

The MONALEESA-2, MONALEESA-3, and MONALEESA-7 studies describe data on dose reduction with ribociclib in a total of 1153 patients. The first reduction was from 600 to 400 mg/day, and the second was from 400 to 200 mg/day. Dose reduction was independent of age and ECOG (Eastern Cooperative Oncology Group) performance status. The exception is patients in the Asian population, among whom the proportion of patients who required a dose reduction was higher than that of patients without dose reduction in the MONALEESA-3 and -7 studies

(MONALEESA-3 — 16.3 and 7.5%; MONALEESA-7 — 40.7 and 28.4%, respectively). In the MONALEESA-2 study, the median relative dose intensity for the ribociclib group was 65.6%, and for the placebo group — 99.3% [14]. Dose reduction was required in 57.4% of patients in the ribociclib group. Moreover, a second dose reduction was required in 40% of patients. The median time to the first dose reduction was 3 months [14]. In the MONALEESA-3 study, the median relative dose intensity for the ribociclib group was 67.8%, and for the placebo group — 99.7% [14]. Dose reduction was required in 38.7% of patients in the ribociclib group. Moreover, a second dose reduction was required in 17.4% of patients. The median time to the first dose reduction was 2.8 months [14]. In the MONALEESA-7 study, dose reduction was required in 37% of patients in the ribociclib group. Moreover, a second dose reduction was required in 27.5% of patients. The median time to the first dose reduction was 2.2 months [14]. Thus, according to the results of clinical studies of ribociclib, dose reduction was required in 38.7–57.4% of patients, which averages 45.8% [14]. Among patients who required a dose reduction of ribociclib, the majority are those patients who required a single reduction (257 out of 375 — 68.5%). The average time to the first dose reduction of ribociclib from the start of the study ranged from 2 to 3 months and generally corresponded in all three studies (MONALEESA-2 — 3.0 months, MONALEESA-3 — 2.8 months, MONALEESA-7 — 2.2 months).

The above data from RCTs and pooled analyses are confirmed by the results of routine practice studies. It should be noted that a significant number of patients receiving CDK4/6 inhibitors required a dose reduction due to toxicity (38.7–57.4%). The results of the dose reduction analysis in real-world clinical practice studies are reflected in Table 2. RCTs are certainly the “gold standard” in terms of the homogeneity of the data obtained, but the above-mentioned studies did not conduct a direct comparison between different drugs in this class. In addition, more detailed data related to dose reduction were not published. Thus, the analysis of real-world clinical practice studies included only those studies in which data for all three drugs in this class — abemaciclib, palbociclib, ribociclib — were simultaneously present, as well as studies in which information on the time to the second dose reduction was provided.

Summarizing the results of real-world clinical practice studies, dose reduction was required in 28.1–57.1% of patients receiving CDK4/6 inhibitors. The time to the first dose reduction ranged from 1 to 3 months, and the time to the second reduction ranged from 4 to 17 months from the start of therapy. The mean relative dose intensity (where it was taken into account) ranged from 0.8 to 0.83.

DISCUSSION

Thus, the need for dose reduction is an urgent fact in a significant proportion of patients (28.1–57.4%) receiving drugs of this class, regardless of menopausal status [14, 30]. Despite the fact that the drug class is well studied, detailed data on dose reduction are rarely found in publications. The few data on the time to dose reduction in RCTs and real-world clinical practice studies have a wide range of values (from 1 month to several years) [14, 30].). At the same time, it is impossible to predict when and in what volume a dose reduction will be required for a particular patient. When starting therapy for patients with drugs in this group, you need to be prepared for the need to reduce the dose at any stage of therapy. A complex dose reduction system can create additional problems when correcting therapy for $HR^+/HER2^-$ breast cancer with CDK4/6 inhibitors. It should be noted that only ribociclib has a dose reduction step of 200 mg, which corresponds to the dosage of the drug, i.e., allows it to be carried out by reducing the dose by one dose [14] (Fig. 2).

For the other two drugs — abemaciclib and palbociclib — dose reduction requires prescribing the drug in a different dosage, which may lead to a decrease in adherence to therapy and depends on the availability of specific drug dosages in the drug supply system [33]. Moreover, the pharmacokinetic parameters of the drugs may further limit the selection of dosages: the absorption process of abemaciclib is obviously saturable, as a result of which a reduction in the daily dose due to taking large doses once a day, instead of twice a day of a reduced dose, may lead to a violation of the therapeutic concentration of the drug in the blood. The conclusion about the undesirability of prescribing abemaciclib once a day is confirmed by the results of mathematical modeling [34]. The relevance of these issues is due to differences in drug dosing regimens. For example, dose reduction of abemaciclib is carried out according to the following scheme: 150→100→50 mg. At the same time, the drug is available in all these dosages. A similar picture is characteristic of palbociclib: dose reduction 125→100→75 mg with the same available dosages[3]. Dose reduction for ribociclib is carried out according to the scheme 600→ 400→200 mg with a single dosage of 200 mg (see Fig. 2).

[3] General characteristics of the drug palbociclib. Available from: https://lk.regmed.ru/Register/EAEU_SmPC

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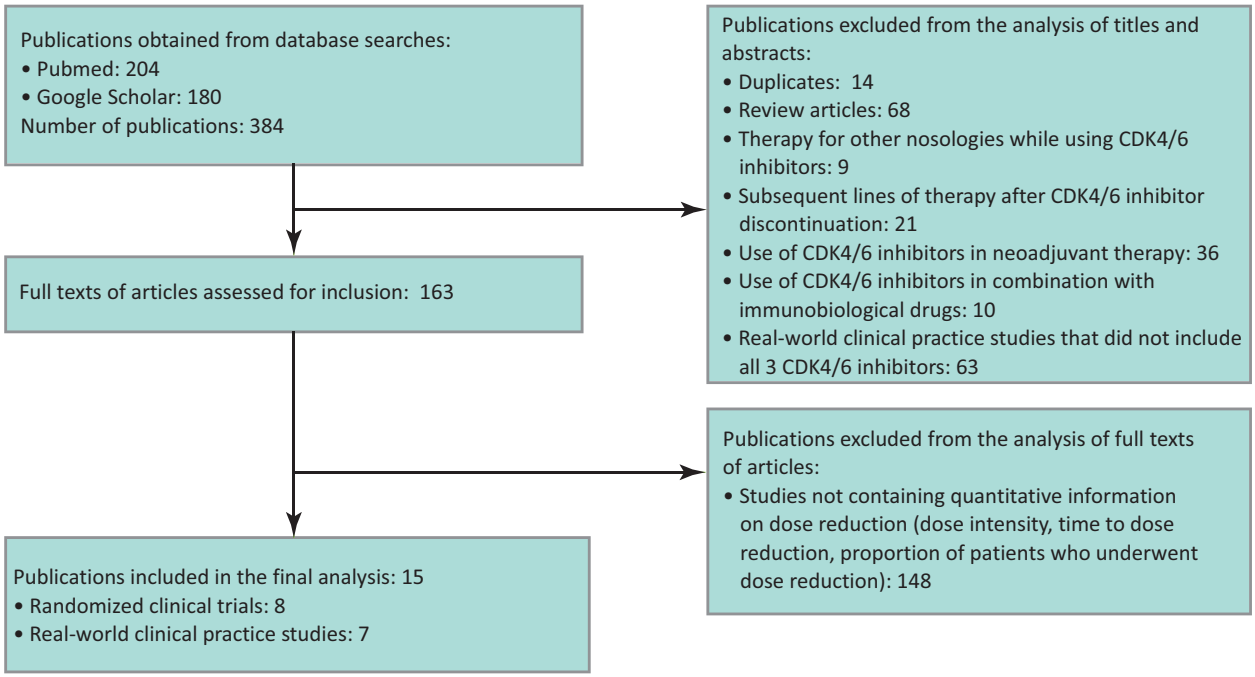

Figure 1 – Publication search strategy.

Table 1 – Dose reduction data extracted from randomized clinical trials

RCT [reference]	Drug and control group, (abs.)	Endocrine partner	Menopausal status	Proportion of patients with dose reduction, % (number of patients)[1]	Median time to first dose reduction, months (min–max)	Proportion of patients with a second dose reduction, % (number of patients)[2]	Median relative dose intensity, % (min–max), drug / placebo[5]
PALOMA-2 [13, 20, 21]	Palbociclib (444) Placebo (222)	Letrozole	Post-	39.4% (175/444)	3.2 (1–28)	36 %) (63/175)	**93.0** (40–110) 99.6 (56–105)
PALOMA-3 [21–23]	Palbociclib (347) Placebo (174)	Fulvestrant	Pre-	42.3% (30/71)	1.2	4.3	**89.8** (22–107) 100 (80–100)
			Post-	31.8% (87/274)			
MONARCH-2 [24]	Abemaciclib (446) Placebo (223)	Fulvestrant	Pre- Post-	42.9% (189/441)3	nd	nd	nd
MONARCH-3 [25]	Abemaciclib (328) Placebo (165)	Anastrozole or letrozole	Pre-	43.6% (142/326)	nd	nd	**86%** 98%
MONALEESA-2 [14]	Ribociclib (334) Palbociclib (334)	Letrozole	Post-	57.4% (192/334)	3.0	40% (77/192)	**65.6% (31.4–99.8%)** 99.3% (50.0–111.9%)
MONALEESA-3 [15]	Ribociclib (484) Fulvestrant (242)	Fulvestrant	Post-	38.7% (92/238)	2.8	17.4% (16/92)	**67.8%** (34.7–99.7%) 98.4% (65.9–131.8%)
MONALEESA-7 [14]	Ribociclib (335) Placebo (337)	Tamoxifen or letrozole or anastrozole	Pre-	37% (91/246)[4]	2.2	27.5% (25/91)	**66.3%** (27.9–98.6%) 98.0% (57.1–104.8%)

Note: RCT — randomized clinical trial; [1] — data is presented in the format — the number of patients who underwent one or more dose reductions and the number of patients who participated in the final data analysis (may not coincide with the total number of randomized patients); [2] — data is presented in the format — the number of patients who underwent a second dose reduction and the number of patients who underwent one or more dose reductions; [3] — the study included patients with both pre- and post-menopause (however, the results do not contain data for each of the subgroups; [4] — the tamoxifen cohort was excluded from the analysis, since the combination with tamoxifen is not registered; [5] — the median relative intensity was calculated as — dose, per patient, divided by the number of days of administration and multiplied by the recommended dosage of the drug.

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Table 2 – Multicenter retrospective real-world clinical practice studies

First author, year [reference]	Number of patients, menopausal status	Dose reduction data[1]		
		Palbociclib	Abemaciclib	Ribociclib
G. Gullick, 2024 [27]	666, pre- and postmenopausal	289/537 (53.8%) • Median cycle number of first dose reduction, *n* (min–max) — 3 (1–63)	50/85 (58.8%) • Median cycle number of first dose reduction, *n* (min–max) — 3 (1–11)	26/44 (59.1%) • Median cycle number of first dose reduction, *n* (min–max) — 2 (1–37)
M. Cejuela, 2024 [28]	206, pre- and postmenopausal	50/96 (52.1%)	30/56 (53.6%)	28/54 (51.9%)
M.M. Queiroz, 2023 [29]	142, menopausal status not specified	34/79 (43%) • Time to first reduction (Me±SD) — 3±8 • Time to second reduction (Me±SD) — 6±5.14	12/21 (57.1%) • Time to first reduction (Me±SD) — 1±2.8 • Time to first reduction (Me±SD) — 17±1.44	18/42 (42.9%) • Time to first reduction (Me±SD) — 2±5.9 • Time to first reduction (Me±SD) — 4±1.54
P. Fedele, 2024 [30]	158, menopausal status not specified	16/57 (28.1%)	19/48 (39.6%)	15/53 (28.3%)
L. Siljander, 2022[4]	2572, menopausal status not specified	Total 1811 Mean relative dose intensity[2] – 0.83	Total 91 Mean relative dose intensity – 0.82	Total 670 Mean relative dose intensity — 0.80
S. Palladino, 2023 [31]	3647, post- and premenopausal	nd/2627 (35%)	nd/291 (44.7%)	nd/729 (22.1%)
K.B. Kristensen, 2021 [32]	128, post- and premenopausal	nd	nd	60/128 (46.8%) • Patients with a second dose reduction — 17/60 (28.3 %)3 • Time to first reduction, Me [min–max] – 2.2 [0.9–17.3] months • Time to second reduction Me [min–max] – 6.5 [1.8–17.5]5

Note: [1] — dose reduction data is presented in the format — number of patients who underwent dose reduction and the total number of patients; [2] — time to second reduction is counted from the start of patient observation; [3] — mean relative dose intensity was calculated as the quotient of the total dose calculated for all patients, divided by the number of days of drug administration, multiplied by the recommended dosage of the drug; [4] — data on the second dose reduction is presented in the format — number of patients who underwent the second dose reduction and the number of patients who underwent dose reduction; [5] — time to second reduction is counted from the start of patient's observation.

Thus, if the dose reduction of ribociclib is multiple and involves reducing the number of tablets with the same dosage per administration, then for abemaciclib and palbociclib, dose reduction requires the use of a new drug package (with a lower dosage[5]). It should be noted that for abemaciclib, in the absence of a dosage for the first dose reduction (100 mg), it can be compensated by taking two 50 mg tablets, which will increase costs, but the therapy regimen will not be violated[6]. In the case of palbociclib, the lack of the required dosage will lead to the cancellation of therapy.

Considering the existing limitations in providing the patient with drugs for anticancer therapy, their availability in the required dosages at any given time may be difficult. Excessive wait time of the patient for the required dosage, or even refusal to take the drug in the initial dosage due to adverse events, or violation of the administration regimen (frequency, rate, etc.) reduces the therapy efficiency. Under this circumstance, the presence of only one dosage in a drug such as ribociclib and the reduction of its dose

[4] Siljander L, Hornemann AT, Møller AH. Real-world relative dose intensity in patients with advanced or metastatic breast cancer treated with cyclin-dependent kinase (CDK) 4/6 inhibitors in Sweden. Presented at the ISPOR Europe Congress 2022, Vienna, Austria and Virtual, 6–9 November 2022. Available from: https://www.ispor.org/docs/default-source/euro2022/isporeusiljander-pdf.pdf?sfvrsn=4e493646_0

[5] General characteristics of the drug ribociclib. Available from: https://lk.regmed.ru/Register/EAEU_SmPC

[6] The State Register of Medicines of the Russian Federation. Instructions for the medical use of the drug abemaciclib. Available from: https://grls.minzdrav.gov.ru/Grls_View_v2.aspx?routingGuid=ca56c862-7110-4a8f-ad46-ecb8008f14f0

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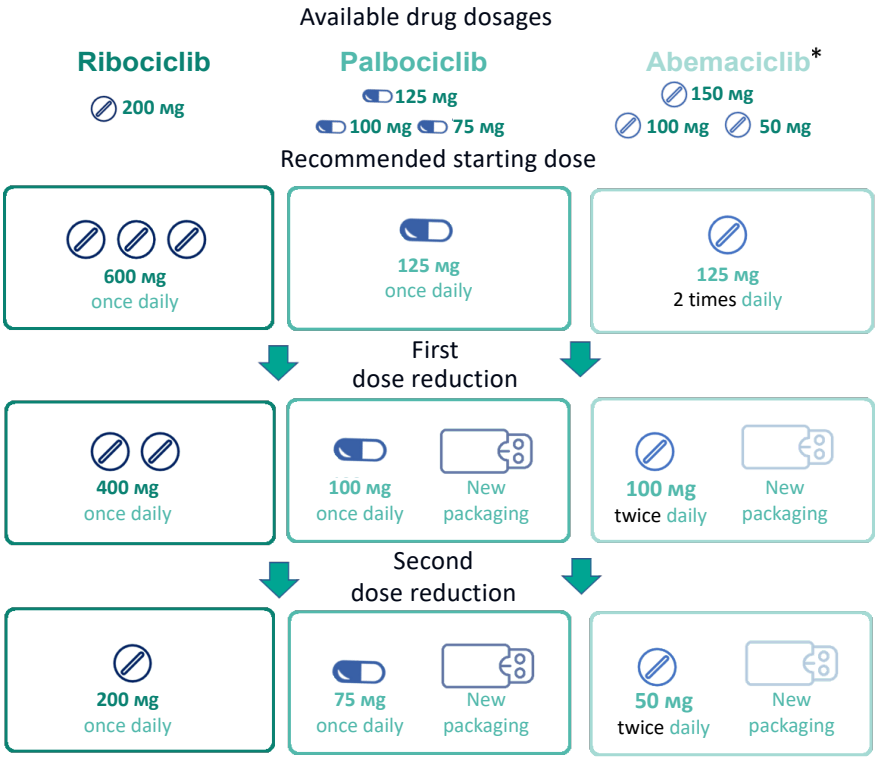

Figure 2 – Use of available dosages of CDK4/6 inhibitors during dose reduction.

