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RESEARCH ARTICLE

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Metabolomics in drugs research on zebrafish-based cardiotoxicity models: endothelial and mitochondrial dysfunction, oxidative stress

N.E. Moskaleva[1], P.M. Rezvanov[1], V.M. Samoylov[1], V.G. Varzieva[1], S.N. Baskhanova[1], V.V. Tarasov[1], E.A. Smolyarchuk[1], D.A. Kudlay[1, 2, 3], S.A. Appolonova[1]

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The aim. To investigate the metabolic profile of zebrafish embryos when exposed to drugs with known risks of cardiotoxicity, such as acetaminophen, carbamazepine, salbutamol, ketorolac, bisoprolol, and metoprolol. The analysis is aimed at detecting changes in the level of amino acids (including branched chain BCAAs), products of carnitine metabolism (acylcarnitines) and related metabolic indices reflecting mitochondrial dysfunction, oxidative stress and disorders of the nitric oxide signaling pathway.

Materials and methods. Zebrafish embryos were incubated with the test substances in a concentration gradient (0.5–10×NOEC). A quantitative targeted metabolomics analysis was performed using high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) with a panel of 98 metabolites (amino acids, nitric oxide metabolism products, vitamins, nucleosides and acylcarnitines). The obtained concentrations of metabolites were compared with the control (0.1% DMSO). Statistically significant deviations were expressed as the ratio of concentration to control on a base 2 logarithmic scale (*log2FC*).

Results. Changes in concentrations of metabolites under the influence of cardiotoxic drugs were revealed. There was an accumulation of BCAAs (the sum of leucine, isoleucine, valine; log2FC≈0.5–2.2; p <0.05) compared with the control, as well as an increase in the level of acylcarnitines, indicating mitochondrial dysfunction: for example, metoprolol and bisoprolol caused an increase in the ratio of the sum of acylcarnitines to free carnitine by more than 4–6 times (*log2FC*=+3.8 for bisoprolol and -1.27 for metoprolol; p <0.01), as well as accumulation of long-chain acylcarnitines. Pronounced changes in indicators related to oxidative stress were noted: in the samples after exposure to beta-1 blockers (bisoprolol, metoprolol) and ketorolac, the concentration of methionine sulfoxide (by 80–130%, p < 0.01), the product of methionine oxidation, and the ratio of methionine sulfoxide/methionine increased, whereas when exposed to salbutamol, on the contrary, the level of methionine sulfoxide decreased (-120%, p <0.01), indicating a multidirectional effect on the oxidative status. Violations of the nitric oxide signaling pathway were reflected in an increase in the level of asymmetric dimethylarginine.

Conclusion. Each of the analyzed compounds produced a specific metabolic “imprint” in Zebrafish samples, reflecting the mechanisms of their cardiotoxicity. An increase in BCAA levels and related indicators indicates a violation of myocardial energy metabolism, the accumulation of long-chain acylcarnitines indicates incomplete beta-oxidation of fatty acids. An increase in the concentration of ADMA is associated with endothelial dysfunction, and an increase in methionine sulfoxide is associated with increased oxidative stress.

Keywords: cardiotoxicity; metabolomics; zebrafish; zebrafish; amino acids; acylcarnitines.

Abbreviations: CTx — cardiotoxicity; ADMA — asymmetric dimethylarginine; BCAA — the sum of branched-chain amino acids (valine+leucine+isoleucine); DMSO — dimethyl sulfoxide; FC (Fold Change) — the ratio of concentration in the sample to concentration in the control; NOEC — concentration having no effect; SDMA — symmetric dimethylarginine; GSG — a combination of amino acids involved in the synthesis of glutathione, glutamate/(serine+glycine); GABR — arginine total availability index, arginine/(citrulline+ornithine); ROS — reactive oxygen species.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Метаболомное исследование кардиотоксичности лекарственных препаратов на зебрафиш в качестве модельного организма: эндотелиальная и митохондриальная дисфункция, окислительный стресс

Н.Е. Москалева[1], П.М. Резванов[1], В.М. Самойлов[1], В.Г. Варзиева[1], С.Н. Басханова[1], В.В. Тарасов[1], Е.А. Смолярчук[1], Д.А. Кудлай[1, 2, 3], С.А. Апполонова[1]

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Цель. Исследовать изменения метаболомного профиля эмбрионов Данио-рерио (зебрафиш) при воздействии лекарственных препаратов с известными рисками кардиотоксичности — ацетаминофена, карбамазепина, сальбутамола, кеторолака, бисопролола и метопролола. Анализ нацелен на выявление изменений в уровне аминокислот (в том числе с разветвлённой цепью — BCAA), продуктов карнитинового обмена (ацилкарнитинов) и связанных метаболических индексов, отражающих митохондриальную дисфункцию, оксидативный стресс и нарушения сигнального пути оксида азота.

Материалы и методы. Эмбрионы Данио-рерио инкубировали с исследуемыми веществами в градиенте концентраций (0,5–10×NOEC). Проводился количественный целевой метаболомный анализ методом высокоэффективной жидкостной хроматографии — тандемной масс-спектрометрии (HPLC–MS/MS) с панелью из 98 метаболитов (аминокислоты, продукты обмена оксида азота, витамины, нуклеозиды и ацилкарнитины). Полученные концентрации метаболитов сравнивали с контролем (0,1% ДМСО). Статистически значимые отклонения выражали как отношение концентрации к контролю в логарифмическом масштабе по основанию 2 (*log2FC*).

Результаты. Выявлены изменения концентраций ключевых метаболитов под влиянием кардиотоксичных лекарственных препаратов. Наблюдалось накопление BCAA (сумма лейцин, изолейцин, валин; *log2FC*≈0,5–2,2; p <0,05) по сравнению с контролем, а также повышение уровня ацилкарнитинов, указывающее на митохондриальную дисфункцию: так, метопролол и бисопролол вызывали увеличение соотношения суммы ацилкарнитинов к свободному карнитину более чем в 4–6 раз (*log2FC*=+3,8 для бисопролола и –1,27 для метопролола; p <0,01), а также накопление длинноцепочечных ацилкарнитинов. Отмечены выраженные изменения показателей, связанных с оксидативным стрессом: в образцах после воздействия β1-блокаторов (бисопролол, метопролол) и кеторолака повышалась концентрация метионин-сульфоксида (на 80–130%, p <0,01) — продукта окисления метионина, и соотношение метионин-сульфоксид/метионин, тогда как при воздействии сальбутамола, напротив, уровень метионин-сульфоксида снижался (–120%, p <0,01), указывая на разнонаправленное влияние на окислительный статус. Нарушения сигнального пути оксида азота отразились в повышении уровня асимметричного диметиларгинина.

Заключение. Каждое из проанализированных соединений вызывало специфический метаболический «отпечаток» в образцах Данио-рерио, отражающий механизмы их кардиотоксичности. Повышение уровня BCAA и связанных показателей указывает на нарушение энергетического обмена миокарда, накопление длинноцепочечных ацилкарнитинов свидетельствует о неполном β-окислении жирных кислот. Рост концентрации ADMA ассоциирован с эндотелиальной дисфункцией, а увеличение метионин-сульфоксида — с усилением оксидативного стресса.

Ключевые слова: кардиотоксичность; метаболомика; Данио-рерио; зебрафиш; аминокислоты; ацилкарнитины

Список сокращений: КС — кардиотоксичность; ЛС — лекарственное средство; ADMA — асимметричный диметиларгинин; BCAA — сумма аминокислот с разветвлённой цепью (валин+лейцин+изолейцин); ДМСО — диметилсульфоксид; FC (Fold Change) — отношение концентрации в образце к концентрации в контроле; NOEC — концентрация, не оказывающая эффекта; SDMA — симметричный диметиларгинин; GSG — комбинация аминокислот, вовлечённых с синтез глутатиона, глутамат/(серин+глицин); GABR — индекс общей доступности аргинина, аргинин/(цитруллин+орнитин); АФК — активные формы кислорода.

INTRODUCTION

Cardiotoxicity (CT) of medicines remains a significant problem in modern pharmacology and clinical practice, as it is the reason for rejection at the stages of development and withdrawal of medicines at the post-marketing stage [1]. CT manifests itself as a result of a complex interaction of various molecular mechanisms of heart damage, such as oxidative stress (OS), mitochondrial and endothelial dysfunction, and impaired ion channel functions [2–5].

Metabolomic analysis provides the possibility of identifying biomarkers of myocardial damage. In particular, profiles of low molecular weight metabolites (amino acids, carnitine metabolism, etc.) in blood plasma reflect characteristic shifts in metabolism during toxic exposure [3–5]. For example, increased levels of branched-chain amino acids (BCAA: leucine, isoleucine, valine) are associated with impaired myocardial contractility [6–8], and the accumulation of long-chain acylcarnitines indicates impaired mitochondrial b-oxidation and energy balance [9–11]. An increased concentration of asymmetric dimethylarginine (ADMA), an inhibitor of NO synthase, correlates with impaired endothelial dysfunction and progression of heart failure [12, 13].

The assessment of CT of medicines is currently carried out on cellular systems using cardiomyocytes and on mammals [14, 15]. Modern methods in vitro screening provides a high level of throughput, but do not reflect the complex interactions occurring in an intact organism [15]. Studies on mammals have better predictive value in relation to clinical results, but have low throughput and high cost [15], which is the reason for the search for other biological models for studying organ toxicity of medicines.

Zebrafish (Danio rerio) demonstrate a high degree of metabolic conservatism and are used to assess the toxicity of various compounds due to genetic homology with mammals, transparency of embryos and high rate of development [16–18]. The most common method for studying CT using zebrafish as a model organism is to identify changes in heart rate or blood flow rate of embryos after incubation with the studied medicines [19].

It would be interesting to supplement the morphological data with information about the mechanisms of toxic action. The use of Danio rerio as a biological model provides the possibility of assessing the systemic effects of medicines in the conditions of an intact organism [20], allows identifying metabolomic markers associated with the mechanisms of the toxic effect of the substance on the myocardium [20], which can be used to identify organ toxicity in the development of new drugs.

THE AIM was to identify specific metabolomic changes that occur when Danio rerio embryos are exposed to various cardiotoxic medicines and to establish a relationship between these changes and the alleged mechanisms of toxicity.

MATERIALS AND METHODS

Reagents and materials

The following reagents and chemicals were used: deionized water (18.2 MΩ×cm) was obtained using a Millipore-Q system (Merck, Darmstadt, Germany), sodium chloride (NaCl), potassium chloride (KCl), calcium (II) chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), methylene blue and ascorbic acid (qualification “pure”) (Prime Chemical Group, Moscow, Russia); acetonitrile, methanol, formic acid “for HPLC”, sodium metabisulfite “reagent grade” (Sigma-Aldrich, USA). Also, salbutamol and bisoprolol fumarate (British Pharmacopoeia (BP) Reference Standard, EDQM, USA), acetaminophen, ketorolac tromethamine, cetirizine hydrochloride, metoprolol tartrate (Pharmaceutical Secondary Standard, Supelco, USA), carbamazepine (≥98% (HPLC), Sigma-Aldrich, USA) were used for the study.

E3 medium contained 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 0.001% methylene blue. Substances were dissolved in dimethyl sulfoxide (DMSO) or water, and then diluted in E3 medium to obtain a concentration for incubation (the final concentration of DMSO was 0.1%).

Amino acid standards and compounds containing an amino group (Sigma-Aldrich, Germany) and isotopically labeled standards (Toronto Research Chemicals, Canada) were used for quantitative analysis. Internal isotopically labeled standards (MassChrom Internal Standard Mix, Chromsystems, Germany) were used for the analysis of acylcarnitines.

Conducting studies on Zebrafish

Wild-type Danio rerio were obtained and maintained at the Center for Biopharmaceutical Analysis and Metabolomic Research, Sechenov First Moscow State Medical University (Sechenov University). The fish belonged to the same population and were randomly distributed into spawning groups. The studies were

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conducted with an automatically controlled 24-hour cycle (14 light and 10 dark time), and the temperature was set at 26±2°C. Unfertilized eggs and dead embryos were identified and separated on the 2nd day after fertilization, and live embryos were used for the study. Morphological pathologies, such as tail malformations, pigmentation levels, shape of the chord and somites, as well as heartbeat, were assessed using a light microscope (Leica DM2000, Germany).

Danio rerio embryos 4 days after fertilization (age up to 120 h) were used for the studies, therefore, approval by the Ethics Committee of any level was not required in the case of this study. Embryos were transferred to 12-well plates (20 embryos per biological sample in one well). Then, 5 mL of the testing medicine solution in E3 medium without methylene blue was added to each well. The concentrations of the medicine ranged from 0.5×NOEC to 10×NOEC (Table 1), 0.1% DMSO in E3 medium was added to the control group. NOEC was determined in acute toxicity experiments conducted in accordance with GOST 33774-2016 "Methods for testing chemical products that pose a danger to the environment. Acute toxicity to fish embryos". The plates were incubated for 4 h. To account for variability, each medicine was studied in 3 repeated incubations. The study design is shown in Figure 1.

Sample preparation for HPLC-MS/MS analysis

After incubation, embryos from each well (20 pcs. per sample) were transferred to 1.5 mL microcentrifuge tubes. The aqueous phase was removed and 10 µl of 13 mM aqueous solution of sodium metabisulfite was added to the sample to prevent oxidation of metabolites. Then, 500 µl of cold methanol with a mixture of internal standards was added, sonicated in an ice bath for 15 min and centrifuged (Centrifuge 5418R, Eppendorf AG, Germany) for 5 min at 16 900 rf at 4°C. After that, 450 µl of the supernatant was evaporated to dryness. The residue was reconstituted with 100 µl of methanol, diluted with 100 µl of deionized water, centrifuged for 10 min at 14,000 rpm on the same centrifuge and analyzed by HPLC-MS/MS.

HPLC-MS/MS analysis

HPLC-MS/MS analysis was performed using an Agilent 1290 Infinity II high-performance liquid chromatography system with an Agilent 6470A mass spectrometric detector (Agilent Technologies, USA). Chromatographic separation was performed using an Acquity UPLC BEH C18 analytical column (2.1×50 mm, 1.7 µm) from Waters Corporation, USA at 40°C. The mobile phase consisted of 0.1% aqueous solution of formic acid (A) and 0.1% solution of formic acid in acetonitrile (B), supplied in a gradient elution program at a flow rate of 0.5 mL/min. The total analysis time was 5.0 min. Samples were injected in a volume of 5 µl.

Mass spectrometric analysis was performed in multiple reaction monitoring mode after electrospray ionization. The mass spectrometry parameters were as follows: capillary voltage is 3500 W, gas temperature is 300°C, gas flow rate is 11 L/min. Compound-specific parameters (fragmentor, collision energy) and optimized for the best response of each analyte are published in Kozhevnikova et al. [9]. The initial data were processed using MassHunter Software (Agilent Technologies, USA).

Statistical analysis

Quantitative analysis of compounds containing an amino group was performed by the internal standard method in accordance with the calibration curve. MassHunter Software (Agilent Technologies, USA) was used to construct the calibration curve. The concentrations of acylcarnitines in the samples were determined by calculating the ratio of the peak areas of acylcarnitines and their internal standards (semi-quantitative method) using MassHunter Software (Agilent Technologies, USA).

Data from 3 repeated experiments were ranked and averaged. The Shapiro–Wilk test was used to assess the normality of the data distribution. In case of violation of normality in one of the groups, the non-parametric Mann–Whitney test was used to compare concentrations between the control and test groups. In the absence of deviations from normality, the Welch's t-test was used. Values at p <0.05 were considered significant. Statistical analysis was performed using STATISTICA version 10.0 software (USA).

To identify the trend of changes in the metabolomic profile, the ratio of the metabolite concentration in the test sample to the control (Fold Change) was calculated and expressed on a logarithmic scale to the base **2 (log2FC)** [21].

Metabolite ratios were also calculated [22–24]: GSG index — a combination of amino acids involved in glutathione synthesis — glutamate / (serine+glycine); BCAA — the sum of branched-chain amino acids — valine+leucine+isoleucine; GABR — index of total

arginine availability — arginine / (citrulline+ornithine); Fischer's index — the ratio of the sum of branched-chain amino acids to the sum of aromatic amino acids.

RESULTS

One of the main problems in the use of Danio rerio as a biological model in metabolomic studies remains high biological variability, which can complicate the interpretation of results and reduce reproducibility [25]. Using a strictly defined stage of development, standardization of diet and feeding time reduces metabolic fluctuations [25]. Combining 20 embryos into one biological sample eliminates variability, especially at early stages of development [25]. More reproducible results are also obtained if the ratios of metabolite concentrations in exposure groups compared to the control are interpreted.

Table 2 presents changes in the concentration of metabolomic markers (for the maximum concentration of 10×NOEC), characteristic of various CT mechanisms, which were observed under the influence of the studied substances. In the negative control (cetirizine), no significant shifts in metabolites were observed, the *log2FC* values are close to zero (p >0.05 for all), which confirms the absence of a cardiotoxic effect of this compound. In contrast, cardiotoxic substances caused significant changes in a number of markers. Based on the data obtained, it is possible to identify characteristic patterns of metabolomic changes. Thus, acetaminophen (at a maximum dose of ≈10×NOEC) led to a moderate increase in total BCAA and the ADMA / arginine ratio. Carbamazepine caused the most pronounced increase in ADMA (*log2FC*≈2.94) and the (ADMA / arginine) index — these shifts indicate a violation of the nitric oxide (NO) synthesis pathway and endothelial dysfunction under the action of carbamazepine. Salbutamol (β2-adrenomimetic), on the contrary, sharply reduced the level of L-arginine (–1.832) and ADMA (–1.409), but with an increase in the ADMA / arginine index. Salbutamol is also characterized by the highest increase in BCAA (log2FC≈1.38) with a decrease in acetylcarnitine (C2) and free carnitine (C0), which indicates an increase in carbohydrate catabolism and carnitine consumption. Ketorolac (NSAID) demonstrated the most significant accumulation of BCAA (*log2FC*≈2.18, p=0.001), which may indicate a developing energy imbalance and osmotic stress in cardiomyocytes. Bisoprolol and metoprolol (selective β1-adrenoblockers) caused similar metabolic shifts characteristic of a state of suppression of cardiac function: moderate increase in BCAA (≈+0.59 and +0.89), significant increase in ADMA (+1.32 and +0.61) against the background of a decrease in L-arginine (–1.25 and –1.14) — signs of endothelial dysfunction and a decrease in NO synthesis.