Note: * for abemaciclib, the dosing regimen is shown for combining therapy.

by reducing the number of tablets per administration gives it a significant advantage over other CDK4/6 inhibitors in terms of patient adherence to therapy throughout the entire treatment period.

Limitations of the review

Among real-world clinical practice studies, only those studies that included all three CDK4/6 inhibitors — ribociclib, palbociclib, and abemaciclib — were selected. This limitation was introduced to ensure data comparability and focus on modern treatment standards for hormone receptor-positive, HER2-negative metastatic breast cancer, where these three drugs are the main therapeutic options. It is important to note that a significant portion of the studies do not contain detailed quantitative data on dose reduction, despite the fact that the fact of reduction in some patients is indicated in our publication. Variability in approaches to reporting dose reduction data (for example, methods for calculating relative dose intensity or heterogeneity of time intervals for assessing time to reduction) can make it difficult to standardize and compare results between studies. This variability may be due to differences in clinical protocols and patient characteristics, which limits the possibilities for quantitative data synthesis and increases the risk of ambiguity in the interpretation of results.

CONCLUSION

An analysis of data on dose reduction of CDK4/6 inhibitors in the treatment of $HR^+/HER2^-$ breast cancer, published based on the results of RCTs, as well as real-world clinical practice studies, showed that dose reduction is the only way to manage adverse events and is often required by more than half of patients, regardless of the drug chosen. The limited availability of data on the time of onset of the first and second dose reductions, as well as the wide range of values, makes it difficult to predict its necessity in advance, and therefore complicates the process of planning and organizing drug supply. Therefore, from the point of view of rational pharmacotherapy, justifying the choice of a specific drug of this class requires a comprehensive analysis of clinical efficacy indicators, pharmacokinetic parameters, as well as administration features, taking into account the specifics of the healthcare system. A complex process of providing medicines can lead to limited availability of individual drug dosages, which in turn, for example, when a CDK4/6 inhibitor dose reduction is necessary, can lead to a significant decrease in patient adherence to therapy and, accordingly, its effectiveness. In this regard, the presence of a single dosage of ribociclib eliminates potential problems with changing the drug package if a dose reduction is necessary. Based on this, it is necessary to be guided by the principles of

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rational pharmacotherapy and take into account not only the clinical effectiveness of drugs and their tolerability by patients, but also the availability of the entire range of drug dosages, taking into account the upcoming dose reduction, deciding on the appointment of drug therapy with CDK4/6 inhibitors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHORS' CONTRIBUTION

Ilya N. Dyakov — concept development, search and analysis of literary sources, writing a draft of the manuscript;
Sergey K. Zyryanov — concept development, scientific supervision, writing the manuscript.
All authors confirm their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept, conduct of the research, and preparation of the article, read and approved the final version before publication).

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Assessment of the allergenic and immunotoxic properties of the recombinant non-immunogenic staphylokinase in preclinical and clinical trials

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The aim. To study possible allergenic and immunotoxic properties of the recombinant non-immunogenic staphylokinase molecule with the amino acids replacement Lys_{74}, Glu_{75} and Arg_{77} with alanine in preclinical and clinical studies.
Materials and methods. The allergenic and immunotoxic properties of the recombinant non-immunogenic staphylokinase drug were studied using standard methods in accordance with the Guidelines for the Preclinical Study of New Substances in guinea pigs (*n*=15) and mice (*n*=45) at doses 5, 10 and 20 times higher than therapeutic (for humans). A clinical study was conducted in 100 patients with acute ST-segment elevation myocardial infarction after a single intravenous injection of the drug. The study included the determination of titers of specific antibodies to recombinant non-immunogenic staphylokinase and the study of plasma neutralizing activity.
Results. During the complete set of preclinical studies, it was found that the drug does not affect the cellular and humoral immune response in guinea pigs and mice at doses many times higher than therapeutic doses for humans. It was found that the drug did not cause an immediate-type of hypersensitivity reaction (Weigle index 0) and a delayed type IV (0 points according to S.V. Suvorov) in guinea pigs, did not affect the cellular capacity of popliteal lymph nodes (reaction index 0.91), did not affect the number of nucleated and antibody-forming cells in the spleen of mice. As a result of a clinical study of recombinant non-immunogenic staphylokinase, no allergic reactions were registered. Assessment of the neutralizing activity of the plasma of patients who were administered recombinant non-immunogenic staphylokinase showed that 70% samples did not have neutralizing activity: 30% of the patients' samples were characterized by a minimum neutralizing activity of 0.33±0.02 μg/mL, which is 30–310 times lower than after the use of native staphylokinase. These values are 7.8 times lower than the determined concentration of recombinant non-immunogenic staphylokinase in the blood (2.59 μg/mL). Thus, the drug does not lead to the anti-staphylokinase neutralizing antibodies formation capable to neutralize its effect upon repeated administration.
Conclusion. According to the results of the trials, the absence of allergenic and immunotoxic properties of the recombinant non-immunogenic staphylokinase and its safety in relation to the immune system have been proven.
Keywords: thrombolysis; recombinant non-immunogenic staphylokinase; allergy; immunotoxic studies
Abbreviations: AFC — antibody-forming cells; IL — interleukin; STEMI — acute ST-segment elevation myocardial infarction; PE — pulmonary embolism; RE — ram erythrocytes.

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RESEARCH ARTICLE

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Оценка аллергизирующих и иммунотоксичных свойств рекомбинантной неиммуногенной стафилокиназы в доклинических и клинических исследованиях

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Цель. Изучить возможные аллергизирующие и иммунотоксичные свойства молекулы рекомбинантной неиммуногенной стафилокиназы с заменой аминокислот Lys_{74}, Glu_{75} и Arg_{77} на аланин в доклинических и клинических исследованиях.

Материалы и методы. Исследование аллергизирующих и иммунотоксических свойств препарата рекомбинантной неиммуногенной стафилокиназы проведено по стандартным методикам в соответствии с требованиями Руководства по экспериментальному (доклиническому) изучению новых фармакологических веществ на морских свинках (*n*=15) и мышах (*n*=45) в дозах, в 5, 10 и 20 раз превышающих терапевтические (для человека). Клиническое исследование проведено у 100 пациентов с острым инфарктом миокарда с подъёмом сегмента ST после однократного внутривенного введения препарата. Исследование включало определение титров специфических антител к рекомбинантной неиммуногенной стафилокиназе и оценку нейтрализующей активности плазмы.

Результаты. При проведении полного комплекса доклинических исследований было установлено, что препарат не влияет на клеточный и гуморальный иммунный ответ у морских свинок и мышей в дозах, кратно превышающих терапевтические для человека. Установлено, что препарат не вызывал реакцию гиперчувствительности немедленного типа (индекс по Weigle 0) и замедленного типа (0 баллов по С.В. Суворову) у морских свинок, а также не влиял на клеточность подколенных лимфоузлов (индекс реакции 0,91) и число ядросодержащих и антителообразующих клеток в селезёнке мышей. В результате клинического исследования рекомбинантной неиммуногенной стафилокиназы аллергических реакций не зарегистрировано. Оценка нейтрализующей активности плазмы крови пациентов, которым вводилась рекомбинантная неиммуногенная стафилокиназа, показало, что пробы 70% пациентов не обладают нейтрализующей активностью: 30% проб пациентов характеризовались минимальной нейтрализующей активностью 0,33±0,02 мкг/мл, что в 30–310 раз ниже, чем после применения нативной стафилокиназы. Эти значения в 7,8 раз ниже определяемой концентрации рекомбинантной неиммуногенной стафилокиназфы в крови (2,59 мкг/мл). Таким образом, препарат не приводит к образованию антител, способных нейтрализовать его действие при повторном введении.

Заключение. По результатам проведённых исследований доказано отсутствие аллергизирующих и иммунотоксичных свойств рекомбинантной неиммуногенной стафилокиназы и ее безопасность в отношении иммунной системы.

Ключевые слова: тромболизис; рекомбинантная неиммуногенная стафилокиназа; аллергические реакции; исследования иммунотоксичности

Список сокращений: АОК — антителообразующие клетки; ИЛ — интерлейкин; ОИМпST — острый инфаркт миокарда с подъёмом сегмента ST; ТЭЛА — тромбоэмболия легочной артерии; ЭБ — эритроциты барана.

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INTRODUCTION

Myocardial infarction, ischemic stroke, and pulmonary embolism (PE) continue to be leading causes of death among all cardiovascular diseases [1]. The prevalence of myocardial infarction (per 100,000 people) reaches 500 cases in men and 100 cases in women, ischemic stroke — 460–560 cases, PE — 35–40 cases [1]. Thrombolytic therapy is a pathogenetically sound method for treating acute ST-segment elevation myocardial infarction (STEMI), ischemic stroke, and massive PE, based on dissolving a fibrin clot (thrombus) and restoring blood flow in the occluded vessel. Thrombolysis can reduce the risk of disability and death [1].

Thrombolytic therapy is carried out using drugs based on recombinant proteins, which determines the need to assess their potential impact on the immune system. Staphylokinase is a unique thrombolytic agent with high biological activity and fibrinolytic properties [2]. As a plasminogen activator, staphylokinase initially reacts with a minimal content of plasmin (3 ppm) located on the fibrin clot, followed by activation of γ-plasminogen and the formation of a triple complex "staphylokinase–plasmin–plasminogen", which lyses fibrin clots. Simultaneously, the resulting plasmin enhances the fibrinolytic activity of staphylokinase, and its excess is rapidly inactivated by α_2-antiplasmin. When the "plasmin–staphylokinase" complex is inhibited by α_2-antiplasmin, an active staphylokinase molecule is released for subsequent recycles. The recirculation of staphylokinase helps to reduce the dose used in clinical practice compared to tissue plasminogen activators and makes it independent of the patient's body weight [3].

The inhibition of the "plasmin-staphylokinase" complex in plasma occurs more than 100 times faster compared to the same process in a thrombus. Thus, staphylokinase has high selectivity for fibrin, which prevents the formation of plasmin from plasminogen in the systemic circulation [4]. Due to the high fibrin selectivity of the drug, the use of staphylokinase is characterized by a minimal risk of developing hemorrhagic complications.

As a result of kinetic analysis of the interaction of staphylokinase with plasmin, it was found that the catalytic activity of staphylokinase is 1000 times higher than that of alteplase [5]. The high fibrin selectivity of staphylokinase made it a first-line drug for the treatment of STEMI and ischemic stroke back in the late last century. However, the presence of immunogenic properties of the native staphylokinase molecule hindered its introduction into clinical practice. To create a non-immunogenic staphylokinase, the amino acids Lys_{74}, Glu_{75}, and Arg_{77} were replaced with alanine in the desired molecule. Recombinant non-immunogenic staphylokinase is a single-chain molecule consisting of 138 amino acids, with a molecular weight of 15.5 kDa. It has been established that the fibrinolytic activity of recombinant non-immunogenic staphylokinase is 40% higher than that of the native staphylokinase molecule [6].

THE AIM of this work was to study the allergenic and immunotoxic properties of the recombinant non-immunogenic staphylokinase molecule in preclinical and clinical studies.

MATERIALS AND METHODS

Preclinical studies

The study of the allergenic, immunotoxic, and immunogenic properties of the recombinant non-immunogenic staphylokinase drug was carried out in accordance with the requirements of the Guidelines for Experimental (Preclinical) Study of New Pharmacological Substances (2005) in the Laboratory of Drug Toxicology of the National Medical Research Center of Cardiology named after Academician E.I. Chazov under the guidance of Prof. E.V. Arzamastsev in the period from September 2008 to January 2010. The studies were approved by the Ethics Committee of the National Medical Research Center of Cardiology named after Academician E.I. Chazov (Protocol No. 1 dated September 4, 2008).

Study of the effect of recombinant non-immunogenic staphylokinase on the immediate-type hypersensitivity reaction in guinea pigs

The studies were performed on 15 variegated male guinea pigs with an average body weight of 290±20 g, obtained from the Stolbovaya Laboratory Animal Nursery (Scientific Center for Biomedical Technologies). The animals were kept under standard vivarium conditions in ventilated cages with a 12-hour light/dark cycle, at an air temperature of +20°C and air humidity of 50–60%. The animals had free access to food and water.

Guinea pigs were divided into three groups of 5 animals each: Control — physiological saline, Group 1 — recombinant non-immunogenic staphylokinase at a dose of 0.665 mg/kg, Group 2 — recombinant non-immunogenic staphylokinase at a dose

of 1.33 mg/kg. The doses of the drug used corresponded to 5 and 10 times the highest daily dose recommended for humans (10 mg for humans or 0.133 mg/kg) [2]. The drug was administered intravenously at a dose of 0.665 mg/kg twice a day every other day. The permissive dose was administered intravenously to guinea pigs 14 and 21 days after sensitization. The intensity of anaphylactic shock was assessed using the Weigle index [8].

Study of the effect of recombinant non-immunogenic staphylokinase on delayed-type hypersensitivity reaction in guinea pigs

The studies were performed on 15 variegated male guinea pigs with an average body weight of 290±20 g, which were divided into three groups of 5 animals each. The groups and doses were similar to the previous experiment. The animals were sensitized by 5-fold intramuscular administration of recombinant non-immunogenic staphylokinase at 5-day intervals. On the 10th day after the last sensitization, the animals had their back hair shaved on a 3×3 cm area and were injected intradermally with 0.1 mL of recombinant non-immunogenic staphylokinase solution, and the same volume of saline was injected into another point. The reaction was assessed visually according to the S.V. Suvorov scale of skin tests (1974) in points 4 and 24 hours after intradermal injection of the permissive dose.

Study of the effect of recombinant non-immunogenic staphylokinase on the cellularity of the popliteal lymph node in mice

The studies were performed on 10 male F_1(CBAxC$_{57}$Bl$_6$) hybrid mice with an average body weight of 18±2 g, obtained from the Stolbovaya Laboratory Animal Nursery (Scientific Center for Biomedical Technologies). The animals were kept under standard vivarium conditions in ventilated cages with a 12-hour light/dark cycle, at an air temperature of +20°C and air humidity of 50–60%. The animals had free access to food and water. Mice were injected with 50 µl of recombinant non-immunogenic staphylokinase at a dose of 1.33 mg/kg into the pad of the left hind paw, and saline into the pad of the right hind paw. After 7 days, the cellularity of the left and right popliteal lymph nodes was determined in mice, and then the relative index was calculated by dividing the indicators of the left lymph node by similar indicators of the right lymph node [8].

Study of the effect of recombinant non-immunogenic staphylokinase on the number of nucleated and antibody-forming cells in the spleen of mice

The experiment is based on determining the number of nucleated and antibody-forming cells (AFC) in the spleen according to Jerne in accordance with generally accepted methods [9]. The studies were performed on 35 male F_1(CBAxC$_{57}$Bl$_6$) hybrid mice with an average body weight of 18±2 g, which were divided into 7 groups of 5 animals each. The mice were immunized by intravenous administration of ram erythrocytes (RE) (Microgen, Russia) at a dose of 5×108 cells/mouse. Animals of groups 1 and 2 received recombinant non-immunogenic staphylokinase intraperitoneally at doses of 1.33 and 2.66 mg/kg, respectively, one day before immunization with RE (day -1), groups 3 and 4 — at the same doses 1 h after immunization (day 0), groups 5 and 6 — 24 hours after immunization with RE (day +1). Control mice were injected with saline on “day +1”. On the 5th day after immunization, the spleen was removed from the mice, which was disintegrated in Hanks’ solution (pH=7.4). The cell suspension was separated from the stroma elements by filtration through a two-layer nylon filter, then washed 3 times and centrifuged (ELMI CM-50, Latvia) at 200 g for 5 min. After lysis of erythroid cells with 3% acetic acid, the number of karyocytes was counted in the resulting suspension on a cell counter (Picoscale PS-4M, Hungary).

Study of the effect of recombinant non-immunogenic staphylokinase on delayed-type hypersensitivity reaction in mice

The studies were performed on 35 male F_1(CBAxC$_{57}$Bl$_6$) hybrid mice with an average body weight of 18±2 g, which were divided into 7 groups of 5 animals each. The mice were immunized by subcutaneous injection of RE into the interscapular region at a dose of 2×108 cells/mouse. The scheme of administration of recombinant non-immunogenic staphylokinase and distribution by groups was similar to the previous experiment. On the 5th day after immunization, all animals received a resolving injection of RE into the left hind paw at a dose of 1×108 cells/mouse in a volume of 50 µl (“experimental paw”). Saline solution was injected into the pad of the contralateral paw (“control paw”). The reaction results were recorded after 24 hours by

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weighing the “control” and “experimental” paws. The reaction index (R.M. Khaitov, 2000) was calculated as the ratio of the difference in mass between the “experimental” and “control” paws to the mass of the “control” paw.

Statistical analysis

Statistical analysis was performed using R 4.2 (R Foundation, USA). For continuous variables, the mean and standard deviation (M±SD) are given. Comparison of distributions in independent groups for continuous parameters was made using the Mann–Whitney test, for discrete parameters — using Fisher’s exact test. Differences were considered statistically significant at p <0.05.

Clinical studies

In accordance with the permission of the Ministry of Health of Russia No. 261 dated May 16, 2014, a multicenter open randomized comparative trial of the efficacy and safety of a single bolus administration of recombinant staphylokinase (15 mg) and tissue plasminogen activator tenecteplase (30–50 mg) in patients with STEMI was conducted from October 2014 to August 2016 at 11 leading medical institutions in Russia [10, 11]. Before inclusion in the study, all patients signed an informed consent. The study was approved by the Ethics Council of the Ministry of Health of Russia (Protocol No. 81 dated April 15, 2014) and Local Ethics Committees at research centers.

Clinical study of the effect of recombinant non-immunogenic staphylokinase on the antibodies formation

382 patients participated in the study. *Inclusion criteria:* diagnosis of STEMI with ST segment elevation of more than 1 mm in two or more consecutive limb leads and/or more than 2 mm in chest leads in the first 12 h from the onset of the disease. *Exclusion criteria:* bleeding, hemorrhagic stroke, ischemic stroke in the preceding 6 months, diseases with an increased risk of bleeding. A complete list of inclusion and exclusion criteria, as well as criteria for evaluating the effectiveness and safety of thrombolytic therapy, have been published previously [10, 11].

Patients were randomized into two groups (n=191 each) to receive recombinant non-immunogenic staphylokinase (Fortelyzin®, SupraGen LLC, Russia) or tenecteplase (Metalyse®, Boehringer Ingelheim International, Germany). Randomization was carried out by the envelope method, in blocks of 4 drugs (2 — recombinant non-immunogenic staphylokinase and 2 — tenecteplase). The sequence of randomization numbers was generated by an independent biostatistician. Recombinant non-immunogenic staphylokinase was administered at a dose of 15 mg, regardless of body weight, as a bolus over 10–15 seconds, tenecteplase — as a bolus at a dose of 30–50 mg, depending on body weight, according to the instructions for medical use[1]. Blood was collected from 100 patients from the recombinant non-immunogenic staphylokinase group to determine the antibody titer of to recombinant non-immunogenic staphylokinase and the neutralizing activity of plasma before its administration, as well as on days 7, 14 and 30 after.

The antibody titer was determined at the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences using a solid-phase indirect enzyme immunoassay according to the standard method [12]. The studied plasma samples obtained from patients after a single intravenous administration of the drug were diluted with a buffer solution (20 mM Tris-HCl, 150 mM NaCl, 0.005% Tween-20, pH=8.0) 1:100, 1:400, 1:1600, 1:6400, 1:25600, 1:102400. 100 µl of dilutions of the test samples were added to the wells. The plate was incubated for 60 min at 37°C. After incubation, the liquid was removed and the wells were washed three times with a buffer solution (20 mM Tris-HCl, 150 mM NaCl, 0.005% Tween-20, pH=8.0). 100 µl of rabbit secondary antibody solution to human IgG labeled with horseradish peroxidase (1:1000, pH=8.0) (Sigma, USA) was added to each well and incubated for 60 min at 37°C. After incubation, the wells were washed again with a buffer solution. 100 µl of substrate (0.07% orthophenylenediamine, 0.06% H_2O_2, pH=5.0) was added to each well. Incubated at room temperature for 3–5 min until staining appeared in the negative control sample. 50 µl of stop reagent (10% sulfuric acid solution) was added to each well. 15 min after the addition of the stop reagent, the optical density in each well was measured at 490 nm on an ImmunoChem-2100 microplate photometer (USA). The maximum dilution of the sample was determined, at which the optical density value of the positive wells exceeded the corresponding value of the negative wells by a factor of three. The antibody titer to recombinant non-immunogenic staphylokinase was calculated using the formula:

[1] Metalyse®. The State Register of Medicines of the Russian Federation. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=123f0609-001e-4e9c-8830-34e99ca499df

$$T = \frac{1}{P_{max}},$$

where P_{max} — the maximum dilution at which the optical density value in positive wells exceeds the optical density value in negative wells by a factor of three.

Clinical study of the neutralizing activity of plasma in patients after administration of recombinant non-immunogenic staphylokinase

The neutralizing activity was determined in accordance with the method of D. Collen [13]. Dilutions of the test samples were prepared similarly to the previous experiment. 5 µl of the test sample and 5 µl of the drug solution in the concentration range of 0.2–100 µg/mL were added to microcentrifuge tubes, mixed and 10 µl of thrombin solution (5 NIH units/mL, pH=7.4) was added. The samples were incubated for 20 min at 37°C. The concentration range of recombinant non-immunogenic staphylokinase at which the moment of thrombus lysis and time were observed was determined. For each test sample, a graph of the dependence of thrombus lysis time (min) on the concentration of the drug (µg/mL) was plotted. Using the graphs, the neutralizing activity was determined — the concentration of the drug at which the thrombus lysis time was 20 min.

Statistical analysis

Statistical analysis was performed using R 4.2 (R Foundation, USA). Continuous variables are described as mean and standard deviation (M±SD) or median and quartiles (Me [Q1; Q3]). Categorical variables are represented by absolute and relative frequencies. The Mann-Whitney *U*-test was used to compare continuous variables, and the two-sided Fisher's exact test was used to compare categorical variables. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Preclinical studies

Study of the effect of recombinant non-immunogenic staphylokinase on the immediate-type hypersensitivity reaction (anaphylactic shock) in guinea pigs

It was found that after a double intraperitoneal administration to guinea pigs on the 14th day of sensitization with recombinant non-immunogenic staphylokinase at a dose of 1.33 mg/kg (10 times the highest daily therapeutic dose for humans), anaphylactic shock was not induced (Table 1). No changes in the behavior of animals — their general condition, indicators of vital functions compared with control animals receiving saline — were revealed (Weigle index is 0). Thus, recombinant non-immunogenic staphylokinase did not cause anaphylactic shock in guinea pigs.

Study of the effect of recombinant non-immunogenic staphylokinase on the delayed-type hypersensitivity reaction in guinea pigs

After sensitization of guinea pigs with recombinant non-immunogenic staphylokinase for 25 days of intradermal injection of the drug, no cases of hyperemia, signs of edema or inflammation were found in any of the animals in the experimental groups (0 points on the S.V. Suvorov scale). The behavior and condition of the animals corresponded to those of the control group.

Study of the effect of recombinant non-immunogenic staphylokinase on the cell content of the popliteal lymph node in mice

7 days after the administration of recombinant non-immunogenic staphylokinase into the pad of the left hind paw of a mouse at a 10-fold higher daily dose recommended for humans, the cell content of the right and left popliteal lymph nodes was determined in comparison with the control. The calculation results are presented in Table 2. No significant differences were found.

The cell count index of the popliteal lymph nodes of the "experimental" and "control" paws was 0.91[2]. Thus, recombinant non-immunogenic staphylokinase did not affect the cell count of popliteal lymph nodes and did not have allergenic properties.

Study of the effect of recombinant non-immunogenic staphylokinase on the number of nucleated and antibody-forming cells in the spleen of mice

The drug was administered to mice 1 day before, on the day of RE administration, or on the day RE immunization in doses 10 and 20 times higher than the highest daily dose for humans. The results of the study are presented in Table 3.

The data presented in Table 3 show that a single intraperitoneal administration of recombinant non-immunogenic staphylokinase to mice did not affect the cell count of the spleen.

[2] The index of 1.00 indicates the equality of the cellular parameters of the right and left lymph nodes. The smaller it is, the greater the difference.

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From the results of counting the number of AFCs in the spleen of mice on day 5 after immunization, presented in Table 4, it is clear that there were no significant differences in the number of AFCs between the groups of animals that received recombinant non-immunogenic staphylokinase in various doses at different times relative to RE immunization and the control animals.

Thus, the drug did not affect the number of nucleated cells and AFCs in the spleen of mice immunized with RE, and, therefore, did not affect the cellular and humoral immune response.

Study of the effect of recombinant non-immunogenic staphylokinase on the delayed-type hypersensitivity reaction in mice

After immunization of mice with RE and recombinant non-immunogenic staphylokinase, a permissive injection of RE was administered into the left hind paw, and physiological saline was administered into the pad of the contralateral paw. The reaction index of animals in different groups is presented in Table 5.

Analysis of the data presented in Table 5 indicates that the drug, at the tested doses of 1.33 and 2.66 mg/kg and sensitization regimens, did not affect the formation of cellular immunity.

Thus, during preclinical studies of the allergenic and immunotoxic properties of recombinant non-immunogenic staphylokinase, it was found that the drug, when administered to guinea pigs and mice at doses 5, 10, and 20 times higher than the therapeutic doses for humans, does not affect cellular and humoral immunity. A complete set of preclinical studies of recombinant non-immunogenic staphylokinase, including the assessment of acute and subacute (subchronic) toxicity, mutagenicity, genotoxicity, embryotoxicity, reprotoxicity, and teratogenicity, also demonstrated its safety and absence of toxic properties [15], which allowed obtaining permission for clinical trials of the drug as a fibrinolytic agent in patients with STEMI.

Clinical studies

Clinical study of the effect of recombinant non-immunogenic staphylokinase on the antibodies formation and neutralizing activity of patient plasma

The study included 100 patients with STEMI who received a single bolus dose of 15 mg of recombinant non-immunogenic staphylokinase. Demographic, anthropometric, medical history data, clinical characteristics, and time intervals are presented in Table 6.

76% of the study participants were male. The average age was 58.9±9.9 years. The proportion of patients with arterial hypertension was 75%, previous myocardial infarction — 12%, lipid metabolism disorders — 86%. Inferior myocardial infarctions predominated in the study population (56%).

Assessment of the effectiveness and safety of thrombolytic therapy is presented in Table 7.

ST segment reduction by 50% from baseline after 90 min was observed in 80% of patients. Restoration of coronary blood flow according to TIMI 2+TIMI 3 criteria was observed in 70%. All-cause mortality was 3%. No intracranial hemorrhages were reported with the use of recombinant non-immunogenic staphylokinase. No major bleeding or hemorrhagic stroke was observed in any patient with cardiogenic shock in the groups. No allergic reactions were reported as a result of the study.

In a study of specific antibody titers in patients with STEMI who were administered recombinant non-immunogenic staphylokinase, it was found that 70 (70%) patients had no detectable specific antibodies. In 30 (30%) patients, specific antibodies were detected with a low titer — in the range of 1/100–1/800.

When determining the neutralizing activity of blood plasma in patients with STEMI who were administered recombinant non-immunogenic staphylokinase, it was found that samples from 70 (70%) patients did not have neutralizing activity. Samples from the remaining 30 (30%) patients were characterized by neutralizing activity at a dose of 0.33±0.02 μg/mL. It has previously been shown that the average values of neutralizing activity of native staphylokinase were many times higher, in the range of 9–93 μg/mL [14–16].

Thus, the average value of the neutralizing activity of blood plasma of patients after administration of recombinant non-immunogenic staphylokinase is 30–310 times lower than after administration of native staphylokinase.

Considering that the determined concentration of recombinant non-immunogenic staphylokinase in the blood with a single bolus administration at a dose of 15 mg is 2.5 μg/mL, which is 7.8 times higher than the neutralizing activity of blood plasma samples (0.33 μg/mL), observed only in 30% of patients, it can be concluded that the administration of the drug does not lead to the formation of antibodies capable of neutralizing its effect upon repeated administration.

Table 1 – Effect of recombinant non-immunogenic staphylokinase on the immediate-type hypersensitivity reaction in guinea pigs

Animal groups, doses	Weigle, M±SD	*p*
Control	0.0±0.00	–
Group 1, 0.665 mg/kg	0.0±0.00	1.00
Group 2, 1.33 mg/kg	0.0±0.00	1.00

Table 2 – Effect of recombinant non-immunogenic staphylokinase on the cell count of popliteal lymph nodes in $F_1(CBAxC_{57}Bl_6)$ hybrid mice

Animal groups, doses	Cell count of popliteal lymph nodes, million/mL, M±SD	*p*
Control	0.78±0.07	–
Group 1, 1.33 mg/kg	0.71±0.07	0.95

Table 3 – Effect of recombinant non-immunogenic staphylokinase on the cell count of the spleen of $F_1(CBAxC_{57}Bl_6)$ mice immunized with ram erythrocytes

Animal groups, doses	Number of karyocytes, 10^7/spleen, M±SD	*p*
Control	26.88±1.67	–
Group 1, 1.33 mg/kg «day -1»	25.64±0.93	0.89
Group 2, 2.66 mg/kg «day -1»	26.62±2.77	0.98
Group 3, 1.33 mg/kg «day 0»	28.44±1.84	0.91
Group 4, 2.66 mg/kg «day 0»	24.48±2.96	0.82
Group 5, 1.33 mg/kg «day +1»	27.71±1.45	0.85
Group 6, 2.66 mg/kg «day +1»	28.48±1.81	0.95

Note: Animals in groups 1 and 2 received recombinant non-immunogenic staphylokinase intraperitoneally at doses of 1.33 and 2.66 mg/kg, respectively, one day before ram erythrocytes immunization ("day -1"), groups 3 and 4 — at the same doses 1 hour after immunization ("day 0"), groups 5 and 6 — 24 hours after ram erythrocytes immunization ("day +1").