Figures 2–5 show the dose-dependent changes in the concentration of some markers. For example, for BCAA (Fig. 2), it is clear that cardiotoxic substances led to an increasing (with dose) increase in the total concentration. The increase in BCAA is especially noticeable when exposed to ketorolac and salbutamol — their curves increased sharply, reaching a 3–4-fold excess of the control at maximum drug concentrations. The ADMA / arginine ratio (Fig. 3) demonstrated differences in behavior: for carbamazepine, the ADMA / arginine ratio was proportional to the dose (an indicator of NO synthase suppression), whereas for ketorolac and salbutamol, the ratio increased sharply with the addition of a minimum amount of the drug (0.5×NOEC), and then did not change. The methionine sulfoxide/methionine ratio (Fig. 4) remained unchanged under the action of cetirizine and salbutamol (the latter even slightly reduced it), but increased with increasing concentrations of β1-blockers and ketorolac, reflecting an increase in oxidative stress in the heart tissue. Finally, the (C16+C18) / C0 ratio (Fig. 5) — an indicator of the accumulation of long-chain acylcarnitines — practically did not change under the action of acetaminophen, carbamazepine and salbutamol, but increased sharply for bisoprolol and metoprolol when transitioning to high concentrations. These graphical dependencies make the statistical data complete, confirming the presence of characteristic metabolic "fingerprints" in different mechanisms of CT.

DISCUSSION

The metabolomic profile of Danio rerio changed after exposure to cardiotoxic drugs, mimicking the metabolic consequences for the heart in mammals. According to literature data, an increase in the concentration of BCAA in human blood plasma is associated with a deterioration in myocardial contractility and a worsening prognosis in heart failure [9], and the accumulation of long-chain acylcarnitines correlates with the risk of developing arrhythmias and adverse outcomes in cardiomyopathies [26]. These data are also contain the obtained results: medicines known to cause energy deficiency in the myocardium (ketorolac,

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β-blockers) [27] caused the greatest increase in BCAA and acylcarnitine levels, reflecting the inhibition of the tricarboxylic acid cycle and β-oxidation of fatty acids in cardiac tissue. A likely mechanism was the overload of mitochondria with fatty acids while simultaneously inhibiting their oxidation — in the case of β-blockers, due to a decrease in ATP demand against the background of bradycardia and a decrease in coronary blood flow, in the case of ketorolac, due to the suppression of mitochondrial biogenesis in chronic inflammation. This imbalance led to the accumulation of long-chain acylcarnitines, which can disrupt the functioning of ion channels and provoke arrhythmogenic effects [11].

Another important factor of CT is ED due to impaired NO signaling system [8, 9]. A Significant increase in ADMA (NO synthase inhibitor) levels under the influence of a number of medicines was observed, especially carbamazepine and β-blockers. It is known that ADMA competitively displaces L-arginine from the active center of endothelial NO synthase, reducing NO production and causing vasoconstriction and tissue ischemia [12]. An increase in ADMA concentration in plasma is considered a risk factor for ED and cardiovascular disorders [12]. In our studies, the increase in ADMA was accompanied by an increase in the ADMA / Arg index and a decrease in L-arginine levels (for example, with metoprolol), which indicates a possible depletion of NO-dependent vasodilation.

Salbutamol reduced the concentration of ADMA, probably by stimulating NO synthesis (adrenaline and β2-agonists increase eNOS activity [28, 29]). However, at the same time, a sharp drop in the level of L-arginine was observed, which together led to an imbalance in ADMA / Arg. Nevertheless, salbutamol increased the level of citrulline (a by-product of eNOS), which may indicate activation of NO synthesis; the decrease in arginine is probably associated with its redistribution to other pathways (for example, the ornithine cycle or creatine synthesis). In general, the data demonstrated that various mechanisms (an increase in ADMA, a deficiency of L-arginine, or their combination) contribute to a decrease in NO bioavailability under the action of toxic doses of medicines, which potentially leads to a deterioration in myocardial perfusion and increases its vulnerability to ischemia and arrhythmias [12].

Disorders of nitrogen metabolism of amino acids under the influence of toxicants also manifested in the accumulation of aromatic amino acids — phenylalanine, tyrosine, tryptophan — in almost all exposures. An increase in phenylalanine levels and a decrease in the tyrosine/phenylalanine ratio are typical for conditions of heart failure and may indicate a decrease in the activity of phenylalanine hydroxylase in the liver (for example, in hypoxia) [30]. The tyrosine/phenylalanine ratio increased significantly under the influence of β-blockers, which can be interpreted as enhanced conversion of phenylalanine to tyrosine in situ or increased breakdown of catecholamines (forming tyrosine) during prolonged β-blockade. In any case, the accumulation of phenylalanine — a marker of liver or kidney dysfunction — possibly reflects the systemic effect of medicines on the entire body, even with short exposure. Similarly, an increase in tryptophan may be associated with suppression of its metabolism via the kynurenine pathway, which is often observed in stressful conditions and inflammation.

OS is recognized as a key link in CT of many medicines [31], and in this study, it was observed under the influence of β-blockers — a significant accumulation of methionine sulfoxide indicates increased formation of reactive oxygen species (ROS) and oxidation of methionine. This is consistent with clinical data that overdose of selective β1-adrenoblockers leads to cardiogenic shock with tissue ischemia, provoking the release of free radicals and lipid oxidation [27]. Acetaminophen generates reactive metabolites in the liver [32, 33]; in the heart, its pro-oxidant effect was weak (there was only a tendency to decrease the level of methionine sulfoxide), possibly due to compensatory activation of antioxidant enzymes. Unexpectedly, a decrease in oxidation markers was observed with salbutamol — this phenomenon may be explained by the fact that acute β-adrenergic exposure activates protective pathways (for example, an influx of Ca2+ can stimulate the synthesis of NO, which has antioxidant properties, and also include the expression of NRF2-dependent antioxidant defense genes). It is more likely that salbutamol simply does not cause such a pronounced OS, since increased blood flow and metabolism in conditions of β-stimulation do not allow reduced equivalents to accumulate (maintaining a high level of NAD^+ / NADH). In turn, ketorolac moderately increased the concentration of methionine sulfoxide and taurine, which is consistent with data on nephropathies and cardiomyopathies caused by NSAIDs through the formation of ROS and depletion of cellular antioxidants [34].

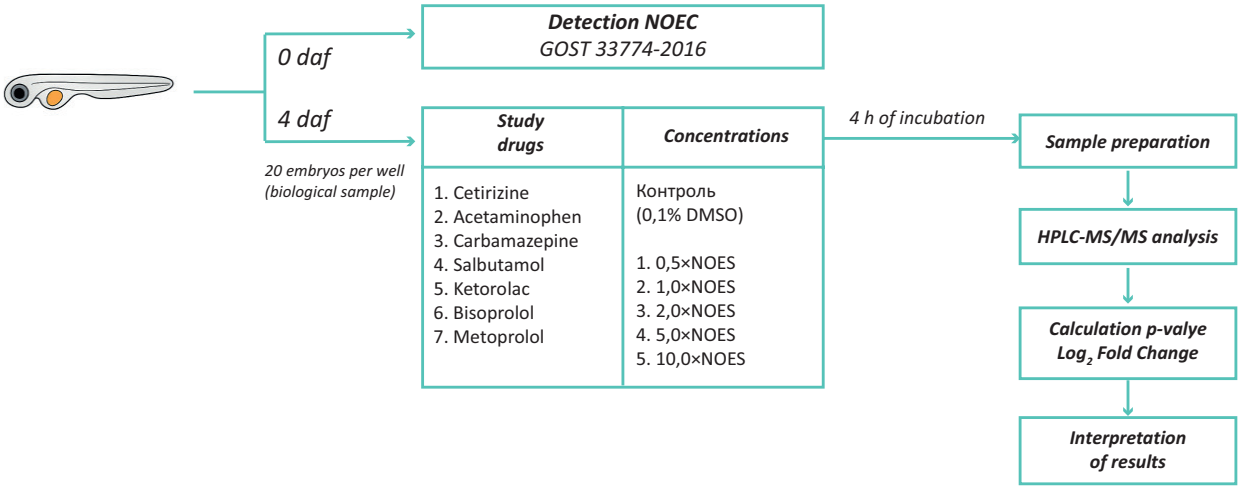

Figure 1 – Study design.

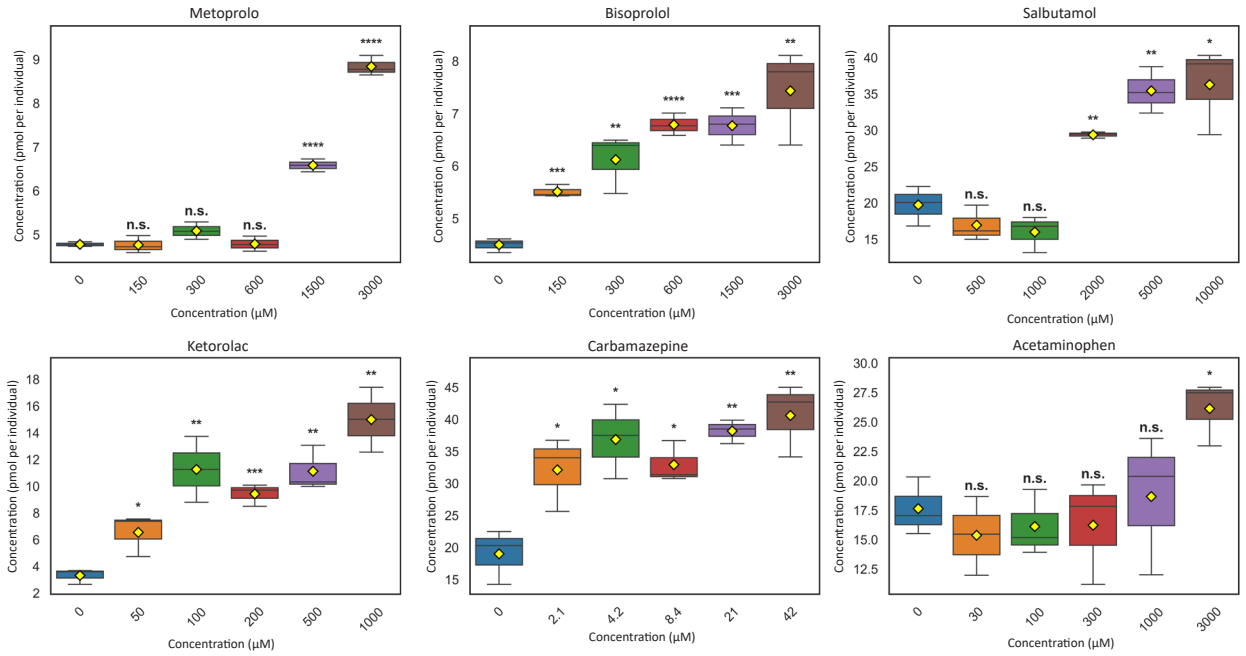

Figure 2 – Dependence of changes in the level of BCAA (pmol/individual) in Danio rerio embryos on the concentration of the studied drugs (μM).

Note: The median value is marked with a yellow rhombus, n.s. — p >0.05, * — p <0.05, ** — p <0.01, *** — p <0.001, **** — p <0.0001.

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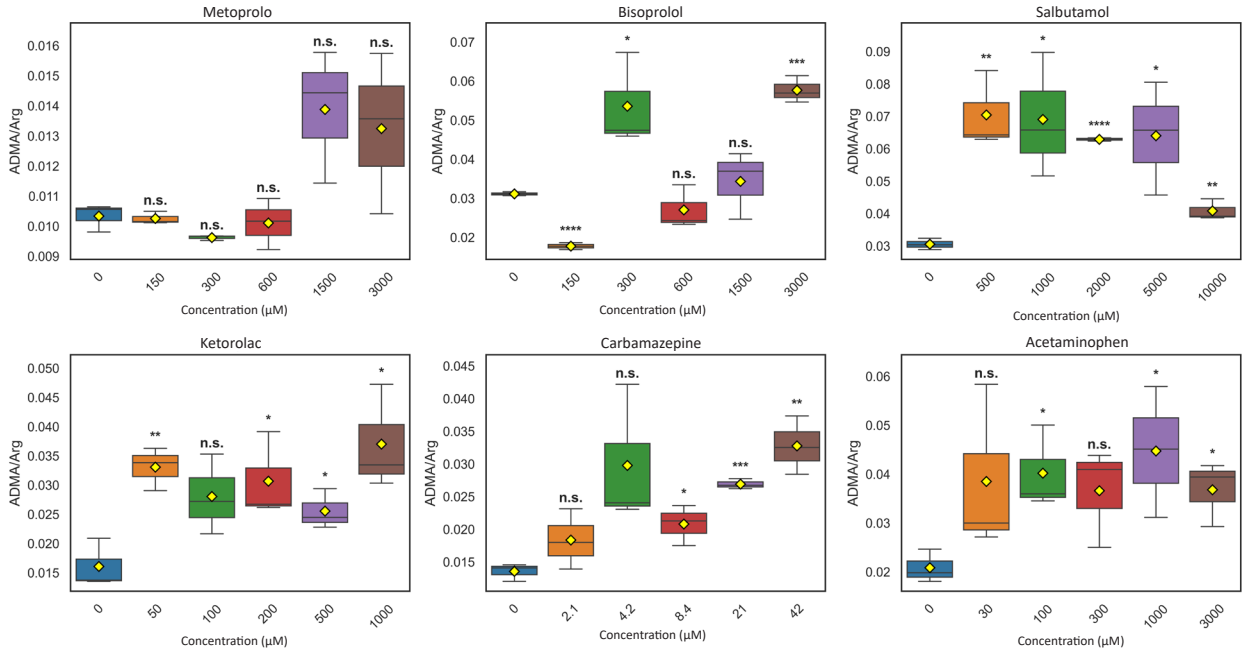

Figure 3 – Dependence of the ratio of ADMA / arginine concentrations in Danio rerio embryos on the concentration of the studied drugs (μM).

Note: The median value is marked with a yellow rhombus, n.s. — $p > 0.05$, * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$, **** — $p < 0.0001$.

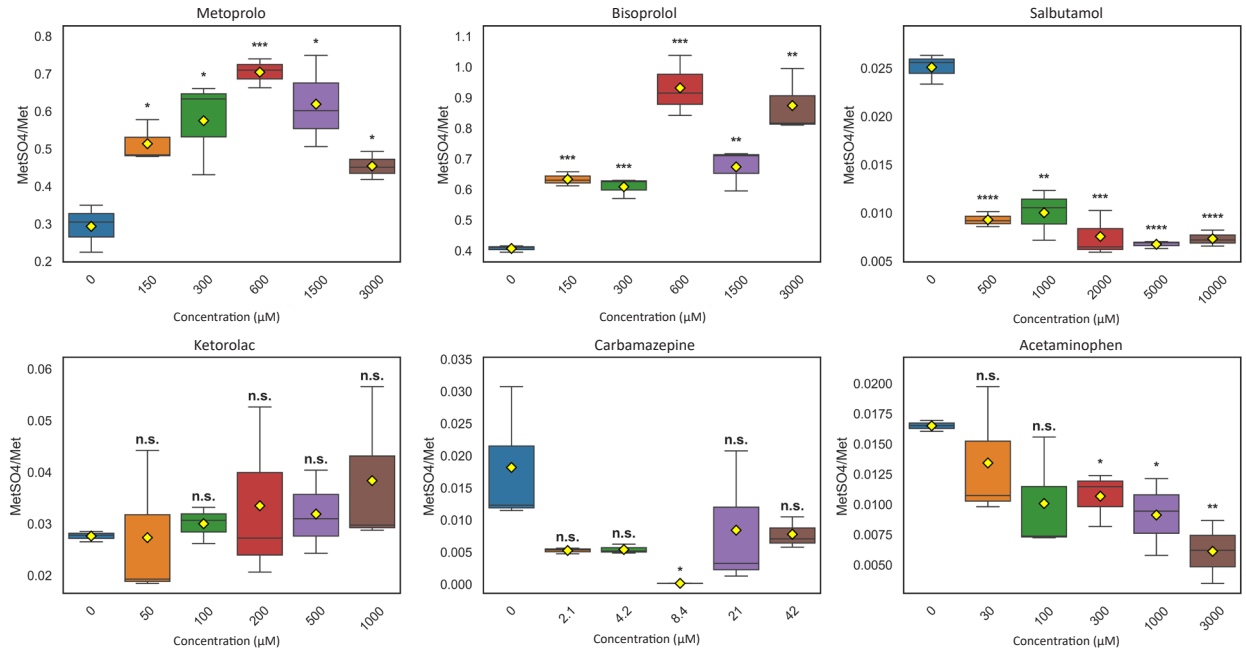

Figure 4 – Dependence of the ratio of methionine sulfoxide (MetSO4)/methionine concentrations in Danio rerio embryos on the concentration of the studied drugs (μM).

Note: The median value is marked with a yellow rhombus, n.s. — $p > 0.05$, * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$, **** — $p < 0.0001$.