Table 4 – Effect of recombinant non-immunogenic staphylokinase on the number of antibody-forming cells in $F_1(CBAxC_{57}Bl_6)$ mice immunized with ram erythrocytes

Animal groups, doses	Number of antibody-forming cells in the spleen, 1×104, M±SD	*p*
Control	8.66±1.73	–
Group 1, 1.33 mg/kg «day -1»	10.29±1.32	0.72
Group 2, 2.66 mg/kg «day -1»	8.65±2.22	0.95
Group 3, 1.33 mg/kg «day 0»	9.39±1.41	0.80
Group 4, 2.66 mg/kg «day 0»	9.03±1.14	0.82
Group 5, 1.33 mg/kg «day +1»	9.96±1.95	0.78
Group 6, 2.66 mg/kg «day +1»	10.12±0.68	0.75

Note: Animals in groups 1 and 2 received recombinant non-immunogenic staphylokinase intraperitoneally at doses of 1.33 and 2.66 mg/kg, respectively, one day before ram erythrocytes immunization ("day -1"), groups 3 and 4 — at the same doses 1 hour after immunization ("day 0"), groups 5 and 6 — 24 hours after ram erythrocytes immunization ("day +1").

Table 5 – Effect of recombinant non-immunogenic staphylokinase on the development of delayed-type hypersensitivity reaction in $F_1(CBAxC_{57}Bl_6)$ mice

Animal groups, doses	Reaction index, M±SD	*p*
Control	19.81±2.43	–
Group 1, 1.33 mg/kg «day -1»	17.96±2.64	0.75
Group 2, 2.66 mg/kg «day -1»	19.91±2.59	0.96
Group 3, 1.33 mg/kg «day 0»	24.52±3.85	0.12
Group 4, 2.66 mg/kg «day 0»	23.48±2.77	0.35
Group 5, 1.33 mg/kg «day +1»	22.16±1.62	0.59
Group 6, 2.66 mg/kg «day +1»	20.46±0.94	0.79

Note: Animals in groups 1 and 2 received recombinant non-immunogenic staphylokinase intraperitoneally at doses of 1.33 and 2.66 mg/kg, respectively, one day before ram erythrocytes immunization ("day -1"), groups 3 and 4 — at the same doses 1 hour after immunization ("day 0"), groups 5 and 6 — 24 hours after ram erythrocytes immunization ("day +1").

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Table 6 – Baseline characteristics of patients

Characteristics	Indicator (*n*=100)
Gender, male / female	76/23 (76%/23%)
Age, years	58.9±9.9
Patients older than 75 years	6 (6%)
Weight, kg	83.8±14.2
Body mass index, kg/m^2	28.5±4.5
Myocardial infarction	12 (12%)
Arterial hypertension	75 (75%)
Diabetes mellitus Type II	14 (14%)
Dyslipidemia	86 (86%)
Smoking	39 (39%)
STEMI, mm	3.58±1.96
SBP, mm Hg .	118.6±8.2
DBP, mm Hg	74.7±7.2
HR, bpm	75.9±14.7
STEMI localization:	
anterior	42 (42%)
inferior	56 (56%)
other	2 (2%)
Type of heart failure according to Killip:	
I	87 (87%)
II	8 (8%)
III	3 (3%)
IV	2 (2%)

Table 7 – Assessment of the effectiveness and safety of thrombolytic therapy

Criterion	Indicator (*n*=100), abs. (%)
ST segment reduction by 50% after 90 min	80 (80%)
Restoration of coronary blood flow according to TIMI criteria:	
0	24 (24%)
1	6 (6%)
2	32 (32%)
3	38 (38%)
2+3	70 (70%)
Death from all causes	3 (3%)
Cardiogenic shock	4 (4%)
Recurrent myocardial infarction	4 (4%)
Major bleeding	1 (1%)
Intracranial hemorrhage	0 (0%)
Minor bleeding	3 (3%)
Allergic reactions	0 (0%)

DISCUSSION

In accordance with the Rules of Good Clinical Practice approved by the Eurasian Economic Commission, the assessment of allergenic, immunotoxic, and immunogenic properties of original molecules is one of the most important stages of research necessary for further clinical trials and registration of a medicinal product. Immunogenicity can provoke such serious side effects from the immune system as anaphylactic shock and Quincke's edema. According to the Clinical Guidelines of the Ministry of Health of Russia "Anaphylactic Shock" (2020)[3], anaphylactic shock is understood as "acute circulatory failure, manifested by a decrease in systolic blood pressure below 90 mm Hg. or by 30% from the working level and leading to hypoxia of vital organs." It should be emphasized that such a

[3] Clinical Guidelines of the Russian Ministry of Health «Anaphylactic shock» (2020). Available from: https://cr.minzdrav.gov.ru/view-cr/263_2

diagnosis without pronounced hemodynamic disorders is illegal and should be considered as anaphylaxis. Anaphylactic shock refers to a type I hypersensitivity reaction that occurs with the participation of class E immunoglobulins fixed on the surface of basophil and mast cell membranes[4]. Anaphylactic shock should be distinguished from angioedema (Quincke's edema) — a localized transient acute edema of the skin or mucous membranes, as well as urticaria — a group of diseases characterized by the development of itchy blisters or angioedema[5].

This article presents the results of preclinical and clinical studies of the allergenic, immunotoxic, and immunogenic properties of the recombinant non-immunogenic staphylokinase molecule. During a set of preclinical studies, it was found that the drug does not affect the cellular and humoral immune response in guinea pigs and mice at doses 5, 10, and 20 times higher than therapeutic doses for humans. Recombinant non-immunogenic staphylokinase does not cause anaphylactic shock and the development of edema in experimental animals with intravenous, intramuscular, and subcutaneous administration. In experiments on inhalation administration of recombinant non-immunogenic staphylokinase to mice with acute respiratory distress syndrome [17], it was found that the drug also did not cause allergic reactions and did not have an irritating effect on the respiratory tract, reduced the deposition of fibrinogen in the lungs and had a normalizing effect on the concentration of pro-inflammatory cytokines — IL-1α, IL-17A, IL-6.

A clinical study of the neutralizing activity of blood plasma of patients who were injected with recombinant non-immunogenic staphylokinase showed that samples taken from 70% of patients with STEMI do not have neutralizing activity against the drug. In the remaining 30% of patients, the average values of neutralizing activity of blood plasma after a single administration of the drug did not exceed 0.3 μg/mL, which is 30–310 times lower than after the administration of the native staphylokinase molecule. These values are significantly lower than the concentration of the drug in the blood, so they are not able to neutralize its effect in case of repeated administration.

In 2012, based on the results of preclinical and clinical studies, the Ministry of Health of Russia registered the original thrombolytic drug of recombinant protein containing the amino acid sequence of staphylokinase — Fortelyzin® (produced by SuperGene LLC, Russia; registration certificate No. LP-001941 dated December 18, 2012) for the treatment of patients with STEMI.

According to the safety and efficacy monitoring registers of recombinant non-immunogenic staphylokinase, since 2012, the drug has been used in more than 50 thousand patients with STEMI [18] and in more than 20 thousand with ischemic stroke. According to IMS Health as of March 2025, the recombinant non-immunogenic staphylokinase drug has been used in more than 200 thousand patients. During this period, the automated information system of Roszdravnadzor registered 18 (0.009%) reports of the development of anaphylactic shock after using the drug. This is reflected in the "Side effect"[6] section in the instructions for medical use of the drug, which indicates that such disorders of the immune system as anaphylactoid reactions are very rare (<1 in 10 thousand cases). Thus, the absence of immunogenic properties of recombinant non-immunogenic staphylokinase and its high safety with respect to the immune system are confirmed by many years of experience in use in a wide range of patients.

In clinical studies of recombinant non-immunogenic staphylokinase, no serious adverse events such as anaphylactic shock, urticaria, or Quincke's edema were reported.

In the FRIDOM1 trials [10, 11], recombinant non-immunogenic staphylokinase at a dose of 15 mg was no less effective than tenecteplase in terms of restoring coronary blood flow according to coronary angiography (70 *vs* 71%, p=0.76) and ECG (8% *vs* 80%, p=0.81); the absence of intracranial hemorrhages and the high efficacy of recombinant non-immunogenic staphylokinase allowed it to be registered for the treatment of patients with STEMI. Currently, recombinant non-immunogenic staphylokinase is included in the Clinical Guidelines and standards of treatment for patients with STEMI. The Eurasian Clinical Guidelines for the diagnosis and treatment of acute coronary syndrome with ST-segment elevation specifically mention the absence of antigenicity as an advantage of the drug. By Order of the Ministry of Health of Russia No. 1165n dated October 28, 2020, a recombinant protein containing

[4] Ibid.
[5] Ibid.
[6] Fortelizin®. The State Register of Medicines of the Russian Federation. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=9721d84a-5efa-4a24-b940-d711798bd51c

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the amino acid sequence of staphylokinase is included in the mandatory requirements for the equipment of medicines and medical devices for emergency medical care kits and sets.

According to the REGION-IM register of the National Medical Research Center of Cardiology named after Academician E.I. Chazov, 36% of patients with STEMI receive thrombolytic therapy using recombinant non-immunogenic staphylokinase, and at the prehospital stage, this figure reaches 42%. The frequency of using recombinant non-immunogenic staphylokinase in primary vascular departments reaches 51% [19].

The FRIDA study showed that recombinant non-immunogenic staphylokinase is an effective and safe thrombolytic agent for treating patients with ischemic stroke within 4.5 h of the onset of the first symptoms [20, 21]. In terms of the number of patients with good functional recovery (0–1 points on the modified Rankin scale), the drug was at least as effective as alteplase (50 *vs* 41%, p=0.10). Recombinant non-immunogenic staphylokinase is used as a rapid single bolus (10 seconds) at a single dose of 10 mg in patients with ischemic stroke of any body weight. In 2024, the recombinant non-immunogenic staphylokinase drug was included in the Clinical Guidelines for the treatment of ischemic stroke in the first 4.5 hours from the onset of the disease.

The FORPE trials presents the results of using recombinant non-immunogenic staphylokinase in patients with massive PE [22, 23]. Recombinant staphylokinase is not inferior to alteplase in terms of the primary efficacy endpoint “all-cause mortality within 7 days” (2 *vs* 3%, p=1.00) and has a high safety profile. The use of recombinant non-immunogenic staphylokinase was not accompanied by the development of major bleeding, which occurred with the use of alteplase (0 *vs* 3%, p=0.06) and hemorrhagic stroke (0 *vs* 2%, p=0.25). According to CTPA with contrast enhancement of the pulmonary arteries, a significant decrease in thrombotic masses (65.8 *vs* 47.4%, p <0.001) and a reduction in the size of the right ventricle (50 *vs* 39 mm, p <0.001) were shown 24 hours after thrombolysis with recombinant non-immunogenic staphylokinase. Currently, a double-blind placebo-controlled clinical trial of recombinant non-immunogenic staphylokinase in patients with intermediate-high risk PE has begun (permission of the Ministry of Health of Russia No. 106 от 21.03.2024 г., clinicaltrials.gov No. NCT06362746) [24].

A clinical study of recombinant non-immunogenic staphylokinase is being conducted with its intra-arterial intrathrombal administration in patients with thrombosis of the arteries of the lower extremities in comparison with surgical methods of treatment FORAT (researcher-coordinator — Academician I.I. Zatevakhin, permission of the Ministry of Health of Russia No. 184 dated March 18, 2022, clinicaltrials.gov No. NCT05372718) [25].

Along with the Russian Federation, the recombinant non-immunogenic staphylokinase drug is registered in a number of CIS countries (Tajikistan, Turkmenistan) and is currently undergoing registration in the EAEU countries, Azerbaijan, Georgia, and Uzbekistan.

Study limitations

In the presented clinical study, the recombinant non-immunogenic staphylokinase drug was administered in accordance with the instructions for medical use as a single bolus. Studies after bolus-infusion administration of the drug were not conducted. Studies of the neutralizing activity of blood plasma and titers of specific antibodies after repeated use of the drug were also not conducted. Clinical experience with its double use indicates the preservation of efficacy and the absence of neutralizing activity of blood plasma, which was shown in a patient with massive pulmonary embolism against the background of a shrapnel wound [26].

CONCLUSION

Given the annual increase in the number of thrombolysis procedures, the risks of adverse events should be taken into account. In this regard, a comprehensive study of the allergenic and immunotoxic properties of the recombinant non-immunogenic staphylokinase molecule in preclinical and clinical studies is extremely relevant. Based on the results of a full range of tests, it was convincingly proven that recombinant non-immunogenic staphylokinase does not have allergenic, immunogenic, and immunotoxic properties. The data obtained during these studies will undoubtedly contribute to a more active spread of thrombolytic therapy using non-immunogenic staphylokinase, which in the future will improve the quality of medical care for the population.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Sergey S. Markin — formulation of design, editing and final approval of the article; Sergei V. Ivanov — writing the article; Igor P. Beletsky — formulation of design, administration; Marina V. Zakharova — conducting a research, statistical data processing; Eduard A. Ponomarev — conducting a research; Evgenii V. Arzamascev — conducting a research, statistical data processing. All authors confirm that their authorship meets the international ICMJE criteria (all authors have made significant contributions to the development of the concept, research and preparation of the article, read and approved the final version before publication).

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Rationing of iodine content in kelp layers and products based on them. Changing approaches within the framework of a risk-based strategy in drug quality control

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The State Pharmacopoeia of the Russian Federation regulates the lower level of iodine content in Laminaria thalli. However, an excess of iodine is as harmful to the human body as its deficiency.

The aim. To determine the range of permissible iodine content in the medicinal plant raw material “Laminaria thalli” and products based on it within the framework of a risk-oriented strategy in medicine quality control.

Materials and methods. Samples of medicinal herbal preparations, biologically active additives, food products based on Laminaria thalli of various origins, samples of algae collected by the authors on the coast of the White Sea and the Pacific Ocean, as well as literature data on the iodine content in pharmacopoeial species were studied. The iodine content was determined by inductively coupled plasma mass spectrometry after extraction according to GOST EN 15111-2015. The non-carcinogenic risk was calculated in accordance with Guidance R 2.1.10.1920-04.

Results. The average (0.14%) and maximum (0.46%) iodine content in pharmacopoeial species of Laminaria thalli was determined, which correlates with the iodine content norm in Laminaria algae proposed by the U.S. Food and Drug Administration (FDA) — 0.1–0.5%. It was found that at the maximum therapeutic dose and course of treatment with laxative herbal preparations, containing 0.5% of iodine, the level of non-carcinogenic risk falls into the category of maximum permissible. Under similar conditions, treatment, for example, of mastopathy with preparations based on Laminaria thalli with 0.5% of iodine leads to an unacceptable impact of iodine on human health.

Conclusion. The authors recommend, instead of the existing norm of iodine content (not less than 0.1%), to take into account the permissible amount of this element (0.1–0.5%), which corresponds to its real content in pharmacopoeial species of Laminaria thalli.

Keywords: iodine; Laminaria thalli; hazard quotient; risk-oriented strategy; hyperthyroidism; quantitative content

Abbreviations: BAA — biologically active additive; FDA — U.S. Food and Drug Administration; CVD — cardiovascular diseases; MHP — medicinal herbal preparation; MPR — medicinal plant raw material; EPh — European Pharmacopoeia; FCC — Food Chemicals Codex; TMAH — tetramethylammonium hydroxide; HQ — hazard quotient.

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Нормирование содержания йода в слоевищах ламинарии и продуктах на их основе: изменение подходов в рамках риск-ориентированной стратегии в контроле качества лекарственных средств

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В государственной фармакопее Российской Федерации нормируется нижний уровень содержания йода в слоевищах ламинарии. Однако переизбыток йода так же вреден для организма человека, как и его дефицит.

Цель. Определить диапазон допустимого содержания йода в лекарственном растительном сырье «Ламинарии слоевища» и продуктах на его основе в рамках риск-ориентированной стратегии в контроле качества лекарственных средств.