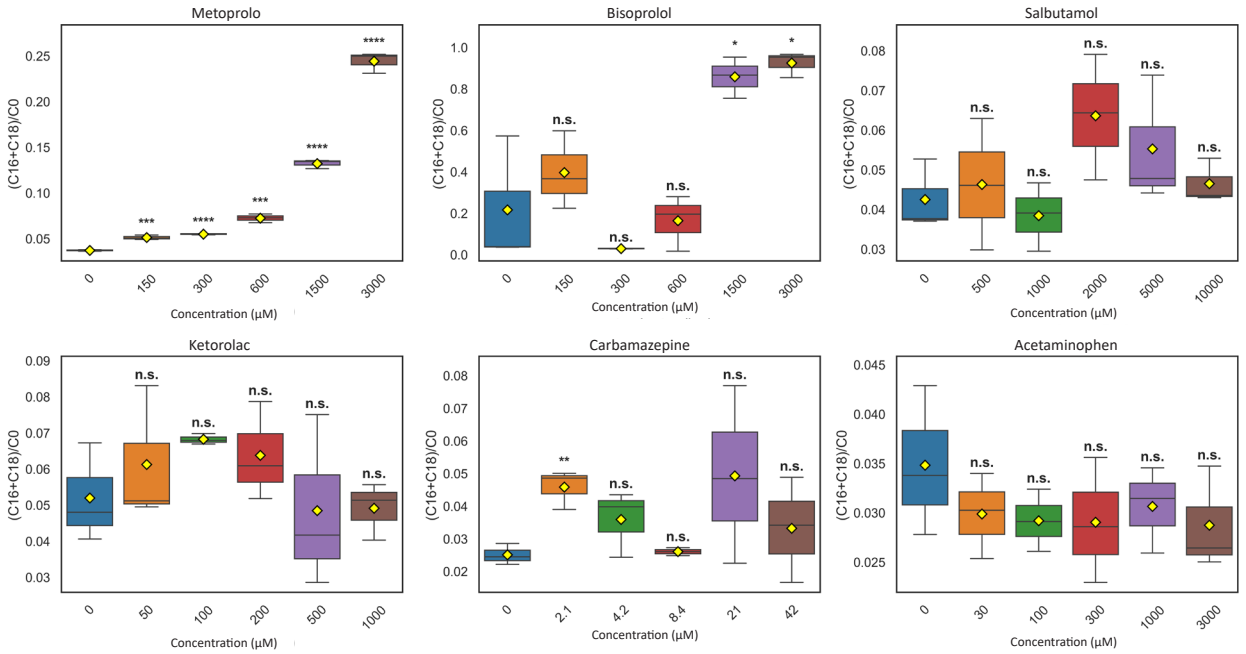

Figure 5 – Dependence of the ratio of (C16+C18)/C0 concentrations in Danio rerio embryos on the concentration of the studied drugs (μM).

Note: The median value is marked with a yellow rhombus, n.s. — $p > 0.05$, * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$, **** — $p < 0.0001$.

Table 1 – Studied substances

Studied substances	Main targets / mechanism of action	Main manifestation of cardiotoxicity[1]	NOEC, μM	Concentrations, μM
Acetaminophen	COX–1/2 inhibitor	Sudden cardiac death (in overdose)	300	150, 300, 600, 1500, 3000
Carbamazepine	Na–channel blocker, enhancement of GABA–ergic transmission	Heart rhythm disorders	4,2	2,1, 4,2, 8,4, 21, 42
Ketorolac tromethamine	COX–2 inhibitor	Heart failure (chronically)	100	50, 100, 200, 500, 1000
Metoprolol (tartrate)	β1–adrenoblocker	Acute myocardial infarction (overdose)	300	150, 300, 600, 1500, 3000
Bisoprolol (fumarate)	β1–adrenoblocker	Heart failure (in high doses)	300	150, 300, 600, 1500, 3000
Salbutamol	β2–adrenomimetic	Tachyarrhythmia (in overdose)	1000	500, 1000, 2000, 5000, 10000
Cetirizine hydrochloride	H1–histamine receptor blocker	No cardiotoxicity	5	2,5, 5, 10, 25, 50

Note: COX — cyclooxygenase; GABA — gamma–aminobutyric acid.

[1] FDA's Adverse Event Reporting System (FAERS). – [Электронный ресурс]. – Режим доступа: https://www.fda.gov/drugs/surveillance/fdas–adverse–event–reporting–system–faers

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Table 2 – *Log2 FC* values for measured metabolites (experiment *vs* control) under the influence of different compounds, when incubated at a concentration of 10×NOEC

Metabolite / Index	Cetirizine	Acetaminophen	Carbamazepine	Salbutamol	Ketorolac	Bisoprolol	Metoprolol
Branched–chain amino acids (BCAA)							
BCAA (Leucine + isoleucine + valine)	–0.233 (0.080)	0.568 (0.016)	**1.096 (0.006)**	**1.379 (0.001)**	**2.181 (0.001)**	0.590 (<0.001)	0.887 (<0.001)
Σ(Leucine + isoleucine)	–0.047 (0.732)	0.615 (0.030)	**1.359 (0.001)**	**1.113 (0.005)**	**2.522 (0.003)**	0.450 (0.002)	0.885 (<0.001)
Isoleucine	–0.216 (0.068)	0.521 (0.063)	0.753 (0.291)	**1.694 (<0.001)**	1.900 (0.012)	0.717 (0.001)	0.889 (<0.001)
Endothelial dysfunction, NO pathway							
Fisher index	–0.182 (0.080)	–0.428 (0.021)	–0.061 (0.841)	**1.164 (0.006)**	1.048 (0.013)	–0.868 (0.003)	–0.060 (0.145)
ADMA	–0.264 (0.255)	0.892 (0.061)	**2.943 (<0.001)**	**–1.409 (<0.001)**	–0.002 (0.996)	**1.318 (0.008)**	0.608 (0.037)
ADMA / Arg	0.280 (0.076)	0.821 (0.021)	**1.273 (0.002)**	0.418 (0.009)	1.204 (0.022)	0.141 (0.560)	0.357 (0.138)
Arginine	0.013 (0.913)	–0.246 (0.499)	**1.637 (0.003)**	**–1.832 (<0.01)**	–1.25 (0.012)	**1.190 (<0.01)**	**–1.137 (<0.01)**
Citrulline / Arg	0.529 (0.029)	0.144 (0.533)	**–0.937 (<0.001)**	**1.655 (0.003)**	1.245 (0.024)	–0.929 (0.263)	0.313 (0.597)
Homoarginine / ADMA	0.214 (0.599)	–0.803 (0.018)	–0.878 (0.058)	–0.390 (0.032)	–0.518 (0.339)	–0.279 (0.297)	–0.514 (0<048)
Homoarginine / SDMA	0.575(0.161)	0.284 (0.381)	1.405 (0.150)	–0.390 (0.018)	–0.371 (0.380)	0.974 (0.003)	–0.037 (0.615)
Homoarginine	–0.090 (0.820)	–0.252 (0.473)	**1.191 (0.002)**	**–1.809 (0.001)**	0.549 (0.020)	0.977 (0.001)	0.060 (0.388)
SDMA	**–0.648 (0.013)**	0.559 (0.058)	1.032 (0.249)	–1.422 (0.007)	**–0.150 (0.565)**	0.007 (0.828)	0.097 (<0.001)
GABR	0.006 (0.952)	–0.260 (0.281)	0.780 (<0.001)	**–1.345 (0.001)**	–0.823 (0.068)	**1.256 (<0.001)**	0.578 (0.010)
Oxidative stress							
Methionine	–0.842 (0.012)	0.500 (0.023)	0.825 (0.270)	0.207(–0.202)	0.556 (0.165)	–0.017 (0.856)	0.759 (<0.001)
Methionine sulfoxide	**–1.205 (0.001)**	–0.877 (0.055)	–0.279 (0.403)	**–1.579 (<0.001)**	0.927 (0.006)	0.700 (<0.001)	**1.389 (0.001)**
Methionine sulfoxide / Met	–0.352 (0.102)	**–1.434 (0.002)**	0.011 (0.963)	**–1.776 (<0.001)**	0.474 (0.304)	0.727 (0.003)	0.631 (0.019)
Glutamine	–0.998 (0.005)	–0.661 (0.035)	0.280 (0.418)	–0.733 (0.005)	0.038 (0.897)	**1.502 (0.003)**	–0.875 (0.001)
Glutamate	–0.441 (0.028)	–0674 (0.037)	0.011 (0.963)	–0.733 (0.005)	0.277 (0.453)	1.829 (0.016)	–0.815 (0.002)
Glutamine / Glut	–0.569 (0.044)	–0.027 (0.922)	0.213 (0.386)	–1.662 (0.003)	–0.192 (0.423)	–0.176 (0.666)	–0.060 (0.158
GSG	–0.433 (0.222)	–0.055 (0.466)	–0.233 (0.542)	**1.448 (0.007)**	0.251 (0.013)	1.109 (0.043)	0.834 (0.025)
Taurine	0.094 (0.475)	–0.321 (0.124)	0.017 (0.930)	–0.339 (0.224)	0.644 (0.016)	0.056 (0.657)	0.693 (0.002)
Mitochondrial dysfunction							
C0	0.454 (0.241)	–0.735 (0.01)	0.080 (0.329)	0.442 (0.081)	–0.394 (0.203)	**–1.243 (<0.01)**	**–1.866 (<0.01)**
C2	1.267 (0.049)	–0.607 (0.064)	**1.258 (0.004)**	0.322 (0.226)	0.429 (0.030)	1.366 (0.133)	**1.901 (<0.001)**
(C2+C3) / C0	1.009 (0.015)	–0.501 (0.090)	**1.205 (0.001)**	0.060 (0.830)	0.735 (0.007)	1.603 (0.076)	**2.394 (<0.001)**
C2 / C0	1.510 (0.108)	–0.748 (0.051)	1.347 (0.016)	0.623 (0.059)	–0.003 (0.992)	1.154 (0.215)	**1.304 (<0.001)**
(C16+C18) / C0	0.164 (0.420)	0.826 (0.021)	1.438 (0.056)	0.327 (0.014)	0.282 (0.122)	**3.809 (0.009)**	**1.268 (<0.01)**
Lipid metabolism							
C18:1 / C18	0.454 (0.241)	–0.735 (0.01)	0.080 (0.329)	0.442 (0.081)	–0.394 (0.203)	–1.243 (<0.01)	–1866 (<0.001)
(C16+C18:1) / C2	1.267 (0.049)	–0.607 (0.064)	**1.258 (0.004)**	0.322 (0.226)	0.429 (0.030)	1.366 (0.133)	**1.901 (<0.001)**
C16 / C0	1.009 (0.015)	–0.501 (0.090)	**1.205 (0.001)**	0.060 (0.830)	0.735 (0.007)	1.603 (0.076)	**2.394 (<0.001)**
C18:1 / C0	1.510 (0.108)	–0.748 (0.051)	1.347 (0.016)	0.623 (0.059)	–0.003 (0.992)	1.154 (0.215)	**1.304 (<0.001)**
Σ(acylcarnitines)/C0	0.164 (0.420)	0.826 (0.021)	1.438 (0.056)	–0.327 (0.014)	–0.282 (0.122)	**3.809 (0.009)**	**–1.268 (<0.01)**
C3 / C2	0.637 (0.067)	0.372 (0.062)	0.115 (0.781)	0.263 (0.233)	–0.188 (0.344)	–0.463 (0.063)	0.355 (<0.001)
C4 / C2	0.243 (0.290)	–0.020 (0.958)	0.348 (0.228)	0.221 (0.160)	–0.871 (0.002)	–0.625 (0.008)	**3.594 (<0.001)**
C8 / C10	–0.595 (0.082)	–0.414 (0.075)	0.190 (0.684)	0.493 (0.139)	0.248 (0.423)	0.552 (0.373)	–1.881 (0.331)
Σ(C2–C5)	–0.446 (0.007)	–0.022 (0.879)	0.381 (0.058)	–0.406 (0.043)	–0.001 (0.997)	**1.367 (0.008)**	**1.095 (<0.001)**
Σ(C6–C12)	–0.295 (0.011)	**–1.731 (0.001)**	0.295 (0.435)	0.138 (0.070)	–0.023 (0.873)	0.023 (0.876)	–0.120 (0.085)
Σ(C14–C18)	0.066 (0.247)	–0.937 (0.005)	0.523 (0.029)	0.488 (<0.001)	0.137 (0.345)	–0.352 (0.001)	**1.547 (<0.001)**
Aromatic amino acids							
Phenylalanine	–0.031 (0.633)	**1.115 (<0.01)**	**1.157 (<0.01)**	0.027 (0.887)	**1.129 (<0.01)**	**0.559 (<0.01)**	**0.617 (<0.01)**
Threonine	–0.501 (0.011)	0.043 (0.816)	–0.161 (0.232)	–0.546 (0.022)	–0.050 (0.855)	**1.128 (<0.01)**	**2.561 (<0.01)**
Tryptophan	–0.520 (0.047)	0.695 (0.015)	0.454 (0.623)	0.104 (0.443)	0.877 (0.026)	1.684 (<0.01)	1.032 (<0.001)
Tyrosine	0.302 (0.120)	1.078 (0.021)	1.306 (**<0.01**)	0.429 (0.033)	**1.287 (<0.01)**	**2.312 (<0.01)**	**1.364 (<0.01)**
Tyrosine / Phenylalanine (Tyr / Phe)	0.340 (0.170)	0.025 (0.715)	–0.025 (0.963)	0.149 (0.502)	0.105 (0.750)	1.758 (0.001)	0.738 (0.006)

Note: Data are presented as *log2 FC* (*p*–value). Changes with |*log2FC*| >1 at *p* <0.01 are highlighted in bold. C0 — carnitine; C2 — acetylcarnitine; C3 — propionylcarnitine; C4 — butyrylcarnitine; C5 — isovalerylcarnitine; C6 — hexanoylcarnitine; C8 — octanoylcarnitine; C10 — decanoylcarnitine; C12 — dodecanoylcarnitine; C14 — tetradecanoylcarnitine; C16 — hexadecanoylcarnitine; C18 — stearoylcarnitine; C18:1 — linoleylcarnitine

Table 3 – Relative contribution of the studied drugs according to three criteria (mitochondrial dysfunction, oxidative stress, impaired NO signaling pathway)

Substance	Mitochondrial dysfunction	Oxidative stress	Impaired NO signaling pathway
Cetirizine	–	–	–
Acetaminophen	+	–	+
Carbamazepine	+	–	+++
Salbutamol	+	–	++
Ketorolac	+++	++	++
Bisoprolol	+++	+++	+++
Metoprolol	+++	+++	+++

Note: "+++" — maximum, "++" — strong, "+" — moderate, "–" — no contribution.

Table 3 shows a comparative assessment of the contribution of each of the studied medicines to the development of key mechanisms of cardiotoxicity — mitochondrial dysfunction, oxidative stress, and disorders of the NO signaling pathway.

As it can be seen from Table 3, the most significant mitochondrial dysfunction was observed under the action of β1-adrenoblockers (bisoprolol, metoprolol) and ketorolac — these drugs caused the maximum accumulation of acylcarnitines and related indicators. Regarding OS, the same β1-adrenoblockers are in the lead (significant increase in methionine sulfoxide), ketorolac makes a slightly smaller contribution, while acetaminophen and salbutamol practically did not cause pro-oxidant effects. Impairment of the NO signaling pathway is most pronounced in carbamazepine (maximum increase in ADMA) and β1-adrenoblockers (significant increase in ADMA with a simultaneous decrease in arginine); ketorolac, acetaminophen, and salbutamol had a moderate effect on this pathway. Thus, systemic metabolomic analysis revealed a complex of interrelated mechanisms of CT in zebrafish embryos after exposure to drugs. The accumulation of ADMA and acylcarnitines in cardiac tissue reflects an energy imbalance.

Model adequacy

The use of zebrafish embryos to assess the cardiometabolic effects of medicines is justified by the high conservatism of the main metabolic and signaling pathways in fish and mammals. Despite some quantitative differences (for example, the intensity of oxidative metabolism in fish embryos is lower than in the heart of adult mammals), qualitative shifts in the metabolomic profile under the influence of toxicants demonstrate good reproducibility of patterns known in higher organisms. The data are consistent with the results of other studies. Thus, earlier cardiotoxic effects were associated with an increase in the level of BCAA and aromatic amino acids associated with metabolic overload of the heart [9, 35], as well as with the accumulation of long acylcarnitines as a precursor to the development of myocardial dysfunction [36, 37]. It is important to emphasize that zebrafish as an experimental model allows tracking these changes at early stages, even before the appearance of irreversible morphological disorders, which opens up the possibility of their use in screening for cardiotoxicity of new compounds.

Limitations of the study

The limitations of this study include the relatively short exposure time (4 h), which simulates acute effects but does not reflect possible compensatory-adaptive reactions with prolonged exposure. For example, with chronic administration of β-blockers, the mammalian body adapts by increasing the density of β-receptors, improving coronary blood flow, which softens metabolic shifts — in fish, similar mechanisms can also reduce toxicity. In addition, the concentrations of drugs we used (up to 10×NOEC) are high enough to detect sublethal effects; in a real clinical situation, such doses correspond to overdoses. However, it is in conditions of overdose that the mechanisms of toxicity manifest themselves "in their pure form", which was required to be demonstrated. In addition, it wasn't taken into account gender differences and the influence of hormonal background (embryos before sexual differentiation).

CONCLUSION

The study showed that metabolomics of Danio rerio embryos reflected the effects of cardiotoxic medicines, revealing specific changes in metabolic biomarkers. In particular, medicines with a risk of arrhythmia (salbutamol, carbamazepine) are characterized by the accumulation of BCAA and an imbalance of NO / ADMA; for cardiodepressants (selective β1-adrenoblockers) — pronounced Mitochondrial dysfunction with the accumulation of acylcarnitines and oxidative stress; for non-steroidal anti-inflammatory drugs (ketorolac) — a combination of moderately pronounced energy and oxidative shifts. At the same time, acetaminophen caused only minor metabolic changes, and the control antihistamine medicine cetirizine did not affect the parameters under consideration, which confirms the absence of cardiotoxicity in this model.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Natalia E. Moskaleva — conducting the experimental part of the work, statistical processing of the results, preparation of a preliminary version of the manuscript; Viktor M. Samoylov — conducting the experimental part of the work, reviewing literary sources; Pavel M. Rezvanov — conducting the experimental part of the work, statistical processing of the results; Valeria G. Varzieva — conducting the experimental part of the work; Sabina N. Baskhanova — statistical processing of the results; Vadim V. Tarasov — approval of the final version of the manuscript; Elena A. Smolyarchuk — analysis of the literature sources and the results obtained in the work; Dmitry A. Kudlay — analysis of the results of the work with their interpretation and conclusions, approval of the final version of the manuscript; Svetlana A. Appolonova — development of the research concept, approval of the final version of the manuscript. All authors confirm that their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept and preparation of the article, read and approved the final version before publication).

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Dynamics of turnover of sugar-lowering drugs in the retail segment of the pharmaceutical market from 2020 to 2024

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A significant part of patients with diabetes purchase sugar-lowering drugs (SLDs) on their own in retail pharmacy chains, and the market trends they generate represent an urgent area for this study.

The aim. To study the turnover of hypoglycemic drugs, including insulins, in the commercial sector of the pharmaceutical market of the Russian Federation (RF) for 2020–2024.

Materials and methods. The data presented in this article is based on a periodic retail audit conducted by the biotech company IQVIA. The period of 5 years (2020–2024) was covered. Data collection covered 33,613 commercial pharmacies in all regions of the RF (about 43% of pharmacies).

Results. In the period from 2020 to 2023, a relatively stable increase in the volume of SLDs purchases was revealed, with small fluctuations, while in 2024 a significant leap in consumption was recorded, both in quantitative (packages) and in value (rubles) terms. During the entire monitoring period, the total volume of SLDs and insulin purchases in the retail market segment increased by more than 1.2 times, from 41.98 million packages in 2020 to 51.70 million in 2024. At the same time, the total procurement budget (at wholesale prices) increased by more than 2 times — from 19.40 to 41.57 billion rubles. The average price per package has also increased significantly, from 462 rubles in 2020 to 923 rubles in 2024, an increase of 1.7 times, reflecting significant inflation in the commercial pharmaceutical sector. In general, the Russian pharmaceutical market offers all variants of modern innovative hypoglycemic drugs (dipeptidyl peptidase-4 inhibitors [iDPP4], sodium-glucose cotransporter 2 inhibitors [iSGLT2], glucagon-like peptide-1 receptor agonists [arGPP-1]), which, according to general trends, demonstrate a steady increase in turnover. Older and less effective sulfonylureas and their combinations with metformin still occupy a significant market share. arGPP-1 group showed the greatest growth in quantity (11 times) and value (5 times), mainly due to the entry of Russian semaglutide analogues into the market. Government measures and investments in import substitution have led to a significant increase in the share of Russian drugs in purchases. Despite the positive dynamics of Russian production, import substitution in the SLDs segment has not achieved complete success and accounts for less than half of the turnover of hypoglycemic drugs.

Conclusion. The growth of SLDs consumption in the retail segment of the RF in 2020–2024 has been revealed, especially due to the innovative drugs arGPP-1 and iNGLT2. Despite maintaining the leading position of metformin, the consumption structure is shifting towards more expensive schemes. The increase in the total procurement budget and the average package price highlights the need to expand preferential security programs. Import substitution has increased significantly over the indicated period, however, the share of domestic drugs is still less than half of the turnover.

Keywords: diabetes; sugar-lowering drugs; hypoglycemic agents; pharmacoepidemiology; insulin

Abbreviations: arGPP-1 —glucagon-like peptide-1 receptor agonists; ATC — Anatomical Therapeutic Chemical classification; GEHI — genetically engineered human insulin; iDPP-4 — dipeptidyl peptidase-4 inhibitors; iSGLT2 — sodium-glucose cotransporter 2 (SGLT2) inhibitors; DM — diabetes mellitus; SLDs — sugar-lowering drugs, FD — federal district; INN — international nonproprietary name; TN — trade name; ADA — American Diabetes Association; IDF — International Diabetes Federation.

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Динамика оборота сахароснижающих лекарственных препаратов в розничном сегменте фармацевтического рынка с 2020 по 2024 гг.

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Значительная доля пациентов с сахарным диабетом приобретает сахароснижающие препараты (ССП) самостоятельно в розничных аптечных сетях, и формируемые ими рыночные тренды представляют собой актуальную область для исследования.

Цель. Изучить оборот гипогликемических препаратов, включая инсулины, в коммерческом секторе фармацевтического рынка Российской Федерации (РФ) за 2020–2024 гг.

Материалы и методы. Данные, представленные в настоящей статье, основаны на периодическом розничном аудите, проводимом биотехнологической компанией IQVIA. Охватываемый период составил 5 лет (2020–2024 гг.). Сбор данных охватил 33 613 коммерческие аптечные организации (АО) на территории всех субъектов РФ (около 43% АО).

Результаты. В период с 2020 по 2023 гг. выявлен относительно стабильный рост объёма закупок ССП, с небольшими колебаниями, тогда как в 2024 году фиксируется значительный скачок потребления, как в количественном (упаковки), так и в стоимостном (рубли) выражении. За весь период наблюдения общий объём закупок ССП и инсулина в розничном сегменте рынка увеличился более чем в 1,2 раза — с 41,98 млн. упаковок в 2020 году до 51,70 млн. в 2024 году. При этом совокупный бюджет закупок (по оптовым ценам) вырос более чем в 2 раза — с 19,40 до 41,57 млрд руб. Средняя цена за упаковку также значительно возросла — с 462 руб. в 2020 году до 923 руб. в 2024 году, увеличившись в 1,7 раза, что отражает существенную инфляцию в коммерческом фармацевтическом секторе. В целом на российском фармацевтическом рынке представлены все варианты современных инновационных гипогликемических лекарственных препаратов (ингибиторы дипептидилпептидазы-4 [иДПП4], ингибиторы натрий-глюкозного котранспортера 2 типа [иНГЛТ2], агонисты рецепторов глюкагоноподобного пептида-1 [арГПП-1]), которые согласно общим тенденциям, демонстрируют стабильное увеличение оборота. Существенную долю рынка до настоящего времени занимали более старые и менее эффективные препараты сульфонилмочевины и их комбинации с метформином. Наибольший рост в количественном (в 11 раз) и стоимостном (в 5 раз) выражении показала группа арГПП-1, главным образом благодаря выходу на рынок российских аналогов семаглутида. Государственные меры и инвестиции в отношении импортозамещения привели к значительному увеличению в закупках доли отечественных препаратов. Несмотря на положительную динамику отечественного производства, импортозамещение в сегменте ССП не достигло полного успеха и составляет менее половины оборота гипогликемических лекарственных средств.

Заключение. Выявлен рост потребления ССП в розничном сегменте РФ за 2020–2024 гг., особенно за счёт инновационных препаратов арГПП-1 и иНГЛТ2. Несмотря на сохранение лидирующих позиций метформина, структура потребления смещается в сторону более дорогостоящих схем. Увеличение совокупного бюджета закупок и средней цены упаковки подчёркивает необходимость расширения программ льготного обеспечения. Импортозамещение заметно выросло за обозначенный период, однако доля отечественных препаратов всё ещё составляет менее половины оборота.

Ключевые слова: сахарный диабет; сахароснижающие препараты; гипогликемические средства; фармакоэпидемиологический анализ; инсулин

Список сокращений: АО — аптечная организация; арГПП-1 — агонист рецептора глюкагоноподобного пептида-1; АТХ — анатомо-терапевтическо-химическая система классификации; ГИЧИ — генно-инженерный человеческий инсулин; иДПП-4 — ингибитор дипептидилпептидазы-4; иНГЛТ2 — ингибитор натрий-глюкозного котранспортера 2 типа; ЛП — лекарственный препарат; ЛС — лекарственное средство; СД — сахарный диабет; ССП — сахароснижающие препараты; ФО — федеральный округ; МНН — международное непатентованное наименование; ТН — торговое наименование; ADA — Американская ассоциация диабета; IDF — Международная диабетическая федерация.

INTRODUCTION

Type 1 and Type 2 diabetes mellitus (T1DM and T2DM) remains a serious global worldwide problem, including the Russian Federation (RF). According to the International Diabetes Federation (IDF), the total number of adults aged 20–79 years suffering from diabetes mellitus (including diagnosed and undiagnosed cases) increased from 537 million (in 2021) to 589 million (11.1% of this age group) in 2025. It is expected that by 2050 this value will reach 852.5 million people [1–3]. January 1, 2024, there were 5 547 879 patients with diabetes mellitus registered in the Russian Federation, 5 168 374 of which had T2DM, 288 020 were adults with T1DM, and 61 318 were children with T1DM under the age of 18 [4]. An important factor contributing to the increase in the incidence of T2DM is the global obesity epidemic. This emphasizes the need to improve the diagnosis and regulation of carbohydrate metabolism, since a significant part of the disorders remains undetected [5].

The high prevalence of diabetes mellitus determines the population's need for sugar-lowering drugs (SLDs). Modern pharmacotherapy of T2DM covers a wide range of medicines that differ in the mechanism of action and type of dosage form. This allows doctors to select individual, personalized treatment regimens, including using rational combinations [6–8]. However, in practice, the availability of modern medicines is often limited.

Patients with diabetes mellitus in the Russian Federation belong to the category of citizens entitled to preferential drug provision. Among the medicines in public procurement, there are also innovative SLDs, however, their share is limited, and the system itself is inert and lags behind current clinical guidelines. In addition, it does not take into account the objective picture and does not cover the real needs of patients [9–11]. In the context of sanctions, there are often cases of supply chain disruptions and shortages of SLDs, and there is a need for compulsory licensing of imported medicines [12–14].

A significant part of citizens are forced to purchase SLDs at their own expense in pharmacies, which emphasizes the relevance of analyzing the structure and volumes of SLDs circulation in the commercial pharmaceutical sector.

THE AIM. To study the circulation of sugar-lowering drugs, including insulin medicines, in the commercial sector of the pharmaceutical market of the Russian Federation for the period from 2020 to 2024. To achieve this aim, the authors formulated the following tasks:

1. Based on data obtained from commercial pharmacies, analyze: the number of purchased SLDs in the context of individual groups and names; the budget allocated for the purchase of these drugs; the weighted average prices per package of drugs;
2. Study the dynamics of the above indicators for 5 years (2020–2024);
3. Identify the main trends in SLDs circulation within pharmaceutical groups and individual names.

MATERIALS AND METHODS

Based on retrospective data, an analysis of SLDs circulation in the retail segment of the pharmaceutical market for 5 years (2020–2024) was carried out.

The initial data were obtained and transferred for research by IQVIA from large pharmacy chains and non-chain POs located in all territorial subjects of the Russian Federation (95 257 pramacies throughout Russia; Table 1) as part of the retail audit project of commercial pharmacies. Data source — database "IQVIA Sell-In Monthly & Quarterly Universe and panel. Retail sector". Classification group — ATC. Selected conditions — ATC3:A10A. Upload dated March 25, 2025. Period — from January 01, 2020 to December 31, 2024.

Information on the number of purchased packages of medicines (pcs), the total budget of purchased medicines by name (rubles) and the weighted average prices for individual medicines (rubles) was analyzed.

During the study, the Anatomical Therapeutic Chemical (ATC) Classification of medicines was carried out.

The representativeness and comparability of the study data are ensured by a unified collection methodology applied by one analytical agency. It is worth noting that during the audit a high coverage of retail pharmacies in the subjects was achieved, on average 43% (see Table 1). The five-year period of

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data analysis was chosen as optimal for assessing the dynamics of sales and the degree of demand for new drugs.

Statistical analysis

Primary data were processed using the MS Office package, namely Microsoft Office Excel 2023 (Microsoft Corp., USA) and presented as arithmetic mean values.

RESULTS

According to the results of the study of the dynamics of retail circulation of SLDs and insulin medicines in Russia over a five-year period, changes in the volume of purchases, total budget and average cost per package for various pharmacological groups were revealed.

In the period from 2020 to 2024, a relatively stable growth in the procurement budget was observed, with slight fluctuations, while in 2024 a significant growth of more than 1.5 times was recorded compared to previous years. Over the entire observation period, the total volume of purchases of SLDs and insulin medicines increased by more than 1.2 times — from 41.98 million packages in 2020 to 51.70 million in 2024. At the same time, the total procurement budget increased from 19.40 to 41.57 billion rubles, which corresponds to an increase of more than 2 times. The average price per package also increased significantly — from 462 in 2020 to 804 rubles in 2024, increasing by 1.7 times, which indicates a pronounced inflationary pressure in the commercial segment of the pharmaceutical market. Table 2 presents the dynamics of retail circulation of SLDs and insulin medicines in Russia for the five-year period from 2020 to 2024.

Insulin drugs

Over the study period, the circulation of insulin medicines increased significantly in quantitative terms from 1.23 million in 2020 to 1.43 million in 2024 (an increase of 16%). Along with this, the total procurement budget increased significantly from 1.38 to 2.22 billion rubles (an increase of 61%), and the average price per package increased from 1 127.36 to 1 557.56 rubles (an increase of 38%).

Analysis of individual subgroups of insulin medicines showed that in 2020, the largest share in terms of the number of packages was occupied by genetically engineered human insulins (GEHI) — 807 820 packages compared to analog insulins (420 097 packages). The lower cost of GEHI preparations allows to significantly reduce the budget (419 444 250 rubles vs 964 850 483 rubles, respectively). In 2024, against the background of the growth of circulation of both groups of medicines, there is a tendency to equalize the number of purchased GEHI and analog insulins in quantitative terms (676 818 and 751 399 packages, respectively; Fig. 1) — while 83% of the budget was accounted for by generics.

It was established that the cost of one package of GEHI and analog insulins is growing comparably. When studying individual international non-proprietary names (INN), it can be assumed that the price remains stable due to the import substitution policy, since a significant increase in the share of domestic medicines was found.

In the subgroup of analog insulins, the most expensive medicine in 2024 were medicines with INN insulin degludec (TN: Tresiba®, manufacturer: Novo Nordisk A/S, Denmark, Novo Nordisk LLC, Russia) and its combination with insulin aspart, (TN: Ryzodeg®, manufacturer: Novo Nordisk A/S, Denmark, Novo Nordisk LLC, Russia); insulin glargine at a dosage of 300 IU/mL (TN: Toujeo Solostar®, manufacturer: Sanofi Vostok JSC, Russia) and its combination with lixisenatide (TN: Soliqua Solostar®, manufacturer: Sanofi-Aventis Deutschland GmbH, Germany, Sanofi Vostok JSC, Russia). At the same time, the largest number of packages purchased drugs with INN: insulin aspart (TN: NovoRapid FlexPen®, manufacturer: Novo Nordisk LLC, Russia) and insulin detemir (TN: Levemir FlexPen®, manufacturer: Novo Nordisk Producao Farmaceutica do Brazil Ltda., Brazil, Novo Nordisk LLC, Russia); insulin glargine at a dosage of 100 IU/mL (TN: Lantus Solostar®, manufacturer: Sanofi Vostok JSC, Russia), insulin lispro (TN: Humalog®, manufacturer: Lilly France, France). Among GEHI, the most expensive medicines in 2024 were medicines with INN: insulin soluble [human genetically engineered] (TN: Rinsulin® R, manufacturer: GEROpharm LLC, Russia) and insulin biphasic [human genetically engineered] (TN: Rinsulin Mix 30/70, manufacturer: GEROpharm LLC, Russia). The largest number of packages purchased SLDs with INN: insulin-isophane [human genetically engineered] (TN: Protaphane® HM Penfill, manufacturer: Novo Nordisk Producao Farmaceutica do Brazil Ltda., Brazil and TN: Biosulin® N, manufacturer: Pharmstandard-UfaVITA OJSC, Russia) and insulin soluble [human genetically engineered] (TN: Actrapid® NM, manufacturer: Novo Nordisk Producao Farmaceutica do Brazil Ltda., Brazil and TN: Biosulin® R, manufacturer: Pharmstandard-UfaVITA OJSC, Russia).

Gliptins

Dipeptidyl peptidase 4 inhibitors (DPP-4 inhibitors) in the form of monopreparations were purchased in

increasing volumes — from 2.45 million packages in 2020 to 4.66 million in 2024 (an increase of 90%). The procurement budget increased from 2.33 to 4.01 billion rubles (an increase of 72%), and the average price per package decreased from 952.79 to 879.11 rubles (a decrease of 7.7%). Purchases are carried out taking into account the following INNs: alogliptin, evogliptin, gozogliptin, linagliptin, saxagliptin, sitagliptin, vildagliptin.

Throughout the entire study period, the original vildagliptin drug (TN: Galvus®, manufacturer: Novartis Neva LLC, Russia) is in the greatest demand, in addition, a high share of purchases is occupied by the preparation with INN alogliptin (TN: Vipidia®, manufacturer: Takeda Ireland Limited, Ireland, Hemopharm LLC, Russia) and generic medicine with INN sitagliptin (TN: Asiglia®, manufacturer: KRKA, d.d., Novo mesto JSC, Slovenia). The highest cost per package was noted for the original DPP-4 inhibitors with INN: linagliptin (TN: Trajenta®, manufacturer: West-Ward Columbus Inc., USA, Dragenopharm Apotheker Püschl GmbH, Germany), and drug with INN saxagliptin (TN: Onglisa®, manufacturer: AstraZeneca UK Limited, Great Britain, AstraZeneca Pharmaceuticals LP, USA).

Glucagon-like peptide 1 receptor agonists (GLP-1 receptor agonists)

A significant increase in the circulation of medicines of the GLP-1 receptor agonist group was revealed. The number of purchases increased by 10.3 times — from 0.20 million packages in 2020 to 2.06 million in 2024. The total procurement budget increased by more than 5.2 times — from 2.30 to 11.90 billion rubles. The average price per package decreased from 11 731.94 to 5 766.36 rubles, which indicates a significant increase in the share of generics in this group.

Among the INNs, it is worth noting medicines with INN: dulaglutide (TN: Trulicity®, manufacturer: Eli Lilly and Company, USA, Eli Lilly Italia S.p.A., Italy), exenatide (TN: Baeta® and Baeta® Long, manufacturer: Baxter Pharmaceutical Solutions LLC, USA, TN: Enestia Belgium N.V., Belgium), liraglutide (TN: Victoza®, manufacturer: Novo Nordisk A/S, Denmark, TN: Saxenda®, manufacturer: Novo Nordisk A/S, Denmark and generic TN: Enligria®, manufacturer: Medsintez Plant LLC, Russia), semaglutide (TN: Ozempic®, manufacturer: Novo Nordisk A/S, Denmark and TN: Rybelsus®, manufacturer: Novo Nordisk A/S, Denmark), as well as generics (TN: Semavik®, manufacturer: GEROpharm LLC, Russia and TN: Kvinsenta®, manufacturer: Medsintez Plant LLC, Russia).