Материалы и методы. Были исследованы образцы лекарственных растительных препаратов, биологически активных добавок, пищевой продукции на основе слоевищ ламинарии различного происхождения, образцы водорослей, собранные авторами на побережье Белого моря и Тихого океана, а также литературные данные о содержании йода в фармакопейных видах. Содержание йода определяли методом масс-спектрометрии с индуктивно-связанной плазмой после экстракции по ГОСТ EN 15111-2015. Расчёт неканцерогенного риска проводили в соответствии с Руководством Р 2.1.10.1920-04.

Результаты. Определено среднее (0,14%) и максимальное (0,46%) содержание йода в фармакопейных видах слоевищ ламинарии, которое соотносится нормой содержания йода в ламинариевых водорослях, предложенных Управлением по санитарному надзору за качеством пищевых продуктов и медикаментов США (U.S. Food and Drug Administration) — 0,1–0,5%. Установлено, что при максимальной терапевтической дозе и курсе лечения слабительными фитопрепаратами, при условии содержания йода в них 0,5%, уровень неканцерогенного риска попадает в категорию предельно допустимого. При аналогичных условиях лечение, например, мастопатии препаратами на основе слоевищ ламинарии с содержанием йода 0,5% приводит к недопустимому воздействию йода на здоровье человека.

Заключение. Авторы рекомендуют вместо существующей нормы содержания йода (не менее 0,1%), принимать во внимание допустимое количество этого элемента (0,1–0,5%), которое соответствует его реальному содержанию в фармакопейных видах слоевищ ламинарии.

Ключевые слова: йод; слоевища ламинарии; коэффициент опасности; риск-ориентированная стратегия; гипертиреоз; количественное содержание

Список сокращений: БАД — биологически активная добавка; FDA — Управление по санитарному надзору за качеством пищевых продуктов и медикаментов США (U.S. Food and Drug Administration); ССЗ — сердечно-сосудистые заболевания; ЛРП — лекарственный растительный препарат; ЛРС — лекарственное растительное сырье; EPh — Европейская фармакопея; FCC — Кодекс пищевых химикатов; ЛС — лекарственное средство; ТМАН — тетраметиламмония гидроксид; HQ — коэффициент опасности.

INTRODUCTION

The implementation of salt iodization programs around the world has reduced the incidence of iodine deficiency-related diseases. However, 30% of the world's population is still at risk[1]. Iodine deficiency in the diet is a growing problem in many countries, including industrialized ones, partly due to changes in eating patterns and food production methods [1, 2].

Iodine intake is extremely important for the functioning of the human body, since the production of thyroid hormones directly depends on the amount of this element. Thyroid hormones are acutely necessary for brain development during intrauterine development, as well as during the first years of life [3]. A deficiency of this element has an adverse effect on

[1] American Thyroid Association Iodine Deficiency. Available from: https://www.thyroid.org/iodinedeficiency/

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the development of mental and physical retardation in children [4], and is also the most common preventable cause of brain damage and the development of neurological diseases [5].

Iodine deficiency in the areas of Ukraine, Belarus and Russia adjacent to the Chernobyl nuclear power station became a factor in the increased uptake of radioactive iodine by the thyroid gland and, after a few years, led to a multiple increase in the incidence of thyroid cancer not only in adults, but also in children [6]. Optimal iodine intake dramatically reduces the risk of thyroid lesions. In Japan, where there is no iodine deficiency (mainly due to the unique dietary characteristics of the population, in particular the active consumption of brown algae), after the accident at the Fukushima nuclear power station, there was no significant increase in the incidence of thyroid cancer in children, even without emergency iodine prophylaxis immediately after the accident [7]. Efforts to prevent and control these diseases are primarily aimed to ensure iodine intake to maintain normal thyroid function [8] (90 μg/day for children, 150 μg/day for people of both sexes over 12 years of age, and 250 μg/day for pregnant and lactating women)[2, 3]. Adequate iodine intake can be achieved by fortifying food with iodine and/or iodine-containing supplements, such as iodates and iodides [9, 10], added as a potassium salt. It should be noted that iodate is more stable in adverse climatic conditions and at elevated temperatures (in particular, during heat treatment of food).

One of the main ways to iodize food is to enrich table salt with iodine additives. The amount of iodine additive is 20–60 mg/kg[4], which, with the norm of table salt consumption (5.0 g/day), is 100–300 μg/day. However, due to the identified relationship between high sodium content and cardiovascular diseases (CVD) [11, 12], hypertension [12–14], urolithiasis and osteoporosis [13], there has recently been a tendency to reduce salt consumption [14, 15]. Replacing table salt with an alternative mixture, according to the latest research, has confirmed the value of a low-salt diet in the prevention of CVD [11, 14]. The best products for the prevention of iodine deficiency are those that are natural sources of iodine: seafood (fish, brown algae, crustaceans), beans, garlic, beets [10]. Brown algae containing iodine in large quantities, are actively used as medicinal herbal products (MHPs) and dietary supplements (DSs) [16–18], in particular, the U.S. Food and Drug Administration approved the use of dietary supplements based on algae of the Laminaria family as a source of this element[5]. These plants contain a large number of useful components (polysaccharides, including alginic acid salts [19], vitamins, polyunsaturated fatty acids and antioxidants, a wide range of essential elements, and most importantly iodine) [20–22]. The concentration of iodine in brown algae exceeds its content in all other living organisms [23], therefore they are a good natural source of iodine for humans [24, 25].

In the State Register of Medicines of the Russian Federation, algae of the *Laminariaciae* family (*Laminaria saccharina, Laminaria japonica*) are listed as such plants, so the State Pharmacopoeia of the Russian Federation XIV edition regulates the lower limit of iodine content (at least 0.1%)[6] in pharmacopeial types of *Laminaria thalli*. The European Pharmacopoeia (EPh) regulates the use of representatives of the Fucus family as medicinal plant raw materials (MPRMs). The U.S. Food Chemical Codex (FCC) recommends the use of representatives of a wide range of algae of the Laminaria family. Data on the types of brown algae used, as well as standards for iodine content, are shown in Table 1.

However, studies show that an excess of iodine is also harmful to the human body, as is its deficiency [26–28]. High iodine intake (1–10 mg/day) when taking MHPs or dietary supplements based on brown algae leads to an increased risk of endemic goiter, which in some cases is accompanied by hyperthyroidism or myxedema [29], and also stimulates autoimmune diseases [30, 31]. In this regard, EPh and FDA provide a range of iodine content in brown algae, and not its lower level (see Table 1).

THE AIM. To determine the range of permissible iodine content in medicinal plant raw materials “Laminaria thalli” and products based on it within the

[2] WHO/NUT/96.13. Recommended iodine levels in salt and guidelines for monitoring their adequacy and effectiveness. Geneva, World Health Organization; 1996. Available from: https://www.who.int/publications/i/item/WHO-NUT-96.13

[3] WHO Secretariat; Andersson M., de Benoist B., Delange F., Zupan J. Prevention and control of iodine deficiency in pregnant and lactating women and in children less than 2-years-old: conclusions and recommendations of the Technical Consultation. Public Health Nutr. 2007;10(12A):1606–1611. DOI: 10.1017/S1368980007361004. Erratum in: Public Health Nutr. 2008;11(3):327.

[4] GOST R 51575-2000 Iodized table salt. Methods for the determination of iodine and sodium thiosulfate.

[5] Office of the Federal Register, National Archives and Records Administration. Food additives permitted for direct addition to food for human consumption: subpart C—coatings, films and related substances – kelp, 21 CFR Sect 172.365. Washington (DC): US Government Printing Office; 2015. Available from: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-172

[6] M.5.0080.18 Laminaria of the stratum. The State Pharmacopoeia of the Russian Federation XIV edition. Available from: https://docs.rucml.ru/ feml/pharma/v14/vol4/999/

framework of a risk-oriented strategy in quality control of medicines. Previously, a risk-oriented approach was mainly used in relation to food contaminants (which include dietary supplements[7, 8]) and environmental objects[9, 10]. Currently, a trend has emerged to assess the carcinogenic and non-carcinogenic risks of contaminants in medicines for a long-time intake: synthetic painkillers [32], MPRMs and MHPs with various pharmacological effects [33, 34].

MATERIALS AND METHODS

Study methodology

The objects of the study were laxative herbal medicines of Russian manufacturing: "*Laminaria thalli* (sea kale)" produced by JSC "Krasnogorskleksredstva" (I-p), LLC "FITO-BOT" (II-p) and CJSC "ST-Medipharm" (III-p); preparations for the treatment of mastopathy "Mammoklam" produced by CJSC MEGA PHARM (IV-p), "Mammolain" produced by CJSC MEGA PHARM (V-p), samples of dietary supplements: "*Laminaria* (sea kale)" produced by CJSC Evalar, Russia (I-b), and "Laminaria SUPERFOOD" produced by LLC "Kron", Russia (II-b), "*Laminaria* for the thyroid gland" LLC PharmOcean Lab. (TM "Doctor More"), Russia (III-b), "Kelp", NOW Internetional, USA (IV-b), "Nalemarin" LLP Biomar, Russia (V-b), food products: Sea kale Kombu Fresh Sakhalin natural (*Laminaria japonica*), Russia (I-e) Arkhangelsk Algae Combine SUPERFOOD AV1918 (*Laminaria Saccharina*), Russia (II-e), as well as samples collected by the authors: thalli of Japanese laminaria (*Laminaria japonica* (L.)) (I-a), collected in the waters of Peter the Great Bay of the Pacific Ocean, and sugar laminaria (*Laminaria Saccharina* (L.)) (II-a), collected in the area of the Bolshoi Solovetsky Island. Self-collected samples were obtained in August 2020, dried in the sun for 2 days, the species identity of the samples was determined using macro- and microscopic analysis. In addition, we used literature data on the iodine content in the thalli of pharmacopoeial species of laminaria from various places of picking [21].

The iodine content in the test samples was determined by inductively coupled plasma mass spectrometry according to GOST EN 15111-2015[11]. Crushed laminaria algae thalli samples, as well as MHPs, were sieved through a sieve with a hole diameter of 1 mm. Then 3 samples of 500 mg each (accurate sample) were taken, followed by iodine extraction with an aqueous solution of tetramethylammonium hydroxide (25% Lot 331635, Sigma-Aldrich, USA, TMAH) according to GOST EN 15111-2015[12]. During the determining of the iodine content in DSs, 20 tablets were crushed, 3 samples of 500 mg each (accurate sample) were taken, then iodine extraction was carried out according to the GOST method. A sample of the test sample was placed in a flask with a capacity of 50 cm^3, 5 cm^3 of water was added and thoroughly mixed. Then 1 cm^3 of 0.5% TMAN solution was added, thoroughly mixed, the tightly closed flask was placed in a drying oven preheated to a temperature of 90±3°C for 3 h. After cooling, the samples were quantitatively transferred to a 25 cm^3 volumetric flask and brought to the mark with water. To remove coarse particles, aliquots were filtered through a membrane filter with a pore diameter of 5 µm. Then the solution of the internal standard of tellurium ions, prepared from a standard sample (SS; R2-TE691015 1000 µg/mL, Inorganic Ventures Lot, USA), was added to the aliquot of the sample extract. Calibration solutions (iodine concentration 5–20–50 µg/dm^3) were prepared by placing the appropriate volume of iodine SS (P2-IOD675953 1000 µg/mL, Inorganic Ventures Lot, USA) and internal standard (tellurium ion solution) in a 50 cm^3 volumetric flask, brought to the mark with 0.5% TMAH solution. A blank sample was prepared similarly to the calibration solution, without adding the SS solution. The iodine content in the test and calibration solutions was determined on an Agilent 7900 instrument (Agilent, USA), the parameters of the experiment are shown in Table 2.

Statistical processing

For each of the tested samples, the average value of measurements obtained from 3 parallel samples in 5 replicates was taken as the measurement result. The measurement results were statistically processed using Microsoft Office Excel 2007. The standard coefficient of variation, confidence interval, and systematic error were determined.

The calculation of non-carcinogenic risk was carried

[7] Technical Regulations of the Customs Union TR CU 021/2011 On Food Safety. Available from: https://docs.cntd.ru/document/902320560. Russian

[8] SanPiN 2.3.2.1078-01, Food raw materials and foodstuffs. Hygienic requirements for food safety and nutritional value; Ministry of Health of the Russian Federation, Moscow; 2002. Available from: https://docs.cntd.ru/document/901806306. Russian

[9] MU 2.3.7.2519-09 Determination of exposure and assessment of the risk of exposure to chemical food contaminants on the population. Methodological guidelines. Moscow: Federal Center of Hygiene and Epidemiology of Rospotrebnadzor; 2010. Russian

[10] R 2.1.10.1920-04 Guidelines for assessing the risk to public health when exposed to chemicals that pollute the environment. Moscow: Federal Center for State Sanitary and Epidemiological Supervision of the Ministry of Health of Russia; 2004. Russian

[11] GOST EN 15111-2015 Food products. Identification of trace elements. Method of iodine determination by inductively coupled plasma mass spectrometry (ICP-MS).

[12] Ibid.

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out according to R 2.1.10.1920-04. "Guidelines for assessing the risk to public health when exposed to chemicals that pollute the environment".

RESULTS AND DISCUSSION

The iodine content in laminaria thalli and products based on them, according to the recommendations for the implementation of analysis methods R 50.2.060–2008[13], the suitability of the GOST-approved method[14] was confirmed using standard sample of SRM 3530 Iodized Table Salt. The results are presented in Table 3.

The recovery rate was 103.1±5%, which corresponds to the requirements of the Eurasian Economic Union Pharmacopoeia for the correctness of analytical methods (recovery [R]=90–110%)[15], the precision (repeatability) of the method (RSD=4.31%) also meets pharmacopoeial requirements (RSD ≤5%)[16].

To determine the concentrations of iodine in the test samples, the calibration curve was used with prepared calibration solutions. The calibration curve, linear regression equation, correlation coefficient (R ≥0.99), detection limit (DL; DL ≥10DL), and background equivalent level (BEC) obtained using the instrument software (MassHunter 4.5) are shown in Figure 1. The calculated values of R^2 (0.9998) and DL (0.02 μg/L) confirmed the suitability of the method for determining the iodine content in samples[17].

At the first stage of the study was the applicability of the iodine content normalization ranges proposed in foreign regulatory documents (RD) to pharmacopoeial types of laminaria thalli (*Laminaria saccharina* L. and *Laminaria japonica* L.)[18], taking into account our own experimental and literature data on the concentration of the element in this herbal medicinal product (Table 4).

Table 4 demonstrates that the average (0.14%) and maximum (0.46%) iodine content in pharmacopoeial types of laminaria thalli correlates with the range of normalization values proposed by the FDA (0.1–0.5%). The lower level of permissible iodine content proposed by EPh (0.03–0.2%) is obviously due to the fact that fucus algae, which are a pharmacopoeial family in European countries, accumulate this element in smaller quantities compared to laminaria algae [42, 43].

At the next stage, the range of iodine content (0.1–0.5%) was evaluated from the point of view of the non-carcinogenic risk of its exposure during oral intake into the body along with the therapeutic dose of MHPs and dietary supplements based on laminaria thalli. Non-carcinogenic risk is understood as an indicator of the expected increase in the incidence of the population due to the toxic properties of chemical substances in the studied objects. When assessing non-carcinogenic risk, it is assumed that there is a threshold of harmful effect, below which toxic effects do not develop. The main quantitative indicator of non-carcinogenic risk is the hazard quotient (HQ), which is equal to the ratio of the average daily dose of consumption of the elemental impurity (ADD) to its safe (reference) exposure level[19, 20]:

$$HQ = \frac{ADD}{RfD},$$

where *RfD* is the reference dose of iodine (0.01 mg/kg[21]).

The value of ADD was calculated using the formula[22]:

$$ADD = \frac{C \times IR \times EF \times ED}{BW \times AT},$$

where C is the concentration of the studied elemental impurity in laminaria thalli, mg/kg; IR is the therapeutic dose of laminaria thalli, kg/day; EF is the frequency of exposure during the year, days; ED is the duration of exposure, years; BW is the average value of human body weight (70 kg[23]) AT is the averaging time of exposure, days.

Information on the values of IR, EF, ED was taken from the instructions for the preparations presented in the State Register of Medicines. The value of AT was equated to the expected human life expectancy (70 years)[24].

[13] R 50.2.060-2008. The state system of ensuring the uniformity of measurements. Implementation of standardized methods of quantitative chemical analysis in the laboratory. Confirmation of compliance with the established requirements (approved and put into effect by Order No. 320-st of the Federal Agency for Technical Regulation and Metrology dated November 25, 2008). Available from: https://docs.cntd.ru/document/1200069291

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Table 1 — Regulation of iodine content in brown algae in some regulatory documents

Regulatory document	Family	Types	Regulated iodine content
SP RF XIV ed.[25]	*Laminariaceae*	*Laminaria saccharina* L. *Laminaria japonica* Aresch.	Not less than 0.1%
EPh ed. 11.3[26]	*Fucaceae*	*Fucus vesiculosus* L. *Fucus serratus* L. *Ascophyllum nodosum* Le Jolis	0.03–0.2%
FCC ed. 9[27]	*Laminariaceae*	*Macrocystis pyrifera* L. *Laminaria digitata* Huds. *Laminaria cloustoni* Edm. *Laminaria saccharina* L.	0.1–0.5%

Note: SP RF — State Pharmacopoeia of the Russian Federation; EPh — European Pharmacopoeia; FCC — Food Chemical Codex.

Table 2 — HPLC analysis conditions

Parameter	Value
High-frequency plasma generator power	1500 W
Plasma gas flow (argon)	15 l/min
Nebulizer gas flow (argon)	1.0 l/min
Signal integration time	0,1 s
Determined isotope (iodine)	127 amu
Internal isotope	125 amu

Table 3 — Evaluation of the suitability of the analysis method

No.	Iodine content, % of nominal value	Metrology
1	107.98	Average value ($\bar{Z}i$) — 103.10%, Systematic error (δ) — 3.1%, Standard deviation (SD) — 4.44 %, Coefficient of variation (RSD) — 4.31%, Confidence interval (P=95%, α=0.05) is ±4,65%
2	102.32	
3	99.09	
4	105.50	
5	96.90	
6	106.82	

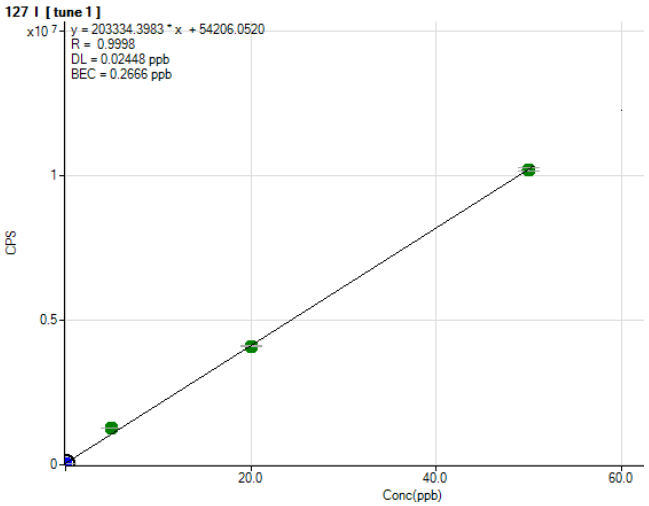

Figure 1 — Calibration curve, characterizing the suitability of the analytical method

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Table 4 — Iodine content in pharmacopoeial types of laminaria thalli

No.	Concentration, mg/kg	
	Laminaria japonica L.	*Laminaria saccharina* L.
	Literary data	
1.	2110 [35]	238 [35]
2.	3040 [36]	957 [38]
3.	3400 [37]	1340 [39]
4.	–	2630 [40]
5.	–	3124 [41]
6.	–	4600 [42]
	Experimental data (Median — 1791 mg/kg, max — 4600 mg/kg)	
1.	1216.74±31.05 (I-m)	716.98±16.94(II-s)*
2.	2390.57±27.22 (II-p)	292.53±10.91 (II-a)*
3.	1473.65±25.96 (III-p)	–
4.	899.30±61.73 (I-e)	–
5.	3700±108.9 (I-a)	–

Table 5 — Value of iodine hazard quotients during oral administration of therapeutic doses of herbal medicines based on laminaria thalli

Herbal medicines	Course of administration	IR, kg	EF, days	C_{med}, 3000 mg/kg		$C_{95\%}$, 5000 mg/kg	
				ADD, mg/kg×day	HQ	ADD, mg/kg×day	HQ
I-m, II-m, III-m	Minimum	0,0015	15	$2,6\times10^{-3}$	0,26	$4,4\times10^{-3}$	0,44
	Maximum	0,003	30	0,01	1,0	0,0176	1,76
IV-m, V-m	Minimum	0,0002	90	$2,1\times10^{-3}$	0,21	$3,5\times10^{-3}$	0,35
	Maximum	0,0006	270	0,02	2,0	0,0317	3,2

Note: the average concentration of the range 0.1–0.5% (0.3% or 3000 mg/kg) was taken as the value of Cmed, and the maximum concentration of the specified range (5000 mg/kg) was taken as C95%. IR — therapeutic dose of laminaria thalli, kg/day; EF — frequency of exposure during the year, days; ED — duration of exposure, years; AT — averaging time of exposure, days; HQ — hazard quotient.

Table 6 – Hazard quotient values for iodine in oral dietary supplements

Supplement	Course of administration	C, mg/kg	IR, kg	EF, days	ADD, mg/kg×day	HQ
I-s	Minimum	1000	0.0002	180	0.0014	0.14
	Maximum			360	0.003	0.28
II-s	Minimum	1000	0.0002	180	0.0014	0.14
	Maximum			360	0.003	0.28
III-s	Minimum	2000	0.0005	30	0.001	0.12
	Maximum					
IV-s	Minimum	100	0.0045	30	0.0005	0.05
	Maximum					
V-s	Minimum*	1200	0.001	30	0.0014	0.14
	Maximum*			60	0.003	0.28
	Minimum**	600	0.0005	30	0.0003	0.04
	Maximum**			60	0.0007	0.07

Notes: * course of administration for adults; ** course of administration for children. Supplement — dietary supplement; C — concentration of the studied elemental impurity in kelp thalli, mg/kg; IR — therapeutic dose of kelp thalli, kg/day; EF — exposure frequency during the year, days; AT — exposure averaging time, days; HQ — hazard quotient.

Table 7 – Iodine content in samples of dietary supplements I-s and II-s

No.	Iodine content, mg/kg	
	I-s	**II-s**
1	3021	3348
2	3094	3407
3	3189	3443
Average	3101 ± 209.1 (2.7)	3399 ± 119.1 (1.4)

The HQ value was calculated at two concentration levels (median and 95th percentile[28]. It should be noted that there are no criteria in Russian and foreign regulatory documents for assessing HQ values from the point of view of the admissibility of the negative impact of a single elemental toxicant. They are presented only for the total hazard index (HI), which is defined as the sum of the hazard quotients of all analyzed contaminants. It is generally accepted that at HI_{med} >1, there is an unacceptable impact of elemental contaminants on human health, requiring appropriate safety measures. The combination of HI_{med} <1 and $HI_{95\%}$ <1 indicate no risk to human health from the action of contaminants. In a situation where HI_{med} ≤1, but $HI_{95\%}$ >1, it is necessary to strengthen control over the content of contaminants with the greatest contribution to exposure. Taking into account the fact that the iodine content in laminaria thalli is significantly higher than the content of heavy metals and inorganic arsenic in them, these criteria were used to assessing HQ values for iodine.

In Russia, MHPs based on laminaria thalli are used to treat chronic atonic constipation (phytopreparation "*Laminaria thalli*, sea kale"), which is crushed and dried pieces of laminaria thalli from various manufacturers (I-m, II-m and III-m) with the same course of administration and dosage) and for the treatment of mastopathy (tablets "Mammoklam" (IV-m) and "Mammolain" (V-m) based on iodine-lipid complex from laminaria thalli with the same course of administration and dosage). In accordance with the instructions for use, the minimum iodine content in all these preparations is 0.1%, which is the same as in the original herbal medicinal product. The values of IR and EF were determined based on the method of administration and therapeutic doses of herbal medicinal products, and ED — by the difference from the average life expectancy (70 years)[29] and the age of starting the medicine:

- I-m, II-m and III-m: half or 1 teaspoon (or 1.5–3 g) for 15–30 days at the age of 12 years[30] (IR=0.0015–0.003 kg; EF=15–30 days, ED=58 years, AT=365×ED);
- IV-m and V-m: 2–6 tablets of 100 mg from 1 to 3 months with a break of 2 weeks to 3 months from 18 years[31] (IR=0.0002–0.0006 kg, EF=90–270 days, ED=52 years, AT=365×ED)

The results of the assessment of non-carcinogenic risk associated with the toxic effect of iodine during oral administration of therapeutic doses of various herbal medicines are presented in Table 5.

HQ was calculated for oral administration of dietary supplements based on laminaria thalli (Table 6). Data on the content, courses of administration (minimum and maximum) of iodine were taken from the information on the packaging or instructions for use of the supplement.

For supplements I-s and II-s, the actual iodine content in the samples was determined according to GOST EN 15111-2015[32]. The results of determining the iodine content in these samples are presented in Table 7.

As a result of the studies, it was found that the iodine content in supplement I-s was 3101±209 mg/kg, and for supplement II-s — 3399±119 mg/kg.

Table 4 demonstrates that with the minimum therapeutic dose and course of treatment of MHPs I-m, II-m and III-m, there is no risk of negative effects of iodine on the human body and control over its content is not required. At the maximum therapeutic dose and course of treatment, the risk level falls into the category of the maximum permissible, which entails the need to control the iodine content in this drug. The intake of MHPs IV-m and V-m, its minimum therapeutic dose and duration of administration are also not associated with a risk to human health. Taking the maximum therapeutic dose during such a course of treatment, as provided for in the instructions for use, leads to unacceptable exposure to iodine on human health. This requires appropriate security measures. As such a measure, we recommend reducing the maximum frequency of drug administration to 140 days per year. In this case, the HQ value at an iodine concentration of 0.5% in MHPs IV-m and V-m and a therapeutic dose of 6 tablets per day will not exceed 1. It is important to note that information on contraindications of taking medicines for the treatment of mastopathy associated with thyroid dysfunction is given in the instructions for these medicines. Both doctors and patients need to carefully assess the risks of using these medicines.

Separately, it is worth focusing on supplements based on kelp thalli. It should be noted that the market for supplements made from algae is developing with unprecedented dynamics [44–46] and they are increasingly being chosen as an easy way to enrich the daily diet with vitamins and minerals. At the same time, the consumer is often mistaken believing that supplements are controlled for the content of contaminants and active substances similarly to medicines due to the similarity of finished forms

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(tablets, capsules, drops, liquid or powder) and the general place of sale (pharmacy). Despite the fact that the norms for the iodine content in supplements based on kelp thalli are indicated on the packaging, state control of the concentration of this element during quality examination in finished products is not carried out, unlike LRP. This is due to the fact that supplements do not have a proven pharmacological effect and information about their exact composition is missing [47].

According to the data obtained (see Table 5), if supplements are taken in accordance with the manufacturer's recommendations, in no case will the HQ exceed 1, provided that their composition corresponds to that indicated on the packaging. It should be noted that the iodine content in tablets of supplements I-s and II-s on the packaging corresponds to its content in 0.1%, while the actual content of this element, determined according to GOST, is more than 3 times higher than the indicated value. The HQ for the real value with daily intake for 1 year is almost equal to one — the value after which the risk becomes unacceptable. It follows from this that the iodine content in supplements based on kelp thalli also needs to be controlled.

CONCLUSION

Medicines based on thalli kelp in Russia are used to treat diseases not directly related to iodine deficiency in the human body. Therefore, with high contents of this element in the initial raw materials, prolonged use of such drugs in the maximum permissible therapeutic doses leads to the risk of developing hyperthyroidism in such patients. Warnings about the possible appearance of hyperthyroidism in the instructions are not enough; it is necessary to change the principle of rationing the iodine content in the pharmacopoeial article "*Laminaria thalli* (sea kale)". It is recommended, to give the range of permissible content of this element (0.1–0.5%), instead of the existing iodine content norm (not less than 0.1%), which corresponds to its real content in pharmacopoeial types of kelp thalli. People with thyroid dysfunction should use supplements based on brown algae with caution due to the high variability of iodine content, as well as the possible difference between the real and theoretical content of this element in supplements.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Victor M. Shchukin — development of research aims and objectives, synthesis of data set out in regulatory documents, collection and processing of primary data, draft writing; Nataliya E. Kuzmina, Natalya D. Bunyatyan — the idea, planning, organization and control of research at stages, processing the results, draft editing; Elena A. Khorolskaya — information and analytical search on the research aim, sample preparation and experiment, draft editing. All the authors confirm their authorship compliance with the ICMJE international criteria (all authors made a significant contribution to the development of the concept, conducting research and preparing the article, read and approved the final version before publication).

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SARS-CoV-2 Main Protease inhibitors in trace constituents from Algerian herbal medicines using *in silico* approaches

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Since antiquity, essential oils are considered as a source of bioactive molecules. Some of them have been shown to possess antiviral activities against various virus strains, among them SARS-CoV-2.

The aim of this study is the search for compounds, among minor components extracted from different aromatic and medicinal plants collected from Algerian pharmacopeia, which may posses possible COVID-19 antiviral activities, by molecular docking in the active site of SARS-CoV-2 main protease.

Materials and methods. Thus, in this study 66 compounds which are declared at traces amount by authors in the composition of the essential oils, and selected from 9 Algerian medicinal plants were docked in the active site of SARS-CoV-2 main protease as possible inhibitors of SARS-CoV-2.

Results. The obtained result shows that only Cembrene constitutes the structure with the best affinity in the binding site of the enzyme with a Bioavailability Score "ABS" equal to 0.55 which confirm non Lipinski violations. However, the compound is predicted not orally bioavailable, because too lipophilic (lipophilicity: Log $P_{o/w}$ (XLOGP3)=6.04>+5.0) and less polar (polarity: TPSA=0.00Å²<20 Å²), and it is also predicted as not absorbed, not brain penetrant and not subject to active efflux from the CNS or to the gastrointestinal lumen.

Conclusion. This result deserves to be more detailed and either confirmed or invalidated with a view to better and rational exploitation.

Keywords: cembrene; pharmacokinetic; COVID-19; bioavailability score; Algerian medicine; molecular docking

Abbreviations: ABS — Abbot Bioavailability Score; ACE-2 — Angiotensin-Converting Enzyme 2; ADME — Absorption, Distribution, Metabolism, Excretion; ADMET — Absorption, Distribution, Metabolism, Excretion, Toxicity, Ala — Alanine; AMES — Assay of the ability of a chemical compound to induce mutations in DNA, Asn — Asparagine; BBB — Blood–Brain Barrier; Caco-2 — Colon Cancer Cell Line; CLogP — Octanol/Water Partition Coefficient; CLpro-3 — Enzyme 3-Chymotrypsin-Like protease; CNS — Central Nervous System; COVID-19 — Coronavirus Disease-19; CYP — Cytochrome; CYS — Cysteine; EOs — essential oils; Gln — Glutamine; Glu — Glutamic acid; Gly — Glycine; HB — Hemoglobin; hERG — human Ether-à-go-go-Related Gene; HIA — Human Intestinal Absorption; HIS — Histidine; HSV-1 — Herpes Simplex Virus type 1; Leu — Leucine; MDCK — Madin-Darby Canine Kidney; Met — Methionine; MlogP — Moriguchi logP; MW — Molecular Weight; MWT — Molecular Weight; OCT — Octanol; pdb code 6LU7 — Protein Data Bank (crystal structure of COVID-19 main protease); Phe — Phénylalanine; PkCSM — Predicting small-molecule pharmacokinetic and toxicity properties; PLpro — Papain Like protease; PGP — Permeability-GlycoProtein; Pi-sigma — sigma (σ) and pi (π) bonds; Pro — Protein; PSA — Polar Surface Area; RdRp — RNA-dependent RNA polymerase; QSAR — Quantitative Structure Activity Relationships , RNA — Ribonucleic Acid; SARS-CoV-2 — Severe Acute Respiratory Syndrome Coronavirus 2; Thr — Threonine; TPSA — Total Polar Surface Area; VDss — volume of distribution; WLOGP — Wildman-Crippen LogP (Water Partition Coefficient (logP)); XLOGP3 — Octanol-Water Partition Coefficient (logP).