The GLP-1 receptor agonist group belongs to the most expensive and most high-tech medicines for the treatment of T2DM. It is worth noting that against the background of the release of domestic semaglutide generics on the market in 2023, the cost of which is more than two times lower than the original medicine, there is a noticeable and rapid increase in the volume of their purchase. If in 2023 only 8 484 packages of generics were purchased (retail sector of the pharmaceutical market), then in 2024 their number counted 1 941 733 packages, the budget increased from 0.41 to 9.90 billion rubles (in wholesale prices). Note that due to the widespread use of drugs with INN semaglutide by patients with obesity and overweight, these data should be interpreted with caution.

Gliflozins

An increase in the circulation of medicines from the group of sodium-glucose cotransporter type 2 inhibitors (SGLT2 inhibitors) was found, for which, as for GLP-1 receptor agonists, clinical research data are accumulating on their pronounced pleiotropic effects [15]. The number of purchases of SGLT2 inhibitors increased from 0.95 million packages in 2020 to 4.24 million in 2024 (an increase of 4.5 times). The procurement budget increased from 2.30 to 11.33 billion rubles (in wholesale prices, an increase of 4.9 times), and the average package price increased from 2 425.00 to 2 670.93 rubles (an increase of 10%). Among the INNs, only original molecules are represented: canagliflozin (TN: Invokana®, manufacturer: Janssen-Cilag S.p.A., Italy), dapagliflozin (TN: Forxiga®, manufacturer: AstraZeneca Industries LLC, Russia), empagliflozin (TN: Jardiance®, manufacturer: Boehringer Ingelheim Pharma GmbH & Co.KG, Germany), ertugliflozin (TN: Steglatra®, manufacturer: Schering-Plough Labo N.V., Belgium), ipragliflozin (TN: Suglat®, manufacturer: Astellas Pharma Europe B.V., Netherlands, Astellas Pharma Inc., Japan).

Significant changes in prices for medicines of the SGLT2 inhibitor group have not been recorded. The most expensive medicine with TN: Jardiance® (manufacturer: Boehringer Ingelheim Pharma GmbH & Co.KG, Germany). At the same time, drug with TN Forxiga® (manufacturer: AstraZeneca Industries LLC, Russia) is consistently leading in terms of the number of purchases.

Sulfonylurea derivatives

Sulfonylurea derivatives are a relatively old group of medicnes, the use of which is associated with the risk of adverse events [16, 17]. Nevertheless, a moderate increase in circulation is also observed in this group. The number of purchased packages of medicines of this group increased from 12.31 million packages in 2020 to 12.37 million packages in 2024. The

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total procurement budget did not change significantly and amounted 2.84 billion rubles both in 2020 and in 2024 (in wholesale prices), and the average price per package did not change significantly — 230.81 and 229.72 rubles in 2020 and 2024, respectively.

Among the INNs are: glibenclamide, gliclazide, glimepiride, to a lesser extent — gliquidone. All medicines of this group have a significant number of generics, including domestic ones. Medicines are distinguished by a low cost, with the exception of the original medicines of glimepiride (TN: Amaryl®, manufacturer: Sanofi S.R.L., Italy) and gliquidone (TN: Glurenorm®, manufacturer: Boehringer Ingelheim Ellas Single Member S.A., Greece). In terms of the number of packages purchased, medicine with INN gliclazide (TN: Diabeton MV®, manufacturer: SERVIER RUS LLC, Russia) and TN: Gliclazide MV®, manufacturer: Ozone Pharm LLC, Russia) is leading.

Metformin and its combinations with other SLDs

Metformin monopreparations remain the most widely prescribed for T2DM. In addition, numerous pleiotropic properties of metformin and the spectrum of its prescriptions are gradually expanding beyond the hypoglycemic effect [18]. During the study period, the number of purchases in quantitative terms increased from 20.68 million packages in 2020 to 23.73 million packages in 2024 (an increase of 15%). The total procurement budget increased from 5.31 to 5.52 billion rubles (in wholesale prices, an increase of 4%), and the average price per package decreased from 256.67 to 232.52 rubles, which indicates the stability of prices for SLDs with INN metformin. The highest price per package was registered for the original metformin (TN: Glucophage® and Glucophage® Long, manufacturer: Merck Healthcare KGaA, Germany, Nanolek LLC, Russia). At the same time, these medicines are leading in quantitative terms in the structure of purchases of medicines with the above INN. The market presents manufacturers of metformin, among which the most famous Russian manufacturers are: Pharmasintez-Tyumen LLC, Ozone Pharm LLC, Avexima Siberia LLC, Biokhimik JSC, Biosintez PJSC, Marbiopharm OJSC, Rafarma JSC, Canonpharma production CJSC, AKRIKHIN JSC, ALSI Pharma JSC, Northern Star NAO, as well as foreign ones: Merck Healthcare KGaA (Germany), BZMZ OJSC (Republic of Belarus), Teva Pharmaceutical Works Private Limited Company (Hungary), Sanofi India Limited (India). As a basic SLDs, metformin is used in a large number of combined SLDs. Thus, metformin in combination with DPP4 inhibitors shows a slight decrease in the volume of purchases in quantitative terms and a slight increase in the volume of purchases — from 1.07 million packages for 1.72 billion rubles in 2020 and 1.03 million packages for 1.79 billion rubles (wholesale prices) in 2024, which indicates an increase in the cost of packaging SLDs of this group.

Among the INNs, combinations of metformin with alogliptin (TN: Vipdomet®, Takeda Ireland Limited, Ireland, Hemopharm LLC, Russia), saxagliptin (TN: Kombogliz® Prolong, manufacturer: AstraZeneca Pharmaceuticals LP, USA), sitagliptin (TN: Asiglia Met®, manufacturer: KRKA, d.d., Novo mesto JSC, Slovenia; TN: Velmetia®, manufacturer: BERLIN-PHARMA CJSC, Russia; TN: Gliptozan Plus®, manufacturer: Grotex LLC, Russia; TN: Forsiglex®, manufacturer: Pharmaceutical Plant "POLPHARMA" SA, Poland) and vildagliptin (TN: Glipvilo Met®, manufacturer: KRKA, d.d., Novo mesto JSC, Slovenia; TN: Galvus Met®, manufacturer: Novartis Neva LLC, Russia, TN: Agarta Met®, manufacturer: Gedeon Richter Romania A.O., Romania). The most popular in this group of drugs is medicine with TN Galvus Met® (manufacturer: Novartis Neva LLC, Russia).

Table 1 – Data on the number and share of pharmacy organizations in the subject of the federation included in the analysis

Name of the federal district	Total pharmacies in the subject, pcs.	Pharmacies that participated in the study, pcs.	Share of pharmacies, %
Central FD	17 241	7 200	42%
Moscow	5 267	3 065	58%
Northwestern FD	4 828	2 054	43%
St. Petersburg	2 505	1 497	60%
Southern FD including North Caucasian	13 258	4 698	35%
Volga FD	16 074	6 910	43%
Ural FD	6 470	3 148	49%
Siberian FD	8 826	3 466	39%
Far Eastern FD	3 964	1 575	40%
Total:	78 433	33 613	43%

Note: FD — federal district.

Table 2 – Dynamics of circulation of the main groups of sugar-lowering drugs in the retail sector of the pharmaceutical market for 2020–2024

Years	2020	2021	2022	2023	2024
All SLDs, including insulin preparations					
Packages purchased (pcs.)	41 983 620	44 092 338	46 606 690	46 452 922	51 703 809
Total budget (rubles, wholesale prices)	19 399 373 986.66	21 575 658 001.09	27 015 257 500.25	25 496 318 883.57	41 565 371 355
Price per package (rubles, wholesale prices)	462.07	489.35	579.64	548.86	804
Insulin					
Packages purchased (pcs.)	1 227 917	1 213 068	1 527 706	1 282 095	1 428 217
Total budget (rubles, wholesale prices)	1 384 304 733.14	1 423 936 878.49	2 057 003 760.68	1 775 871 765.01	2 224 530 834.92
Price per package (rubles, wholesale prices)	1 127.36	1 173.83	1 346.47	1 385.1	1 557.56
DPP-4 inhibitors (gliptin)					
Packages purchased (pcs.)	2 445 705	2 803 887	3 211 027	3 584 223	4 655 476
Total budget (rubles, wholesale prices)	2 330 243 158.94	2 628 006 414.58	3 079 503 746.92	3 280 956 644.31	4 092 691 843.
Price per package (rubles, wholesale prices)	952.79	937.27	959.04	915.39	879.11
GLP-1 receptor agonists					
Packages purchased (pcs.)	195 933	370 570	630 597	170 268	2 064 523
Total budget (rubles, wholesale prices)	2 298 674 359.95	3 306 719 107.73	6 079 632 584.45	2 130 251 215.79	11 904 776 932
Price per package (rubles, wholesale prices)	11 731.94	8 923.33	9 641.07	12 511.17	5 766.36
SGLT2 inhibitors (gliflozin)					
Packages purchased (pcs.)	948 178	1 346 277	1 930 851	2 846 658	4 241 580
Total budget (rubles, wholesale prices)	2 299 796 632.77	3 167 490 854.00	4 777 290 026.99	7 218 593 997.40	11 328 945 678.92
Price per package (rubles, wholesale prices)	2 425.49	2 352.78	2 474.19	2 535.81	2 670. 93
Sulfonylurea					
Packages purchased (pcs.)	12 306 257	12 580 594	12 613 142	12 279 617	12 372 406
Total budget (rubles, wholesale prices)	2 840 385 182.58	2 607 827 913.07	2 713 904 977.01	2 603 542 377.05	2 842 238 539.74
Price per package (rubles, wholesale prices)	230.81	207.29	215.16	212.02	229.72
Metformin (monotherapy)					
Packages purchased (pcs.)	20 675 298	21 840 725	23 042 793	22 871 633	23 734 275
Total budget (rubles, wholesale prices)	5 306 793 180.00	5 379 580 277.70	4 979 623 537.18	4 963 045 484.13	5 518 641 395.36
Price per package (rubles, wholesale prices)	256.67	246.31	216.10	217.00	232.52
Metformin + DPP-4 inhibitor					
Packages purchased (pcs.)	1 071 421	1 205 614	1 195 155	1 210 329	1 028 425
Total budget (rubles, wholesale prices)	1 719 096 182.76	1 841 016 497.35	1 916 666 004.09	1 949 071 449.41	1 788 161 670.71
Price per package (rubles, wholesale prices)	1 604.50	1 527.04	1 603.70	1 610.36	1 738.74
Metformin + SGLT2 inhibitor					
Packages purchased (pcs.)	78 646	126 104	163 055	195 530	240 374
Total budget (rubles, wholesale prices)	212 852 776.79	347 603 865.15	494 669 209.18	626 600 101.21	778 116 415.33
Price per package (rubles, wholesale prices)	2 706.47	2 756.49	3 033.76	3 204.62	3 237.101
Metformin + sulfonylurea					
Packages purchased (pcs.)	2 928 318	2 517 229	2 161 929	1 767 018	1 573 278
Total budget (rubles, wholesale prices)	985 339 508.09	853 480 931.40	855 044 745.64	724 262 736.02	702 344 128.84
Price per package (rubles, wholesale prices)	336.49	339.06	395.50	409.88	446.42
Another SLDs (repaglinid)					
Packages purchased (pcs.)	105 947	86 520	99 248	119 763	185 021
Total budget (rubles, wholesale prices)	21 888 271.64	20 149 707.81	23 080 289.19	33 646 962.94	68 832 682.91
Price per package (rubles, wholesale prices)	206.60	232.89	232.55	280.95	372.03

Note: GLP-1 receptor agonists — glucagon-like peptide-1 receptor agonists; DPP-4 inhibitors — dipeptidyl peptidase-4 inhibitors; SGLT2 inhibitors — sodium-glucose cotransporter type 2 inhibitors; SLDs — sugar-lowering drugs; TN — trade name.

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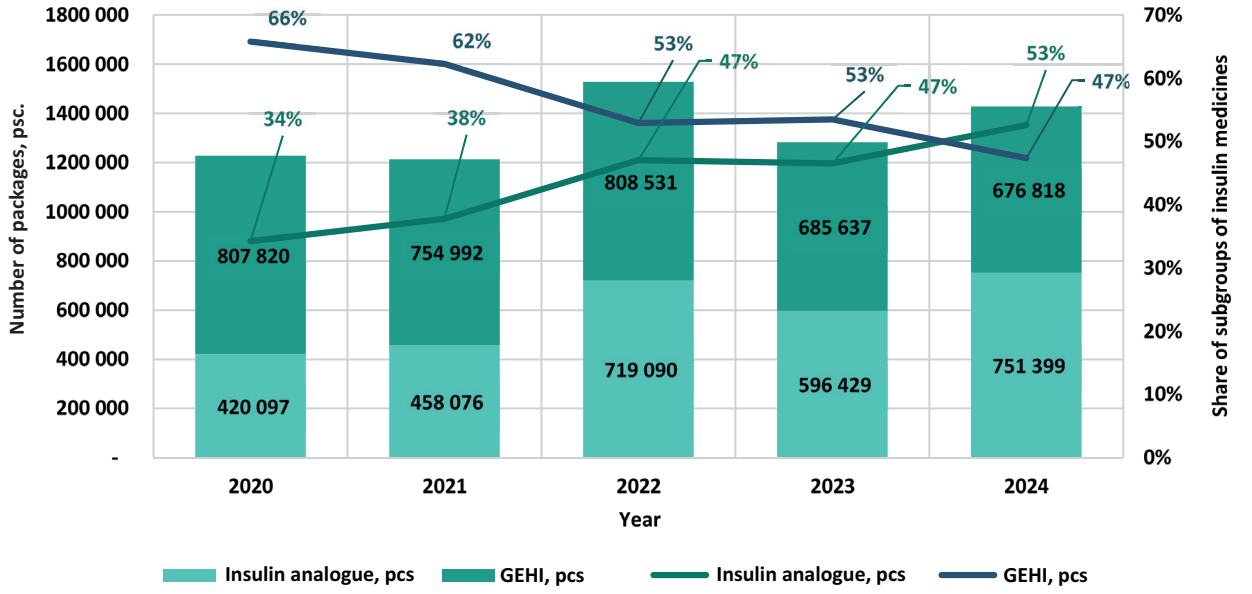

Figure 1 – Dynamics of sales of insulin drugs in the retail segment of the market in quantitative terms for the period from 2020 to 2024

Note: GEHI — genetically engineered human insulins.

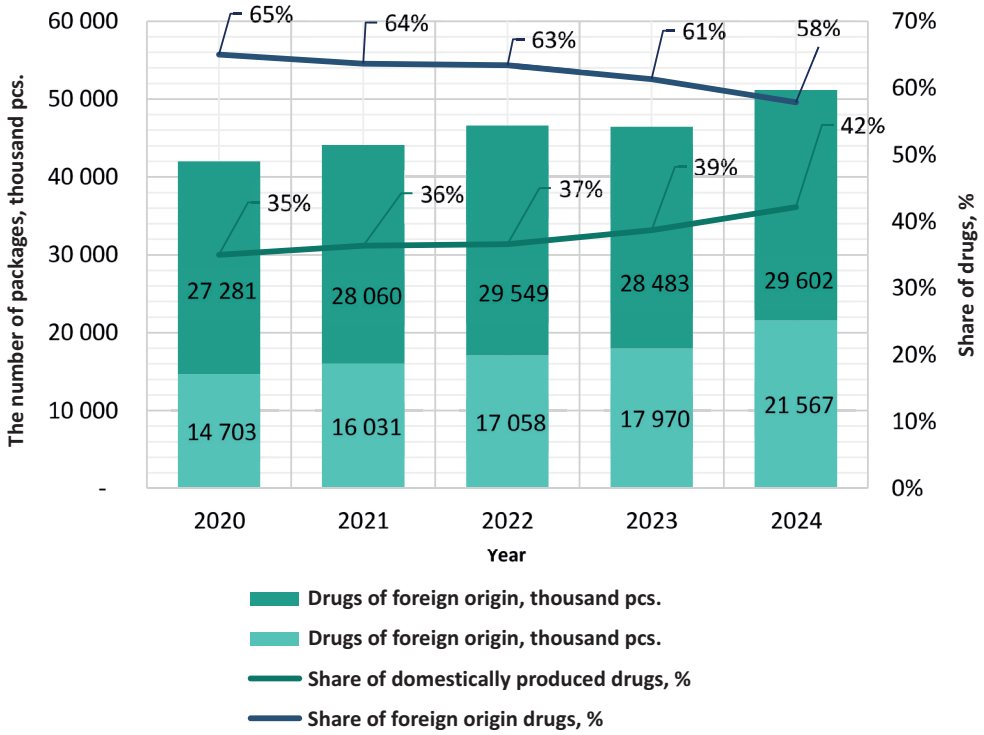

Figure 2 – Dynamics of purchase volumes of sugar-lowering drugs and insulin drugs by country of origin in quantitative terms for the period from 2020 to 2024.

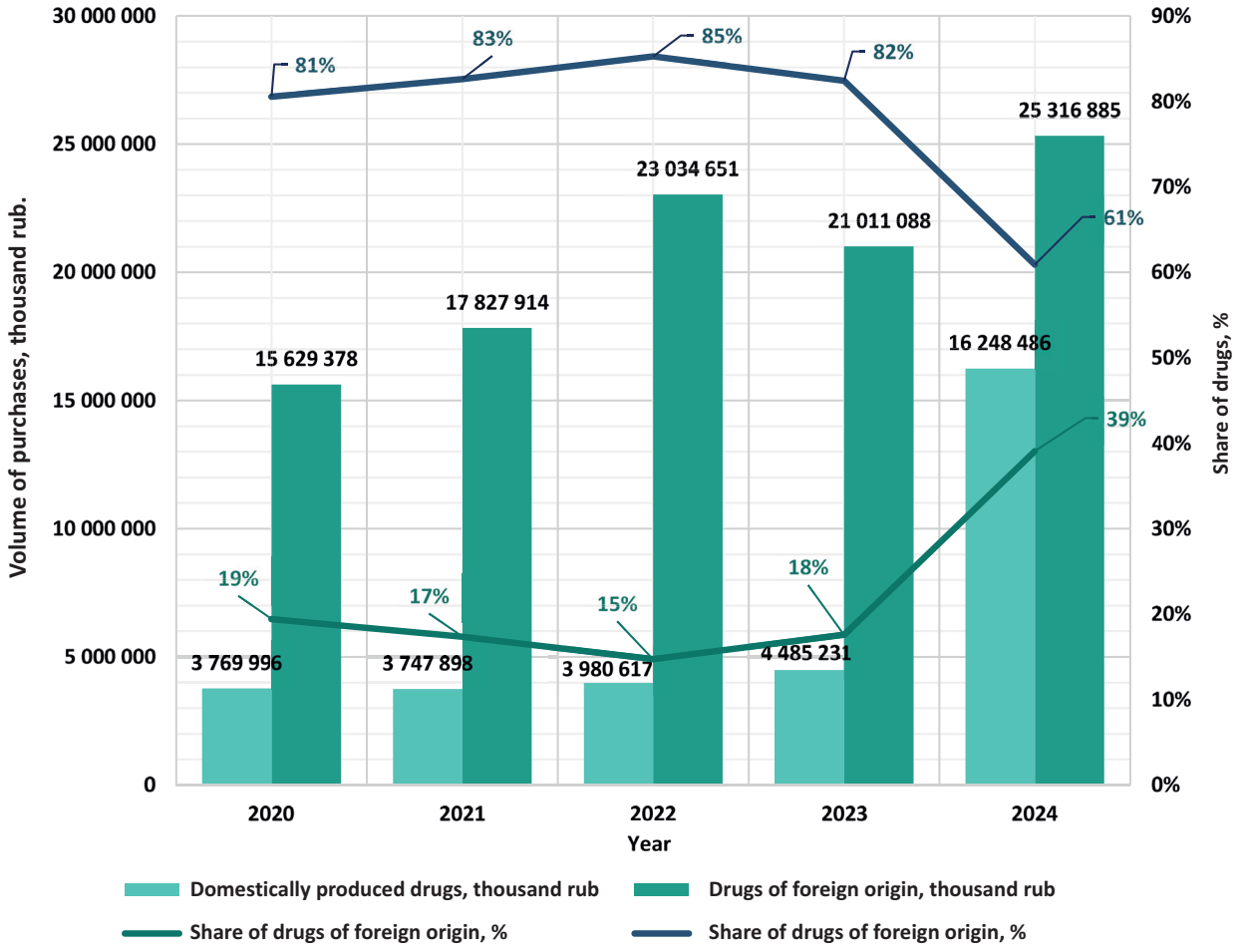

Figure 3 – Dynamics of purchase volumes of sugar-lowering drugs and insulin drugs by country of origin in value terms for the period from 2020 to 2024.

The group of medicines of the combination metformin+SGLT2 inhibitors, namely metformin+dapagliflozin (TN: Sigduo Long®, manufacturer: AstraZeneca Pharmaceuticals LP, USA), metformin + empagliflozin (TN: Synjardy®, manufacturer: Boehringer Ingelheim Ellas Single Member S.A., Greece) occupies a very small market share. Purchases of medicines of this group increased significantly from 78.65 thousand packages in 2020 to 240.37 thousand in 2024 (an increase of 3 times). The total procurement budget increased from 212.85 to 778.12 million rubles (in wholesale prices; an increase of 3.7 times), and the average price per package increased from 2 706.47 to 3 237.10 rubles.

Combined medicines containing metformin and sulfonylurea are still used today, but their share in the market is falling. Purchases decreased from 2.93 million packages in 2020 to 1.57 million in 2024 (a decrease of 46%), and the procurement budget fell from 985.34 to 702.34 million rubles (wholesale prices, a decrease of 29%). There are combinations of metformin with glimepiride (TN: Amaryl® M, manufacturer: Handok Inc, Korea; TN: Glidica M®, manufacturer: Canonpharma production CJSC, Russia), gliclazide (TN: Glimecomb®, manufacturer: AKRIKHIN JSC, Russia) and glibenclamide (TN: Glucovance®, manufacturer: Merck Sante S.a.S., France; TN: Glibomet®, manufacturer: Berlin-Pharma CJSC, Russia; TN: Gluconorm, manufacturer: M.J. Biopharm Pvt. Ltd, India, Pharmstandard-Tomskkhimpharm OJSC, Russia; Russian generics of the above combinations of drugs TN: Glibenfage®, manufacturer: Pharmasintez-Tyumen LLC, Russia; TN: Glibenfor®, manufacturer: Biosintez PJSC, Russia and others. The highest price per package among this group is distinguished by drug with TN Amaryl® M (manufacturer: Handok Inc, Republic of Korea), and the largest volume of purchases in quantitative terms falls on medicine with TN: Glibomet® (manufacturer: Berlin-Pharma CJSC, Russia).

Other hypoglycemic agents

Other SLDs in the commercial sector of the

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pharmaceutical market are represented by medicines with INN repaglinide (TN: Iglinid®, manufacturer: Pharmasintez-Tyumen LLC, Russia; TN: Diaglinid®, manufacturer: AKRIKHIN JSC, Russia; drug with TN: Subetta® (manufacturer: NPF MATERIA MEDICA HOLDING LLC, Russia), a combination of pioglitazone with alogliptin (TN: Incresync®, manufacturer: Takeda Ireland Limited, Ireland). The turnover of SLDs of this group is characterized by a significant increase both in quantitative and in value terms. In general, purchases increased from 105.95 thousand packages in 2020 to 185.02 thousand in 2024 (an increase of 74%), and the total purchase budget of this group increased from 21.89 to 68.82 million rubles (an increase of 75%). The average price per package also increased significantly — from 206.60 to 372.03 rubles.

Ratio of Domestic and Foreign Manufacturers

In addition to studying the dynamics of sales of SLDs and insulin drugs (IDs) by individual groups, the authors considered it important to conduct a comparative analysis of purchases of domestically produced drugs (including manufacturers with a full cycle of drug creation and those whose production is located in the Russian Federation, but the substance is of foreign origin) and foreign manufacturers. It was found that in the period from 2020 to 2024, the volume of purchases of drugs from foreign manufacturers in quantitative and value terms dominates, accounting for more than half of the volume. At the same time, the share of domestically produced drugs, including companies with a full production cycle and those whose production is located in the Russian Federation, but the substance is produced outside of Russia, increased from 35% in 2020 to 42% in 2024. The share of SLDs and IDs produced in a full cycle increased in quantitative terms from 3.6 to 11% (Fig. 2).

Over the same period, budget expenditures on domestic drugs also increased significantly — from 3.8 billion rubles in 2020 to 16.2 billion rubles in 2024. Despite this, their share in the total amount of purchases, including companies with a full production cycle and with a substance of foreign origin, remained lower than that of foreign manufacturers, increasing from 19 to 39% (Fig. 3). The share of full-cycle drugs in monetary terms increased from 1.2% in 2020 to 16% in 2024.

Thus, purchases of domestic drugs (including companies with a full production cycle and those whose production is located in the Russian Federation, but the substance is of foreign origin) increased almost 1.5 times in the number of packages and 4.3 times in terms of budget. Despite the positive dynamics in the growth of the domestic segment, the share of foreign manufacturers remains high.

The results of the analysis of the circulation of SLDs and IDs in the retail sector for the period from 2020 to 2024 allow us to draw a number of key conclusions regarding current trends in pharmacotherapy of diabetes mellitus in Russia and their compliance with both national and international clinical guidelines.

A comparative study of the structure of consumption of SLDs with current clinical guidelines shows significant changes in the management strategy of patients with type 1 and type 2 DM. According to the Russian recommendations of the Endocrinology Research Center, metformin should be preferred as first-line drugs in patients with type 2 diabetes mellitus, and if there are additional indications, SGLT2 inhibitors and GLP-1 receptor agonists [6]. At the same time, international guidelines (American Diabetes Association, ADA) pay considerable attention to a personalized approach, where the choice of drug depends on the presence of cardiovascular diseases, chronic kidney disease, and risk factors for hypoglycemia [19].

The analysis of the sales dynamics of the above-mentioned group of drugs indicates a continuation of the trend towards a gradual abandonment of drugs with a high risk of hypoglycemia (INN: sulfonylurea) in favor of more modern classes [20]. For 2020–2024, the volume of purchases of SGLT2 inhibitors increased 4.5 times in quantitative terms and 4.9 times in value terms, and drugs of the GLP-1 receptor agonist group — 10.5 times in quantitative terms and 5.2 times in value terms, which indicates a wider use of these classes in clinical practice. This growth corresponds to current global treatment standards, in which these drugs are considered preferable for patients with high cardiovascular risk. Nevertheless, despite the general trend towards the use of drugs developed in the last two decades, drugs with the INN metformin remain the most in demand, which in turn fully corresponds to Russian and foreign (ADA) recommendations [6, 19], in which it is designated as the “basis” of the first line. Its share in the total volume of purchases remains at a high level, and consumption increased by 15% in quantitative terms over 5 years. One of the most noticeable trends is a significant increase in the total volume of purchases, which doubled — from 19.40 billion rubles in 2020 to 41.57 billion rubles in 2024. This is due to both an increase in the total volume of consumption and a change in the structure of consumption in favor of more expensive drugs. The largest contribution to the increase in purchases was made by drugs of the SGLT2 inhibitor and

GLP-1 receptor agonist groups, which are among the most expensive drugs. The average price of a package of drugs of the GLP-1 receptor agonist group decreased from 11 731.94 to 5 766.36 rubles, and for drugs of the SGLT2 inhibitor group, on the contrary, increased from 2 425.00 to 2 670.93 rubles. The price of drugs with classic INNs (for example, INN metformin) remained relatively stable.

Among the factors influencing the increase in costs for SLDs and IDs, the following can be distinguished:

- an increase in the number of patients with type 2 diabetes mellitus [1, 4]: according to epidemiological data, the incidence of diabetes mellitus in Russia continues to grow, which increases the need for SLDs;
- a shift in clinical preferences in favor of modern drugs [15]: the inclusion of GLP-1 receptor agonist and SGLT2 inhibitor drugs in the structure of public procurement and their wider use in the practice of doctors;
- an increase in the cost of drug packaging: new classes of drugs remain significantly more expensive than traditional SLDs, which increases the share of costs in the budget [20, 21]. Expansion of the commercial segment: despite the inclusion of new drugs in public procurement programs, a significant number of patients continue to purchase them on their own, which is reflected in the growth of costs / revenue of the retail sector.

Thus, the analysis of the structure of consumption of SLDs confirms the adaptation of clinical practice to modern recommendations, but there remain problems with the availability of expensive drugs, which requires optimization of mechanisms for preferential drug provision.

The data obtained demonstrate a sharp increase in the consumption of SLDs and IDs in Russia, especially in the segment of modern SLDs. Synthetic drugs of the GLP-1 receptor agonist and SGLT2 inhibitor groups show the greatest increase in purchase volumes, both in value and in quantitative terms, which indicates a change in clinical protocols in favor of more innovative treatment methods. Metformin remains the leading drug in the treatment of type 2 diabetes mellitus, but its share in the total turnover is decreasing relative to combined treatment regimens.

A significant increase in the average purchase budget indicates an increase in the cost of treating diabetes mellitus, which may be associated with both the spread of the disease and a change in the structure of pharmacotherapy. High growth rates of budgets for innovative drugs emphasize the need to expand state funding programs to ensure their accessibility to patients.

Government measures and investments in import substitution have led to a significant increase in purchases of domestically produced drugs [22–25]. Despite the positive dynamics of domestic production, import substitution in the SLDs segment has not achieved complete success. In 2020–2024, the share of foreign manufacturers remains steadily high, both in quantitative and in value terms. Thus, for further strengthening of the position of domestic manufacturers, additional measures of state support, investments in the production and development of new drugs, as well as stimulating consumers to switch to domestic products are necessary.

Limitations of the study

Our study covers a significant part of the volume of retail sales of SLDs in Russia, but does not cover the entire drug market of this group. It should be taken into account that information on drug purchases is dynamic, subject to change, and can be difficult to accurately collect and analyze. The calculations are made in wholesale prices, that is, in the purchase prices of drugs by pharmacies. The results obtained can be considered reliable, since the absolute majority of the drugs under consideration are included in the List of Vital and Essential Medicines, therefore, retail markups on them are strictly limited and controlled by the state. Certain variations in the presented data are possible due to the indicated aspects. This analysis is aimed at identifying key trends and general directions of market development, but does not claim to be exhaustive or absolutely accurate in all quantitative indicators.

CONCLUSION

In the period from 2020 to 2024, there is a relatively stable growth in the volume of purchases of SLDs and IDs, with slight fluctuations, while in 2024 there is a significant jump in consumption, both in quantitative and in value terms. Over the entire observation period, the total volume of purchases of SLDs and IDs increased by more than 1.2 times — from 41.98 million packages in 2020 to 51.70 million in 2024. At the same time, the total purchase budget increased from 19.40 to 41.57 billion rubles, which corresponds to a 2-fold increase. The average price per package also increased significantly — from 462 rubles in 2020 to 804 rubles in 2024, increasing by 1.7 times, which reflects significant inflation in the commercial sector of the pharmaceutical market.

In general, the Russian pharmaceutical market

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presents all options for modern innovative SLDs (DPP-4 inhibitors, SGLT2 inhibitors, GLP-1 receptor agonists), which, according to general trends, show a steady increase in turnover. A significant share of the market to date is occupied by older and less effective sulfonylurea drugs and their combinations with metformin. The largest growth in quantitative and value terms of purchases was shown by the GLP-1 receptor agonist group (by 10.3 and 5.2 times, respectively), mainly due to the entry into the market of Russian generics of drugs with the INN semaglutide. Government measures and investments in import substitution have led to a significant increase in purchases of domestically produced drugs. Despite the positive dynamics of Russian production, import substitution in the SLDs segment has not achieved complete success and accounts for less than half of the turnover of sugar-lowering drugs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Denis V. Kurkin — idea, content planning, design of the graphic material, editing and approval of the final version of the manuscript; Ekaterina V. Makarova — writing a draft of a manuscript; Valentina I. Zvereva, Nazar A. Osadchenko, Anastasia R. Makarova, Dmitry A. Bakulin, Olga V. Marincheva, Yuliya V. Gorbunova, Ivan S. Krysanov, Ksenia N. Koryanova, Daria A. Galkina — collecting of the material; Yury A. Kolosov — editing the final version of the manuscript; Roman V. Drai, Igor E. Makarenko, Anna S. Shuvaeva — consultations on highly specialized issues, approval of the final version of the manuscript. All the authors have made an equivalent and equivalent contribution to the preparation of the publication. All authors confirm that their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept and preparation of the article, read and approved the final version before publication).

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The effects of kappa opioid agonist RU-1205 on local field potentials and behavior in the discriminative stimulus paradigm

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Kappa opioid receptors play a pivotal role in regulating both physiological and cognitive processes. RU-1205, a benzimidazole derivative acting as a specific kappa opioid receptor agonist, has demonstrated the capacity to modulate neuronal activity. However, the nuanced effects of RU-1205 on neuronal activity remain incompletely understood.

The aim of the study was to identify and elucidate the effects of the kappa opioid agonist RU-1205 on local field potentials and behavior in the discriminative stimulus paradigm.

Materials and methods. The experiments were conducted in male rats weighing 260–280 g. The animals were surgically implanted with cortical electrodes (F — frontal, O — occipital, P — parietal) as well as deep electrodes in specific brain regions, including the medial prefrontal cortex (mPFC), hippocampus (Hipp), nucleus accumbens (NAc), ventral tegmental area (VTA) and amygdala (Amy). LFP signals were obtained and analyzed after the administration of the compound RU-1205 (350 µg/5 µl intracerebroventricular injections) using spectral and coherence analysis methods. Drug discrimination paradigm was employed to evaluate the similarity of the compound RU-1205 to the selective kappa opioid receptor agonist U-50488 and the p38 MAPK inhibitor SB203580 (including in combination with the opioid receptor blocker naloxone).

Results. Electrophysiological changes observed include an increase in power of theta frequencies (4–8 Hz) in F, P and mPFC leads, along with a reduced power of delta frequencies (0.5–4 Hz) in O and Hipp)leads, and a suppression of gamma activity (30–50 Hz) in F and mPFC leads, all with statistical significance (p <0.05). Post-administration of RU-1205 resulted in a decreased coherence between pairs of electrodes: P–O, P–F, F–O, and mPFC–Hipp (all p <0.05). It was found that the compound RU-1205 is similar to U-50488 and does not exhibit p38 inhibitory activity in the discriminative stimulus paradigm.

Conclusion. Compared to the selective kappa-opioid agonist U-50488, the compound RU-1205 induces less significant changes in LFP activity without electrophysiological and behavioral signs of beta-arrestin pathway activation. The overall data suggest that RU-1205 is a functionally selective agonist of kappa-opioid receptors.

Keywords*:* kappa opioid receptors; RU-1205; electrophysiology; brain bioelectrical activity; spectral analysis; coherence analysis; discriminative stimulus paradigm; drug discrimination

Abbreviations: CNS — central nervous system; ACSF — artificial cerebrospinal fluid; BLA — basolateral amygdala; dHPC — dorsal hippocampus; Hipp — hippocampus; LFP — local field potential; NAc — nucleus accumbens; p38 MAPK — p38 mitogen-activated protein kinase; PrL — prelimbic cortex; VTA — ventral tegmental area.

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Влияние каппа-опиоидного агониста РУ-1205 на локальный полевой потенциал и поведение в модели дискриминации стимула

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Каппа-опиоидные рецепторы играют ключевую роль в регуляции физиологических и психических процессов. Было показано, что производное бензимидазола РУ-1205, специфический агонист каппа-опиоидных рецепторов, модулирует активность нейронов. Однако комплексное воздействие соединения РУ-1205 на нейрональную активность остается недостаточно изученным.