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Определение ингибиторов основной протеазы SARS-CoV-2 в следовых количествах компонентов алжирских растительных лекарственных средств с использованием методов *in silico*

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С древних времен эфирные масла считались источником биологически активных соединений. Было доказано, что некоторые из них обладают противовирусной активностью в отношении различных штаммов вирусов, в том числе SARS-CoV-2.

Целью данного исследования стал поиск соединений среди второстепенных компонентов, выделенных из различных ароматических и лекарственных растений, которые могут обладать возможной противовирусной активностью против COVID-19 путем молекулярного докинга в активном центре основной протеазы SARS-CoV-2.

Материалы и методы. Авторами исследованы 66 соединений, содержащихся в следовых количествах в составе эфирных масел. Соединения получены из 9 лекарственных растений, произрастающих на территории Алжира. Исследуемые соединения были включены в активный центр основной протеазы SARS-CoV-2 в качестве возможных ингибиторов SARS-CoV-2.

Результаты. Полученные результаты показывают, что только чембрен представляет собой структуру с наилучшей аффинностью в сайте связывания фермента с показателем биодоступности, равным 0,55, что подтверждает отсутствие нарушений правила Липински. Однако прогнозируется, что соединение не будет обладать биодоступностью при пероральном приёме, в связи с избыточной липофильностью (липофильность: Log $P_{o/w}$ (XLOGP3)=6,04>+5,0) и низкой полярностью (полярность: TPSA=0.00Å²<20 Å²). Также следует отметить, что чембрен не всасывается, не проникает в мозг и не подвергается активному оттоку из ЦНС или в просвет ЖКТ.

Заключение. Представленные результаты заслуживают более подробного описания, подтверждения, либо аннулирования с целью более эффективного и рационального использования.

Ключевые слова: чембрен; фармакокинетика; COVID-19; биодоступность; алжирская медицина; молекулярный докинг

Список сокращений: ЛС — лекарственное средство; ЛРП — лекарственный растительный препарат; БАС — биологически активные соединения; ACE-2 — ангиотензинпревращающий фермент 2; ADME — всасывание, распределение, метаболизм, экскреция; ADMET — всасывание, распределение, метаболизм, экскреция, токсичность; Ala — аланин; AMES — анализ способности химического соединения индуцировать мутации в ДНК, Asn — аспарагин; ГЭБ — гематоэнцефалический барьер; Caco-2 — линия клеток рака толстой кишки; ClogP — коэффициент разделения октанола и воды; CLpro-3 — фермент 3-химотрипсиноподобная протеаза; ЦНС — центральная нервная система; CYP — цитохром; CYS — цистеин; ЭМ — эфирные масла; Gln — глютамин; Glu — глутаминовая кислота; Gly — глицин; Hb — гемоглобин; hERG — ген специфических калиевых каналов сердца; HIS — гистидин; HSV-1 — вирус простого герпеса 1-го типа; Leu — лейцин; MDCK — клетки Мадин-Дарби почки собаки; Met — метионин; ММ — молекулярная масса; OCT — октанол; pdb-код 6LU7 — кристаллическая структура основной протеазы COVID-19; Phe — фенилаланин; PkCSM — прогнозирование фармакокинетических и токсических свойств низкомолекулярных соединений; PLpro — папаиноподобная протеаза; PGP — гликопротеин P; ПМПМ — площадь молекулярной полярной поверхности молекул; RdRp — РНК-зависимая РНК-полимераза; QSAR — соотношение количественной структуры и активности; РНК — рибонуклеиновая кислота; Thr — треонин; TPSA — общая площадь полярной поверхности; VDss —объем распределения; WLOGP — коэффициент разделения воды (logP); XLOGP3 — коэффициент разделения октанола и воды (logP).

INTRODUCTION

The SARS-CoV-2 virus, causative agent of the most dangerous pandemic till now, COVID-19, is the seventh coronavirus [1] appeared in less than twenty years. The structure of this virus is greatly established [2], and well described [3]. This emerged pandemic has raised great public health and socioeconomic concern all around the world [4]. As on February 2021, there have been over 100 million cases and more than 2 million deaths reported since the start of the pandemic [5]; which mean that the pandemic spread very quickly and the numerous

routes of virus transmission have been described in the literature [3]. Knowing the mechanisms of virus infection, penetration into the host cell [1, 6], endocytosis then membrane fusion [7], and its replication cycle [8]; several antiviral strategies have been studied and proposed, among other inhibition of entry of SARS-CoV-2 into the host cell [9], Inhibition of the protease of SARS-CoV-2 [10], Inhibition of the synthesis (replication) of viral RNA [1]. These have constituted potential targets, in probable therapeutic treatments of Covid-19, for drug molecules. Based on previous experiences, drugs have been suggested as promising therapies for the treatment; among which and the most studied, we cite, by way of example: Hydroxychloroquine and chloroquine are used to inhibit SARS-CoV-2 binding to the ACE-2 receptor and impedes membrane fusion [4], or to block the replication of enveloped viruses by inhibiting the glycosylation of envelope proteins) [11]; Remdesevir is designed to inhibit viral RdRp, an enzyme that is integral to viral RNA replication. Without viral RNA replication, the virus is unable to multiply and spread to the infected host's other cells and reduces viral load [4]. As protease inhibitors, Lopinavir in combination with ritonavir may inhibit the action of 3CLpro [12], enzyme 3-chymotrypsin-like protease which plays a crucial role in processing the viral RNA, by disrupting the process of viral replication and release from host cells [13]; and others, in the process of testing and experimentation. However, none of these drugs are immune to side effects (unwanted), to contraindications, to precautions, and to drug interactions; in addition, not all researchers agree on the same opinion on the use of these drugs in the treatment of the pandemic, the pros and cons.

For these reasons and for others, the return to nature is required. Thereby, according to some authors [14] herbal medicines and medicinal plant-based natural compounds offer considerable potential for the development of new agents effective against infections currently difficult to treat and provide a rich resource for novel antiviral drug development. For example, some natural medicines have been shown to possess antiviral activities against various virus strains [14]. So, plants have been utilized for the isolation of novel bioactive compounds as they synthesize a vast number of chemical compounds with complex structures. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new antiviral drugs, since their chemical diversity provides unmatched availability [15]. Indeed, over 70% of therapies have a natural origin or were motivated by natural product chemistry [16]. Therefore, León-Méndez et al [17] consider essential oils "EOs" (complex mixtures of odorous and volatile compounds naturally produced by plants as secondary metabolites and stored in special fragile secretory structures, with low molecular weights and diverse chemical structures, and which bears tens to hundreds of varieties of molecules) as a source of bioactive molecules.

Biological properties of EOs are highlighted [17]. The effectiveness of EO has been attributed mainly to the presence of bioactive compounds in their composition [18]. These biological activities are attributed, in some cases, both to major components and to the minor ones present in these oils, but generally the essential oil, in its totality, acted less than the major constituents [19]. According to Pengelly [20], it is often the unique chemical combination rather than a single component that is responsible for any therapeutic activity. Antiviral activity is one of the other biological activities, which was document. Thus, Ma and Yao [21] summarizes the antiviral properties of EOs from different aromatic plants and EO-derived components on different virus and Tariq et al. [22] enumerates the major constituents of Medicinal and aromatic plants along with their antiviral activities. There, many studies reporting antiviral activity of natural products or isolates against human coronavirus strains are summarized by others [23].

The results of several studies concerning the antiviral efficacy of essential oils from a wide range of plant species led Ma and Yao [21] to draw the following conclusions, for each study: for a study, antiviral efficacy of the EO could be ascribed to its principle; for another, component minor components may be more bioactive than the primary component; among others, either minor or primary are responsible for EO bioactivity; others studies suggest that individual terpene in EO may not contribute equally to the antiviral efficacy of the EO mixture; and concluded that the antiviral effectiveness of EOs can be contributed unequally to the active components, either minor or principle ones, and underlying synergism. It is necessary to point out that to search for potential and specific inhibitors of Coronavirus, the virtual screening is mostly carried out to identify novel phytochemicals against SARS-CoV-2 from different plants. In addition, Wani et al. [24] noted

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that the data available on anti-COVID-19 activity of essential oils is mostly based on in vitro studies and computer aided docking techniques. In this way, four proteins (spike proteins, RdRp "RNA-dependent RNA polymerase", 3CLpro "chymotrypsin-like protease", and PLpro "papain like protease") which are essential for the pathogenicity of virus [14] constitute the molecular targets of natural products against coronavirus [23]. As an example, spike protein was selected for virtual screening [25], main protease [26], PLpro [27], RdRp [28], and 3CLpro [27] all most In Silico screening. Moreover, it has been shown that enveloped viruses respond sensitively to essential oils [15].

Thus, in continuation with our previous works [29], about minor components, extracted from different aromatic and medicinal plants collected from Algerian pharmacopeia, which were docked in the active site of SARS-CoV-2 main protease as possible inhibitors of SARS-CoV-2, so we consequently docked another minor's one, declared as in trace amount by authors, to main protease to look for possible CoVid-19 antiviral agents.

THE AIM of this study is the search for compounds, among minor components extracted from different aromatic and medicinal plants collected from Algerian pharmacopeia, which may posses possible COVID-19 antiviral activities, by molecular docking in the active site of SARS-CoV-2 main protease.

MATERIALS AND METHODS

Data collection

66 compounds were selected from nine medicinal plants growing wild in Algeria namely, *Artemisia arborescens* L. (1 compound), *Pinus halepensis* Mill. (4 compounds), *Eucalyptus* spp. (1), *Juniperus oxycedrus* L. (16), *Myrtus communis* (4), *Ocimum basilicum* (1), *Ocimum gratissimum* (2), *Thymus munbyanus* (28), *Teucrium polium* (10). On the one hand, all these plants are known, in the traditional Algerian pharmacopoeia, to treat pulmonary diseases and in general diseases of the respiratory system. On the other hand, compounds selected were those which are declared at traces amount by authors in the composition of the essential oils of these plants.

Molecular Docking

We performed a docking of studied compounds in the binding pocket of SARS-CoV-2 main protease (pdb code 6LU7) [30], to determine binding affinity and study the intermolecular interactions of studied molecules in the specific target. Molecular docking was implemented by means of the AutoDock program. Autodock vina was used for docking of ligand [31] and Autodock tools 1.5.6 to analysis the resuls [32]. Discovery Studio 2016 program was used to obtain the binding site of crystallographic structure of SARS-CoV-2 main protease (pdb code 6LU7) [33]. The active site of SARS-CoV-2 main protease (pdb code 6LU7) with coordinates (x=-10.782, y=15.787 and z=71.277) has been determined on the basis of the co-crystallized ligand N3 [34]. The grid box parameters were 20×34×20 xyz points with a grid spacing of 1 Å, the grid box was made keeping active site in the center of the box and cover the folic acid binding site in the enzyme (generated using the co-crystallized ligand (N3) as the center for docking) [34]. To prepare ligand and enzyme, an extended PDB format, termed PDBQT, was used for coordinate files, which includes atomic partial charges and atom types using Autodock tools 1.5.6. Torsion angles were calculated to assign the flexible and non-bonded rotation of molecules. The docked results were visualized and analyzed using the Discovery Studio program [35]; And calculation were performed according Hernández-Santoyo et al. [36].

Lipinski's Rule of five and ADMET Prediction

According Lipinski et al [37], the rule of five predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight (MWT) is greater than 500 and the calculated Log P (CLogP) is greater than 5 (or MlogP >4.15). That rule drug likeness for orally available drugs was calculated by using pkCSM [38] web servers. Molecules violating more than one of these parameters may have problems with bioavailability and a high probability of failure to display drug-likeness [39].

ADMET is another concept that focuses on absorption, distribution, metabolism, excretion (ADME) and toxicity characteristics in safe medicines. So, in silico approaches were used to predict and model the most relevant pharmacokinetic, metabolic and toxicity endpoints, thereby accelerating the drug discovery process [40, 41]. The computational prediction of the pharmacokinetic parameters/properties of isolated compounds was done using pkCSM [38] web servers.

RESULTS AND DISCUSSION

Molecular Docking

Molecular docking was performed to find the poses and possible types of interactions between the 66 studied natural compounds molecules and SARS-CoV-2

Mpro (pdb code 6LU7). The results are presented in Table 1.

The study shows that Cembrene is the best compound with binds with the pocket of SARS-CoV-2 Mpro; which could have more inhibitory potential against SARS-CoV-2 main protease than the other studied compounds. It is one of elements declared at amount trace in the essential oil of *Juniperus oxycedrus* L. Previous study showed another minor compound (Abietatriene), for the same species, which have potential inhibition against SARS-CoV-2 main protease with an estimated free binding energy of -6.4 kcal/mol [29]. The essential oils of this species revealed antiviral activity against SARS-CoV and HSV-1 replication in vitro; the effectiveness was assessed by visually scoring of the virus induced cytopathogenic effect post-infection [42]. Also, it is reported in the literature that this species is used in folk medicine in the treatment of many infectious diseases [43].

"Cembranes" family are the most widely occurring diterpenes in Nature and from which hundreds have been isolated, mainly from three sources tobacco, Caribbean gorgonians, and Pacific soft corals [44]. Cembrene, the first naturally occurring 14 memhered cyclic diterpene hydrocarbons ($C_{20}H_{32}$, Fig. 1) to be characterized, is found in pine oleoresins [45]. According Han et al. [46], the structure of a compound determines its physical and chemical properties as well as the ADMET. A range of biological activities has been reported for cembranes, against tumors, inflammation, as well as microbial and/or viral infections [47–49].

Cembrene seems be to inhibit viral receptor with a docking score of -6.3 kcal/mol through the Alkyl bond with CYS-145 and Pi-sigma HIS-41 (Fig. 2). Such types of bond help to improve the hydrophobic interaction of the ligand in the binding pocket of the receptor [50]. According the same authors, a large number of Pi-sigma interactions, which largely involves charge transfer, helps in intercalating the drug in the binding site of the receptor and, on the other hand, the complex stability can be linked to the with extra Pi-sigma interaction.

Elsewhere, many other types of hydrophobic/hydrophilic interactions were also perceived comprising Van der Waals, Conventional Hydrogen Bonds, Amide-Pi Staked, Carbon Hydrogen Bond, and Alkyl/Pi-Alkyl types. These interactions were shaped between The N3 co-crystallized ligand with Asn142, Glu 166, His 164, Gly 143, Thr 190, Gln 189, His 163, Phe 140, Leu 141, Met 165, His 172, Leu 167, Ala 191, Met 167, Pro 168, Met 49, His 41 amino acids residues in the active site of studied enzyme, SARS-CoV-2 Mpro (-6.9 kcal/mol) [51].