Цель. Поиск и интерпретация изменений локального полевого потенциала, а также оценка поведения в модели дискриминации стимула под влиянием каппа-опиоидного агониста РУ-1205.

Материалы и методы. Крысам (260–280 г) имплантировали корковые (F — фронтальные, O — окципитальные, P — париетальные), а также глубокие электроды в зону медиальной префронтальной коры (mPFC), гиппокампа (Hipp), прилежащего ядра (NAc), вентральной области покрышки (VTA) и миндалины (Amy). Проводился спектральный и когерентный анализ LFP-сигналов, полученных после введения соединения РУ-1205 (350 мкг/5 мкл интрацеребровентрикулярно). Использовалась модель дискриминации стимула, чтобы оценить сходство соединения РУ-1205 с селективным агонистом каппа-опиоидных рецепторов U-50488 и ингибитором MAPK p38 SB203580 (в том числе в комбинации с блокатором опиоидных рецепторов налоксоном).

Результаты. Зафиксированы изменения: повышение мощности в диапазоне тета-частот (4–8 Гц) на отведениях F, P и mPFC, снижение мощности в диапазоне дельта-частот (0,5–4 Гц) сигналов с O и Hipp отведений, а также подавление гамма-активности (30–50 Гц) на отведениях F и mPFC (p <0,05). После введения РУ-1205 наблюдалось снижение когерентности между парами электродов: P–O, P–F, F–O и mPFC–Hipp (p <0,05). Отсутствие p38-ингибирующей активности РУ-1205 и его сходство с U-50488 подтверждено в модели дискриминации стимула.

Заключение. Установлено, что по сравнению с селективным каппа-опиоидным агонистом U-50488 соединение РУ-1205 вызывает менее выраженные изменения LFP-активности без электрофизиологических и поведенческих признаков активации бета-аррестинового пути. Совокупность данных свидетельствует о принадлежности соединения РУ-1205 к функционально селективным агонистам каппа-опиоидных рецепторов.

Ключевые слова: каппа-опиоидные рецепторы; РУ-1205; электрофизиология; биоэлектрическая активность мозга; спектральный анализ; когерентный анализ; модель дискриминации стимула; дискриминация лекарственных средств

Список сокращений: ЦНС — центральная нервная система; ACSF — искусственная спинномозговая жидкость; BLA — базолатеральная миндалина; dHPC — дорсальный гиппокамп; Hipp — гиппокамп; LFP — локальный полевой потенциал; NAc — прилежащее ядро; p38 MAPK — p38 митоген-активируемая протеинкиназа; PrL — прелимбическая кора; VTA — вентральная область покрышки.

INTRODUCTION

Kappa opioid receptors are involved in the modulation of various physiological and cognitive functions, including pain perception, stress response, and mood modulation [1]. We have previously demonstrated that the benzimidazole derivative RU-1205, a specific kappa-opioid receptor agonist, modulates neuronal activity [2, 3]. However, the full scope of its effects on neuronal activity remains to be fully elucidated.

Local field potentials (LFPs) reflect the collective electrical activity of large neuronal populations and

are a subject of comprehensive investigation in electrophysiology and neuroscience [4]. The analysis of LFP signals provides invaluable information for understanding the neuropharmacological profiles of pharmaceutical drugs and experimental compounds. Furthermore, the use of intracerebral electrodes enables the study of local changes in neuronal bioelectrical activity within target brain regions. Coherence analysis can also be employed to investigate functional connectivity between different brain areas, allowing for a more detailed examination of the interactions between various frequency components of the signal and the identification of complex dependencies that might be overlooked in spectral analysis alone [5].

Several studies have reported a pronounced influence of kappa-opioid agonists on brain bioelectrical activity. Following the administration of salvinorin-A, an increase in the power of delta (1.3–3.5 Hz) and gamma (35–40 Hz) waves, accompanied by a decrease in alpha wave power (7.5–13 Hz), was observed on the electroencephalogram [6]. A study utilizing a *Salvia divinorum* Epling & Játiva extract revealed ECoG changes characterised by an increased spectral power density in signals from frontal leads and a decreased power in signals from occipital leads [7]. The kappa-opioid agonists enadoline and PD117302 induced a dose-dependent shift in EEG power, particularly within the 4 to 8 Hz frequency range. These effects were abolished by norbinaltorphimine, confirming the involvement of kappa-opioid receptors in the observed effects [8].

Key brain regions associated with the analgesic and aversive effects of kappa-opioid agonists include the cerebral cortex (mPFC), hippocampus (Hipp), nucleus accumbens (NAc), ventral tegmental area (VTA), and amygdala (Amy) [9]. These areas exhibit the highest density of kappa-opioid receptors [10] and were therefore selected for this investigation.

The kappa-opioid receptor agonist RU-1205 is of particular interest due to its unique pharmacological profile. The compound does not produce aversive effects, as determined by the conditioned place preference test, nor does it induce tolerance upon chronic administration, distinguishing it from typical kappa-opioid agonists [11, 12]. To explain these properties, a multitarget mechanism of action was hypothesized, suggesting that the effects of RU-1205 may involve not only the activation of kappa-opioid receptors but also an additional inhibitory effect on p38 MAP kinase. This hypothesis was based on previous experiments demonstrating that the aversive effects of kappa-opioid agonists could be completely prevented by the administration of the p38 inhibitor SB203580 [13]. It was also established that RU-1205 could suppress the aversive effects of the kappa-opioid agonist U-50488 [11]. The hypothesis of p38-inhibitory activity can be tested using a drug discrimination paradigm. This methodology is widely employed in psychopharmacology and behavioral science to assess the perceptual and cognitive effects of various chemical substances, based on the principle that animals can be trained to distinguish between the interoceptive effects of different pharmacological agents.

THE AIM of this study was to evaluate the impact of RU-1205 on LFP activity in the cerebral cortex, hippocampus, medial prefrontal cortex, amygdala, nucleus accumbens, and ventral tegmental area. A further aim was to determine whether RU-1205 possesses p38 MAPK-inhibitory properties by assessing the ability of rats to discriminate its effects from those of the p38 inhibitor SB203580.

MATERIALS AND METHODS

Study design

The study comprised two main stages. At the first stage, LFPs were recorded following intracerebroventricular administration of RU-1205, and the resulting signals were subjected to spectral and coherence analyses. In the second stage, we analyzed the discriminative stimulus properties of RU-1205.

Test compounds

The study used 9-(2-morpholinoethyl)-2-(4-fluorophenyl)imidazo[1,2-a]benzimidazole (RU-1205), synthesized at the Research Institute of Physical and Organic Chemistry of the Southern Federal University (RF Patent No. 2413512 C1, purity ≥ 99,46%), U-50488 (Sigma Aldrich, USA), and SB203580 (Sigma Aldrich, USA).

Study duration and conditions

The study was conducted between July and September 2023. All experimental procedures were carried out at the Laboratory of Electrophysiological Research, Scientific Center for Innovative Drugs of the Volgograd State Medical University.

Ethics approval

Animal experiments were performed in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and

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Other Scientific Purposes, the principles of Good Laboratory Practice (GLP) (GOST 33044-2014, 2021), and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The study protocol was approved by the Local Ethics Committee of Volgograd State Medical University (Registration number IRB00005839 IORG0004900, Minutes No. 2022/096, dated January 21, 2022).

Animals

The experiments were conducted on outbred male albino rats (*n* = 41) weighing 260–280 g. The animals were housed under standard vivarium conditions with a 12-hour light-dark cycle and at the temperature of 22 ± 2 °C, a relative humidity of 40–50%, and *ad libitum* access to food and water.

Surgical procedures

Under 2% isoflurane anesthesia (Laboratories Karizoo, S.A., Spain) administered via a rodent gas anesthesia system (Gas Anesthesia System-21100, Ugo Basile, Italy), stainless steel electrodes (0.1 mm diameter), insulated along their entire length except for the tip, were implanted into the right cerebral hemisphere according to the following stereotaxic coordinates relative to bregma:

Cortical electrodes: F — anteroposterior (AP) = 0,00, mediolateral (ML) = +2.00; P — AP = -4.08, ML = +2.00; O — AP = -7.08, ML = +2.00.

Deep electrodes: prelimbic cortex (PrL) — AP = +2.7 mm, ML = +0.8 mm, dorsoventral (DV) = 3.8 mm; basolateral amygdala (BLA) — AP = -2.8 mm, ML = +5–5.3 mm, DV = 8.8 mm; hippocampus (Hipp) — AP = –4.9 mm, ML = +4.8 mm, DV = 6.0 mm; ventral tegmental area (VTA) — AP = -5.2 mm, ML = +1,0 mm, DV = 8.6 mm; nucleus accumbens (NAc) — AP = +1.8 mm, ML = +1.6 mm, DV = 7.3 mm.

For intracerebroventricular injections, a 21-gauge stainless steel guide cannula was implanted into the left lateral ventricle using the following stereotaxic coordinates from bregma: AP = -0.6 mm, ML = -1.6 mm, DV = 4.0 mm. The electrodes and cannula were secured to the skull using dental acrylic (Protacryl-M, Ukraine) and two stainless steel screws.

Postoperatively, animals were housed individually and given 7 days to recover.

Signal recording

For the electrophysiological experiments, animals were assigned to two groups (*n* = 16): 1) the first group (*n* = 8) received an intracerebroventricular (i.c.v.) injection of 5 µl of artificial cerebrospinal fluid (ACSF); 2) the second group (*n* = 8) received RU-1205 at a dose of 350 µg / 5 µl i.c.v., equivalent to the intraperitoneal ED_{80} determined in analgesic activity assays. LFPs were recorded using a laboratory electroencephalograph (NVX-36, MKS, Russia). LFP activity was recorded in a monopolar montage against a common average reference at a sampling rate of 500 Hz. Thirty minutes after i.c.v. administration of the test substance or ACSF, LFPs were recorded for 10 minutes.

Spectral analysis

The signal was filtered using a basic FIR filter with a passband of 0,5 to 50 Hz. Subsequently, independent component analysis was applied to remove muscle artifacts. Spectral analysis was performed via a direct discrete Fourier transform for the following frequency bands: delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), and gamma (30–50 Hz). The analysis was conducted in Python (v.3.11.3) using the MNE-Python package (v.1.6.1)[1].

Coherence analysis

Magnitude-squared coherence [14] was calculated to quantify phase synchronization in the frequency domain between LFP signals (1-second epochs) for all electrode pairs using the ‘mscohere’ function (parameters: window = 1 s, noverlap, nfft = 500, fs = 500) in MATLAB (R2023b; MathWorks Inc., United States) under an individual license. The analysis was focused on the theta frequency range (4–8 Hz). Fisher’s z-transformation was applied to all coherence values for data normalization to permit parametric statistical testing.

Analysis of the discriminative stimulus properties of RU-1205

The drug discrimination paradigm is used to study the effects and mechanisms of action of pharmacological agents [15–17]. While conditioning can be established using food reinforcement, this typically requires 4–7 weeks of training. To accelerate the conditioning process, electrical stimulation of the ventral tegmental area was employed, which significantly shortened the training period.

For the experiment on the discriminative stimulus properties of RU-1205, intact rats (*n* = 25) were implanted with a stimulating stainless steel electrode (0.1 mm) in the ventral tegmental area using the

[1] Zenodo. MNE-Python (v1.6.1). Available from: https://zenodo.org/records/10519948 2

following stereotaxic coordinates from bregma: AP = -5.2 mm, ML = +1.0 mm, DV = -8.6 mm.

During the initial training phase, animals were placed in an operant chamber with a single available lever (right or left) (Fig. 1). 10 min before the session, animals were injected with either: 1) RU-1205 at a dose of 350 µg / 5 µl i.c.v. (n = 9); 2) a combination of RU-1205 (350 µg) and naloxone (200 µg) in 5 µl ACSF i.c.v. (n = 8); 3) a combination of SB203580 (1 µg) and naloxone (200 µg) in 5 µl ACSF i.c.v. (n = 8); or a control solution of ACSF (5 µl) / naloxone (200 µg). Naloxone was co-administered with SB203580 in the third group to confirm that naloxone itself does not interfere with the discrimination of p38 MAPK-inhibitory activity. Furthermore, its inclusion with RU-1205 in tests against SB203580-trained animals was intended to prevent potential false-negative results, where the kappa-opioid component of RU-1205 might mask its discriminability. For half of the animals in each group, a left lever press was reinforced following control solution administration and a right lever press was reinforced following test compound administration; this was reversed for the other half.

A correct lever press resulted in the delivery of an electrical stimulus (24 biphasic 60 Hz pulses, 2 ms duration, fixed-ratio 1:1) to the reinforcement area via an isolated stimulator (A-M Systems MODEL 4100, USA). The current intensity was individually titrated (80–150 µA) to a level below that which elicited involuntary movements, avoidance behavior, or vocalizations. Over the subsequent 3–4 days, the fixed ratio was gradually increased to 10.

During the discrimination training phase, the fixed ratio was again progressively increased from 1 to 10. Each session lasted 20 min, with both levers available simultaneously. An incorrect lever press reset the response counter, requiring the animal to complete 10 consecutive correct operant actions to receive reinforcement. The administration of ACSF / naloxone or test compounds was randomized across three weekly sessions (Table 1).

The criterion for acquisition of discrimination was defined as an accuracy of ≥ 80% on the drug-appropriate lever in at least 8 of 10 consecutive sessions, after which the animal proceeded to the testing phase [16].

During the testing phase, substitution tests were conducted with ascending doses, as the discriminative stimulus properties of compounds are concentration-dependent, making it impossible to predetermine subjectively equivalent doses. The first group of animals, trained to discriminate RU-1205, received RU-1205 (3.5, 35, or 350 µg, i.c.v.) 10 min before the session. Subsequently, these animals underwent substitution tests with the selective kappa-opioid agonist U-50488 (1, 10, 100 µg, i.c.v.) and the p38 MAPK inhibitor SB203580 (0.01, 0.1, 1 µg, i.c.v.) to test for generalisation to the training drug.

During its testing phase, the third group received naloxone (200 µg, i.c.v.) 15 min prior to the session, followed by SB203580 (0.01, 0,1, 1 µg, i.c.v.) 10 minutes prior. These animals then underwent substitution tests with a combination of RU-1205 and naloxone (3.5, 35, and 350 µg RU-1205 / 200 µg naloxone, i.c.v.). Tests were conducted twice weekly. Between test sessions, animals underwent maintenance training with ACSF / naloxone or the training dose of their respective compound. For each training drug, the mean number of sessions to acquisition (± SD) was calculated, and dose-effect curves were constructed, plotting the percentage of responses on the drug-associated lever. The experimental timeline is depicted in Figure 2.

Statistical data analysis

Statistical analysis was performed using GraphPad Prism 10.1 (Dotmatics, USA). Data were tested for normality using the Shapiro-Wilk test before applying parametric methods. Spectral and coherence analysis data are presented as mean ± standard error of the mean (M ± SEM). Discriminative stimulus data are presented as mean ± standard deviation (M ± SD), expressed as the percentage of responses on the drug-associated lever. An unpaired Student's t-test was used for comparisons between two independent groups in the LFP analysis. Dose-effect curves were analyzed using a one-way repeated measures analysis of variance (ANOVA), followed by Dunnett's post hoc test. A $p < 0.05$ was considered statistically significant.

RESULTS

Analysis of LFP spectral characteristics revealed statistically significant changes following the administration of RU-1205 (350 µg, i.c.v.) compared to vehicle ($p < 0.05$). Specifically, an increase in theta band power and a decrease in gamma band power were observed in frontal cortical recordings (Fig. 3a). In parietal cortical recordings, an increase in theta band power was also detected (Fig. 3b). Concurrently, a decrease in delta band power was observed in occipital cortical recordings (Fig. 3c).

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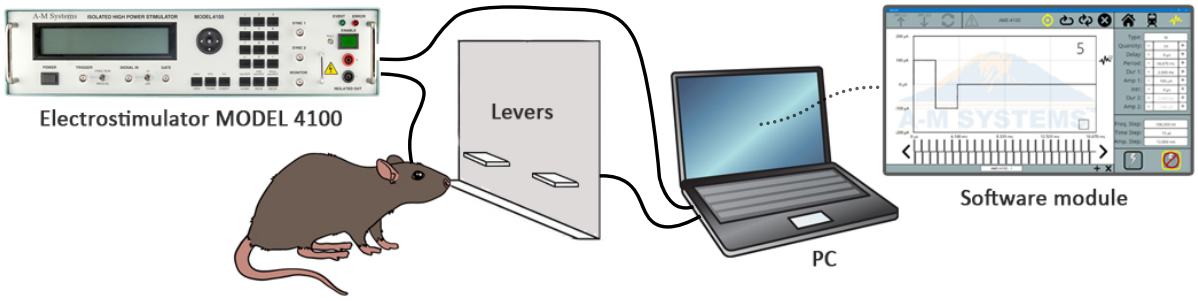

Figure 1 – Operant chamber with two levers, an electrical stimulator, and a personal computer for investigating neuroactive substances in a stimulus discrimination model.

Table 1 – Administration schedule for ACSF (5 µl), naloxone (200 µg), and test compounds during discrimination training.

Group		Week 1	Week 2	Week 3
1	RU-1205 (350 µg i.c.v., *n* = 9)	R-A-R-R-R-A-A	A-R-R-A-R-A-A	A-R-R-R-A-A-R
2	RU-1205 + naloxone (350 µg / 200 µg i.c.v., *n* = 8)	Rn-N-Rn-N-N-Rn-Rn	N-Rn-Rn-N-N-Rn-Rn	Rn-N-N-Rn-Rn-Rn-N
3	SB203580 + naloxone (1 µg / 200 µg i.c.v., *n*=8)	Sn-N-Sn-Sn-N-N-Sn	N-Sn-N-Sn-Sn-N-Sn	Sn-N-Sn-N-Sn-N-N

Note: A — ACSF; N — naloxone; R — RU-1205; Rn — RU-1205+naloxone; Sn — SB203580+naloxone.