Lipinski's Rule of five and ADMET Prediction

The molecular weight and other parameters for cembrene are shown in table 2. Cembrene was found to be fitting well with the Lipinski rule of 5 for drug likeliness, with one violation concerning Log P, whereas the co-crystallized ligand presented three Lipinski Violations. The n-octanol-water partition coefficients, usually expressed as logP values, are used as a measure of lipophilicity and the importance of the use of these values in quantitative structure activity relationships (QSAR) is well established for prediction of biological or pharmacological activity of compounds [52]. The logP is closely related to the transport properties of drugs and their interaction with receptors [53].

These physicochemical parameters are associated with acceptable aqueous solubility and intestinal permeability and comprise the first steps in oral bioavailability [54]. For example, the higher molecular weight compounds are in general less likely to be orally active than lower one; also, rotatable bond count is now a widely used filter following the finding that greater than 10 rotatable bonds correlate with decreased oral bioavailability. In a general way, Oral drugs are lower in MWT and have fewer H-bond donors, acceptors and rotatable bonds [54], which coincides with our result.

The computational prediction of the pharmacokinetic parameters/properties of Cembrene are displayed in Table 3. Pharmacokinetic parameters are derived from the measurement of drug concentrations in blood or plasma [40]. Han et al. [46] attribute each parameter to some factors which depend like that: the absorption of drugs depends on factors including membrane permeability [indicated by colon cancer cell line (Caco-2)], intestinal absorption, skin permeability levels, P-glycoprotein substrate or inhibitor. The distribution of drugs depends on factors that include the blood–brain barrier (logBB), CNS permeability, and the volume of distribution (VDss). Metabolism is predicted based on the CYP models for substrate or inhibition (CYP2D6, CYP3A4, CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4). Excretion is predicted based on the total clearance model and renal OCT2 substrate. The toxicity of drugs is predicted based on AMES toxicity, hERG inhibition, hepatotoxicity, and skin sensitization. For more detail, Waterbeemd and Gifford [40] well described and reviewed the key pharmacokinetic parameters and their importance for the dose regimen and dose size.

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Table 1 – Affinity of the best conformation in the binding pocket of SARS-CoV-2 Mpro

Compounds	Score (kcal/mol)	Compounds	Score (kcal/mol)
Methyl eugenol	-4.9	Phellandrene	-4.5
Tricyclene	-4.0	trans-PinoCarveol	-4.5
Terpine*n*-4-ol	-4.7	Neryl acetate	-5.2
α-Terpinyl acetate	-5.2	α-Bisabolol	-5.7
Manoyl oxide	-5.8	Isoamyl 2-methyl butyrate	-4.4
δ-Terpineol	-5.2	*n*-Nonanal	-3.8
d-3-Carene	-4.4	Z-Thujone	-4.5
n-Octanol	-3.6	E-Verbenol	-4.8
n-Nonanal	-3.8	Thuj-3-e*n*-10-al	-4.9
Terpin-1-ol	-4.8	Geraniol	-4.8
Fenchyl acetate	-4.9	Geranial	-4.6
cis-Carveol	-4.6	α-E-Bergamotene	-5.0
trans-Piperitol acetate	-5.0	14-hydroxy-α- Muurolene	-5.5
trans-β-Damascenone	-4.9	β-Bisabolenol	-5.9
β-Calacorene	-5.8	Dibutyl phthalate	-5.6
7-epi-a-Eudesmol	-5.7	α-Terpinolene	-4.9
Juniper camphor	-5.4	4-Terpineol	-4.7
(E,Z)-Farnesol	-5.3	cis-Linalool oxide	-4.7
β-Bisabolenal	-5.8	*n*-Octanol	-3.6
(Z,E)-Farnesyl acetate	-5.6	6,7-Epoxymyrcene	-4.3
(E,E)-Farnesyl acetate	-5.8	*n*-Nonanal	-3.8
Cembrene	**-6.3**	trans-Thujone	-4.9
(3Z)-Hexenol	-3.8	trans-*p*-Mentha-2,8-dien-1-ol	-4.8
n-Hexanol	-3.6	cis-*p*-Mentha-2-en-1-ol	-4.8
3-Octanone	-3.9	cis-Limonene oxide	-4.9
3-Octanol	-3.9	trans-limonene oxide	-4.6
Isoborneol	-4.6	δ-Elemene	-4.9
Trans-pinocamphone	-4.8	*n*-hexadecanoic acid	-4.4
Neral	-4.6	α-trans-Bergamotene	-5.2
p-Vinylguaiacol	-4.8	cis-Muurola-4(14),5-diene	-5.1
Sesquicineole	-5.7	*n*-Heptacosane	-4.1
α-Calacorene	-5.6	*n*-Nonacosane	-4.3
Cyclocolorenone	-5.5	*n*-Dotriacontane	-4.1

Table 2 – Lipinski's rule of potential inhibitor: Cembrene

	Log P	HB Acceptor	HB Donor	Rotatable bonds	MW, g/mol	Lipinski violations
Rule	***<5***	***≤10***	***<5***	***<10***	***≤500***	***≤1***
Cembrene	6.62	0	0	1	272.47	1

Note: HB — hemoglobine; MW — molecular weight.

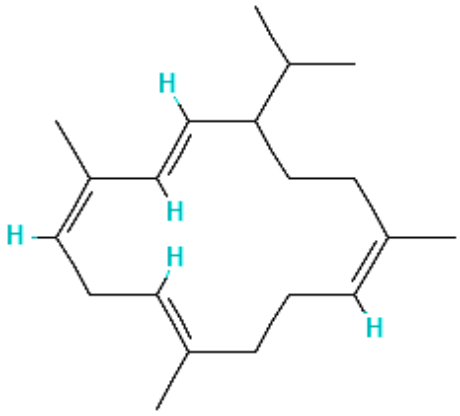

Figure 1 – Structures of Cembrene[1] with the best Affinity in the binding pocket of SARS-CoV-2 Mpro.

[1] Cembrene. PubChem. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Cembrene

Table 3 – *In silico* ADMET prediction of potential inhibitor: Cembrene

Property	Model Name	Unit Numeric/Categorical (Yes/No)	Predicted Value
Absorption	Water solubility	log mol/L	-7.207
	Caco2 permeability	log Papp in 10^{-6} cm/s	1.458
	Intestinal absorption (human)	% Absorbed	94.374
	Skin Permeability	log Kp	-1.675
	P-glycoprotein substrate	Yes/No	No
	P-glycoprotein I inhibitor	Yes/No	No
	P-glycoprotein II inhibitor	Yes/No	No
Distribution	VDss (human)	log L/kg	0.667
	Fraction unbound (human)	Fu	0.107
	BBB permeability	log BB	0.689
	CNS permeability	log PS	-2.206
Metabolism	CYP2D6 substrate	Yes/No	No
	CYP3A4 substrate		No
	CYP1A2 inhibitior		No
	CYP2C19 inhibitior		Yes
	CYP2C9 inhibitior		No
	CYP2D6 inhibitior		No
	CYP3A4 inhibitior		No
Excretion	Total Clearance	log ml/min/kg	1.48
	Renal OCT2 substrate	Yes/No	No
Toxicity	AMES toxicity	Yes/No	No
	Max. tolerated dose (human)	log mg/kg/day	0.269
	hERG I inhibitor	Yes/No	No
	hERG II inhibitor	Yes/No	No
	Oral Rat Acute Toxicity (LD50)	mol/kg	1.512
	Oral Rat Chronic Toxicity (LOAEL)	log mg/kg_bw/day	1.244
	Hepatotoxicity	Yes/No	No
	Skin Sensitisation	Yes/No	Yes
	T.Pyriformis toxicity	log µg/L	2.031
	Minnow toxicity	log mM	-0.448

Note: BBB — blood-brain barrier penetration

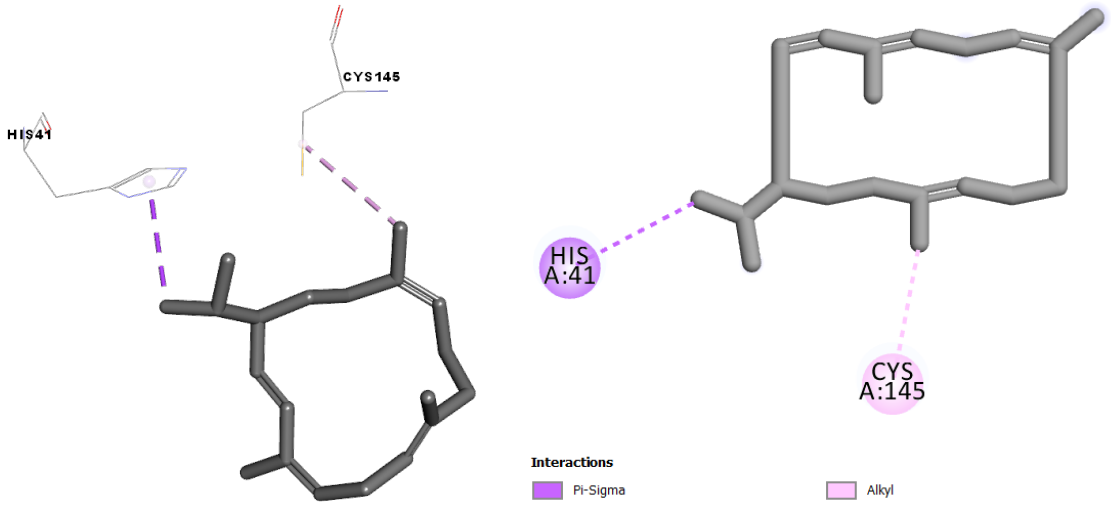

Figure 2 – 2D and 3D presentations of interactions between Cembrene and SARS-CoV-2 Mpro.

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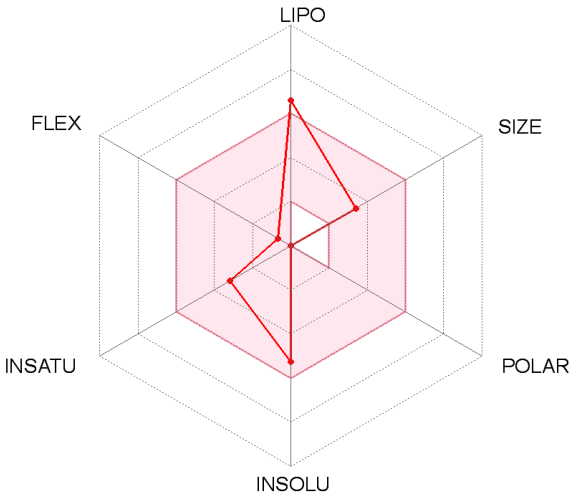

Figure 4 – Bioavailability Radar of Cembrene

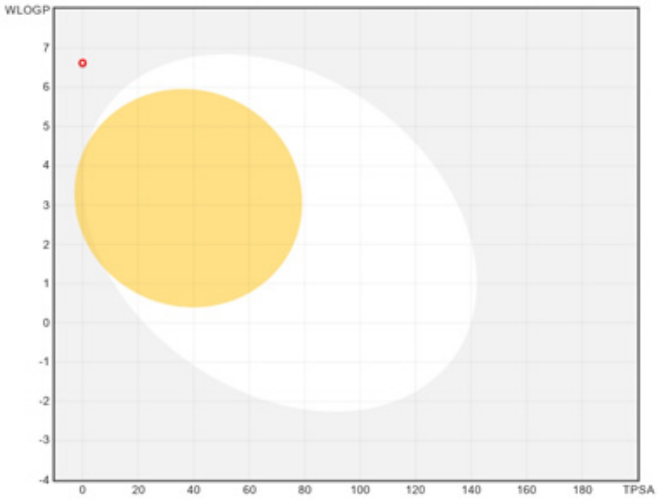

Figure 5 – Boiled-Egg of Cembrene

MWT has a large effect on solubility, our result for solubility (237.281, Moderately soluble) is in agreement with Gleeson [55] for which on average, molecules with MWTs < 300 have solubilities of ≈250 μM (μmol/L), and which is considered as an important component of an orally administered drug, determining the amount freely available to permeate through the gastrointestinal membranes into systemic circulation; also, the increasing of MWT is correlate with decreasing of membrane permeability, according their parameters MDCK or Caco-2.

A Bioavailability Score, ABS identifies poorly- and well-absorbed compounds tested in humans, it is 0.55 for compounds, which pass the rule of five [56]. Our result shows an ABS of Cembrene equal to 0.55 which confirm non Lipinski violations. Considering the bioavailability radar of Cembrene (Fig. 4), the compound is predicted not orally bioavailable, because too lipo (lipophilicity: Log $P_{o/w}$ (XLOGP3)=6.04>+5.0) and less polar (polarity: TPSA=0.00Å²<20 Å²). The molecular polar surface area (PSA) is considered as descriptor that was shown to correlate well with passive molecular transport through membranes and, therefore, allows prediction of transport properties of drugs [57], and lipophilicity as the key physicochemical parameter linking membrane permeability — and hence drug absorption and distribution- with the route of clearance (metabolic or renal) [40]. For instance, it has been reported that target promiscuity as well as toxicity issues like hERG inhibition, phospholipidosis or cytochrome P450 (CYP) inhibitions are more likely to be problematic for compounds with high lipophilicity values; also solubility and metabolism are more likely to be compromised at these high values whereas permeability could be decreased when this property is too low [58].

According Daina and Zoete [59], Gastrointestinal absorption (HIA) and brain penetration (BBB) are two pharmacokinetic behaviors crucial to estimate at various stages of the drug discovery processes. So, to this end, the Brain or Intestinal estimated permeation method (BOILED-Egg) is proposed by Daina and Zoete in 2016 [59] as an accurate predictive model that allows for intuitive evaluation of passive gastrointestinal absorption (HIA) and brain penetration (BBB) in function of the position of the molecules in the WLOGP-versus-TPSA referential and which works by computing the lipophilicity and polarity of small molecules. The colored zone is the suitable physicochemical space for orally bioavailability, the white region in the BOILED-Egg graphical is the physicochemical space of molecules with highest probability of being absorbed by the gastrointestinal tract, the yellow region (yolk) is the physicochemical space of molecules with highest probability to permeate to the brain and blue dots for P-gp substrates (PGP+) and red dots for P-gp non-substrate (PGP-) as described by the same authors. For this, Cembrene is predicted as not absorbed and not brain penetrant (outside the Egg, Fig. 5) and not subject to active efflux from the CNS or to the gastrointestinal lumen (P-gp non-substrate (PGP-), red dot).

CONCLUSION

A virtual screening technique, including molecular docking, and ADMET Prediction was carried out, for the selection of the compounds which could have a potent antiviral treatment of COVID-19. In total, 66 natural compounds, selected from 9 Algerian herbal medicine, were docked in the active site of SARS-Cov-2 main protease. The results of this study

indicates clearly that, among these compounds, only Cembrene constitutes the structure with the best affinity in the binding site of the enzyme and respect the conditions mentioned in Lipinski's rule, except the Log P, a measure of lipophilicity and closely related to the transport properties of drugs and their interaction with receptors. Concerning the pharmacokinetic properties and bioavailability, Cembrene is predicted not orally bioavailable, because too lipophilic and less polar and. It is also predicted as not absorbed and not brain penetrant and not subject to active efflux from the CNS or to the gastrointestinal lumen. This result might be interest researchers confirm or invalidate the results obtained and push the research thoroughly.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Benalia Yabrir — problem statement, development of the research concept, writing and scientific editing the text of the manuscript; Assia Belhassan — processing the study data, analyzing and describing the results; Tahar Lakhlifi, Mohammed Bouachrine — supervision, editing and manuscript revision; Guillermo Salgado Moran, Lorena Gerli Candia — participation in the development of the study design and article concept.
All the authors confirm their authorship compliance with the ICMJE international criteria (all authors made a significant contribution to the development of the concept, conducting research and preparing the article, read and approved the final version before publication).

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