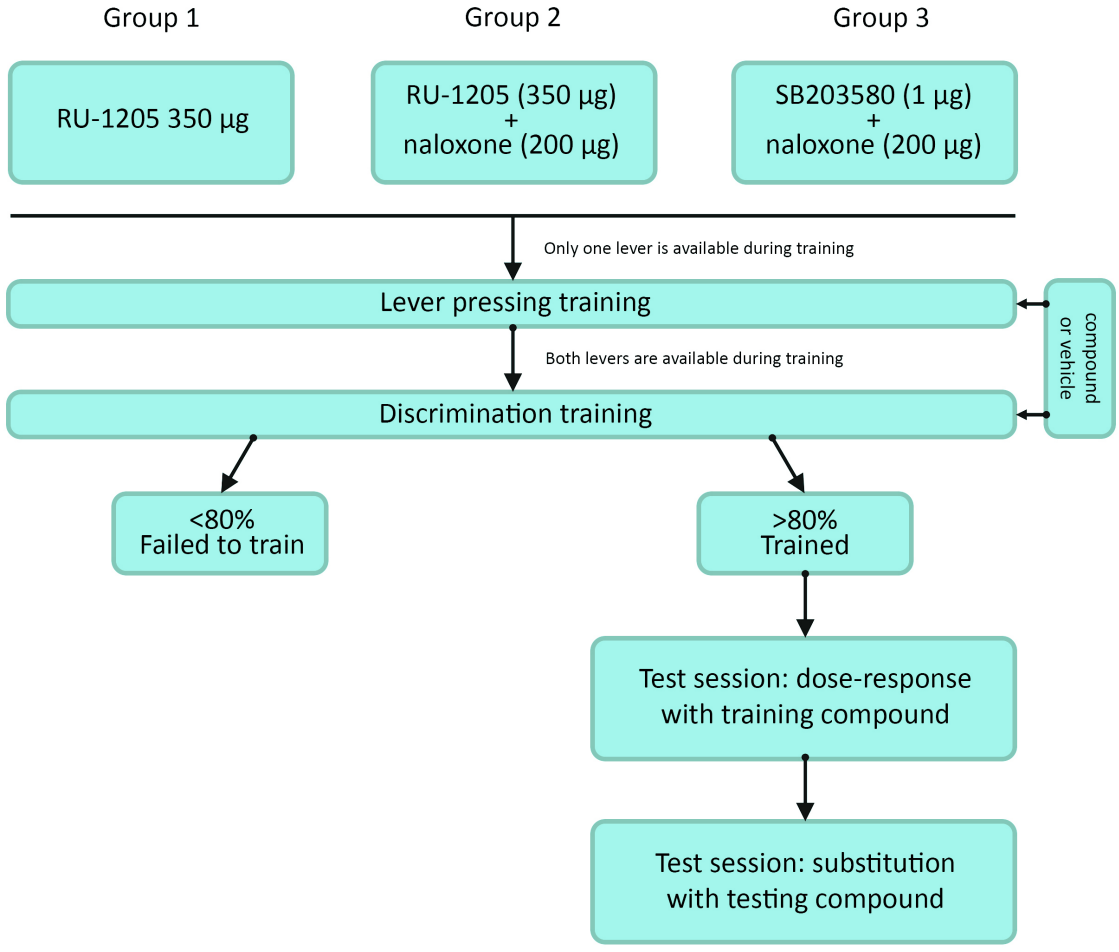

Figure 2 – Schematic of the experimental design for studying the discriminative stimulus properties of the test compounds, encompassing training and testing phases.

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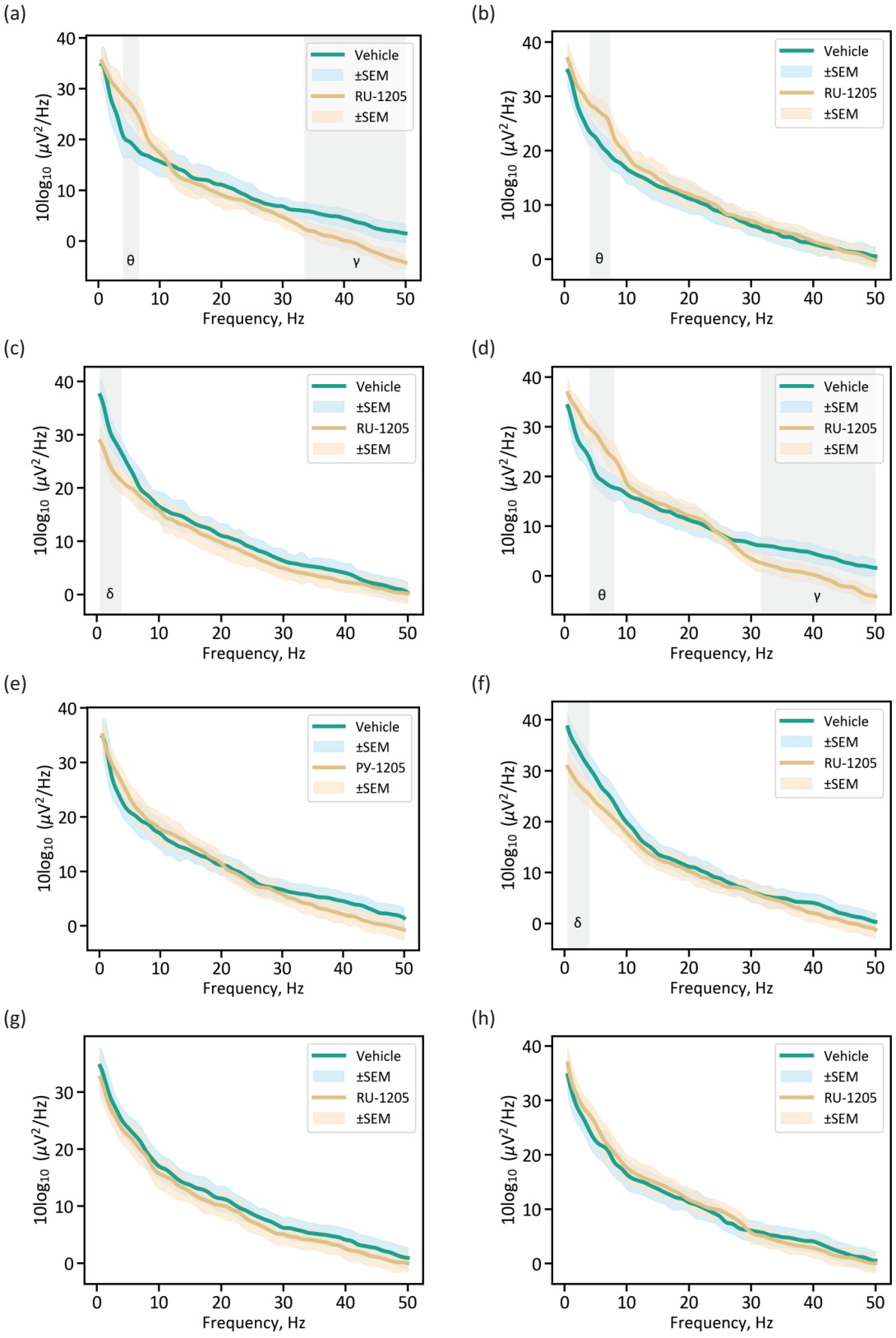

Figure 3 – Spectral power density of LFP oscillations following administration of RU-1205 (350 μg, i.c.v.).
Note: (a) — frontal lead (F); (b) — parietal lead (P); (c) — occipital lead (O); (d) — medial prefrontal cortex (mPFC); (e) — amygdala (Amy); (f) — hippocampus (Hipp); (g) — nucleus accumbens (NAc); (h) — ventral tegmental area (VTA). Each plot displays mean±SEM for spectral power density. Shaded areas indicate frequency bands with statistically significant power deviations relative to vehicle ($p < 0.05$).

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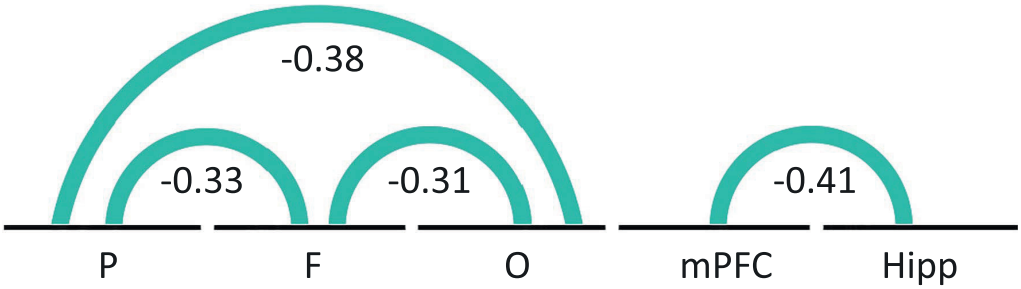

Figure 4 – Effect of RU-1205 on theta band coherence between LFP signals.
Note: the diagram illustrates the connections (coherence values after Fisher's z-transformation) that were statistically significantly altered by RU-1205 (350 µg, i.c.v.) compared to control ($p < 0.05$). F — frontal lead; P — parietal lead; O — occipital lead; mPFC — medial prefrontal cortex; Hipp — hippocampus.

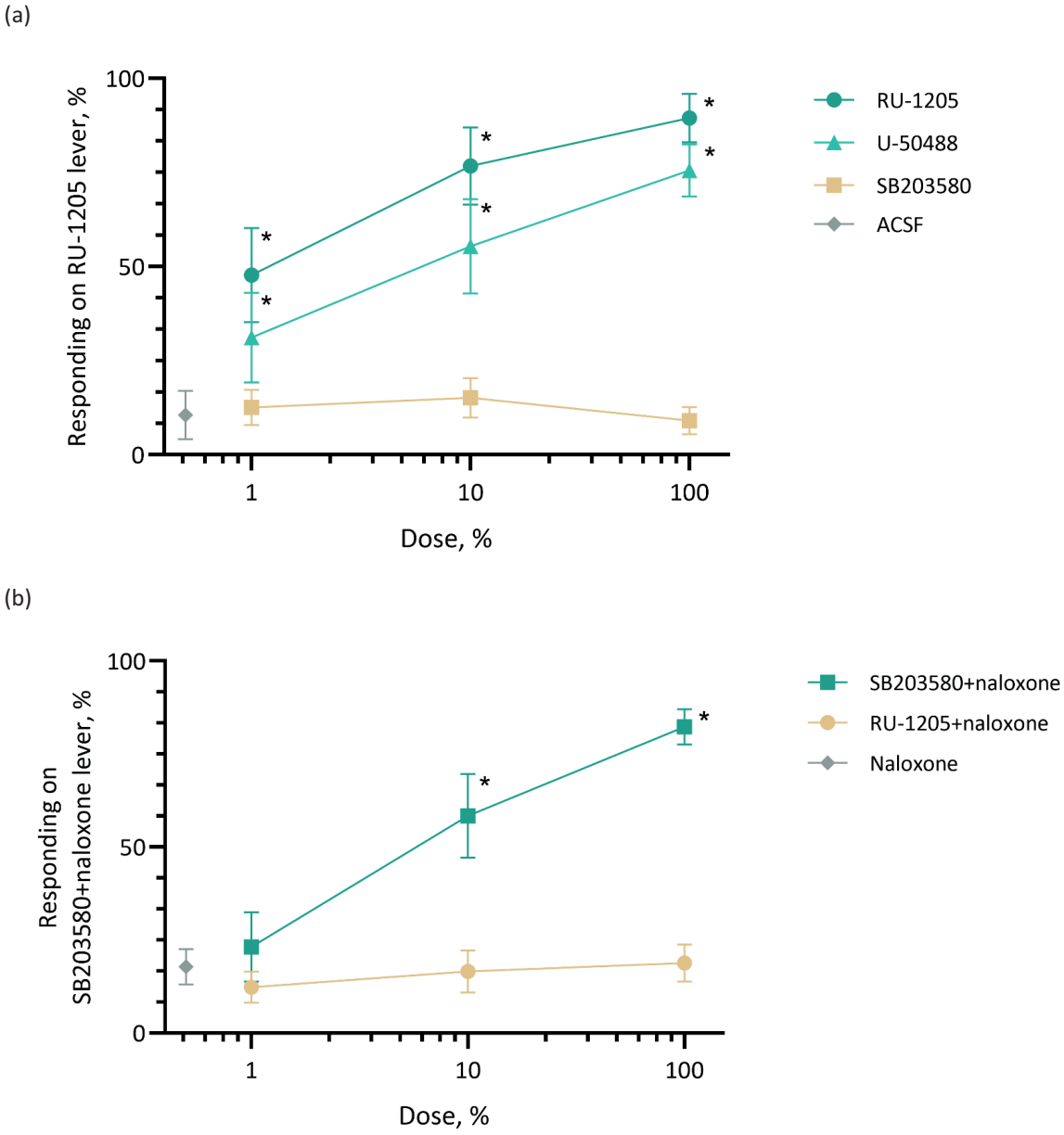

Figure 5 – Effect of test compounds on the percentage of responses on the drug-appropriate lever in the stimulus discrimination paradigm.
Note: (a) — response of rats to RU-1205, U-50488, and SB203580 after training to discriminate RU-1205 (350 µg, i.c.v.); (b) — Response of rats to combinations of SB203580+naloxone and RU-1205 + naloxone after training to discriminate SB203580+naloxone (1 µg / 200 µg, i.c.v.). The highest dose of each test substance was normalized to 100%. Data are presented as mean±SD; $p < 0.05$ compared to control solution.

RU-1205 led to increased theta power and suppressed gamma oscillations in the medial prefrontal cortex (Fig. 3d). A reduction in delta band power was also noted in the hippocampus (Fig. 3f). In contrast, no statistically significant changes were found in the amygdala, nucleus accumbens, or ventral tegmental area.

Next, theta band coherence was assessed across all electrode pairs. RU-1205 significantly decreased coherence for four pairs of leads compared to vehicle ($p < 0.05$), as presented in Fig. 4.

All rats in the first group acquired the discrimination in an average of 18 ± 3.0 sessions. During testing, the training dose of RU-1205 produced 89.44 ± 6.45% responding on the drug-appropriate lever, and RU-1205 dose-dependently increased responding on this lever ($F_{2.060, 16.48} = 208.1$, $p < 0.0001$). U-50488 partially substituted for RU-1205, with rats generalizing to U-50488 up to 75.56 ± 6.95% at the 100 µg dose, 55.34% (± 12.52, SD) at 10 µg, and 31.11 ± 11.89% at 1 µg ($F_{2.221, 17.77} = 201.7$, $p < 0.0001$) (Fig. 5a).

The second group of animals failed to reach the discrimination criterion during the training period.

The third group acquired the discrimination between SB203580 in combination with naloxone and vehicle in 17,5 ± 3,29 days. The discrimination exceeded the 80% criterion ($F_{2.105, 14.74} = 245.7$, $p < 0.0001$), although the effect of the lowest dose (0.01 µg) did not differ significantly from control. In substitution tests, RU-1205 co-administered with naloxone did not significantly alter responding on the lever associated with SB203580, indicating that RU-1205 lacks additional p38 MAPK-inhibitory activity (Fig. 5b).

DISCUSSION

The effects of kappa-opioid agonists on electroencephalographic activity are well-documented in both animal and human studies. This class of compounds induces specific alterations in cortical activity, notably a characteristic increase in power within the 4–8 Hz range (theta activity enhancement) [8]. The pharmaco-EEG profile of the canonical kappa-opioid agonist U-50488 is consistent with a CNS depressant effect [18]. In studies of U-50488's effects on LFP activity, significant changes have been identified in the nucleus accumbens (NAc) and ventral tegmental area (VTA) — key components of the reward system — as well as in the basolateral amygdala (BLA), a region associated with aversive states and depression. The location and nature of these changes align with the spectral correlates of aversive effects. Theta frequency in the prefrontal cortex and limbic areas (amygdala, hippocampus) is closely linked to fear and avoidance behaviors. It has been noted that gamma activity in the amygdala is suppressed during periods of fear [19, 20], while theta power in the mPFC-BLA circuit increases [21]. Stress can enhance theta waves in the dorsal hippocampus (dHPC), BLA, and amygdala, and gamma frequencies in the dHPC, BLA, and the infralimbic (IL) division of the medial prefrontal cortex [22]. Furthermore, studies have revealed abnormal LFP patterns in the NAc of individuals with depression [23]. In animal models of anxiety, an increase in theta band power (4–12 Hz) has been observed in the NAc [24]. Consequently, it is plausible that previously uncharacterized effects of RU-1205 could be revealed through the analysis of its impact on brain bioelectrical activity.

RU-1205 did not induce significant LFP changes in the NAc, VTA [25], or Amy [26], which is consistent with *in vivo* data demonstrating its lack of dysphoric and depressant properties [11, 27]. The deviations in spectral characteristics from the F, P, mPFC, and Hipp leads in the RU-1205 group correspond with those recorded following U-50488 administration. Some of these changes could potentially serve as LFP markers for the analgesic effects of these kappa-opioid agonists. For instance, EEG gamma waves are reportedly associated with nociception, with gamma activity significantly increasing during a painful stimulus [28]. Additionally, data from human EEG studies indicate that chronic pain relief is associated with an increase in theta power over fronto-medial leads [29].

The subsequent phase of this research investigated coherence, a measure of synchrony between two LFP signals. Previous experiments with U-50488 demonstrated pronounced alterations in phase synchronization [18]. A decrease in coherence was observed between cortical electrodes and between the prefrontal cortex, hippocampus, nucleus accumbens, and ventral tegmental area (effects also seen in depression, cognitive impairment, and with opioid analgesic use), while an increase in connectivity with the amygdala was noted (a characteristic sign of aversive action and stress response) [18]. It is well-established that

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the amygdala and hippocampus are involved in pain perception and negative mood [30]. During states of fear, theta oscillations in the basolateral amygdala, hippocampus, and medial prefrontal cortex become synchronized, a phenomenon thought to underlie the response to aversive stimuli [19]. Likhtik et al. also concluded that mPFC-BLA synchronization is a key factor in anxiogenesis [21]. Notably, morphine has been shown to attenuate theta activity and enhance gamma activity in the NAc, while increasing NAc-VTA coherence [31, 32]; opposite effects were observed for U-50488 [18]. Therefore, these changes in synchrony may represent electrophysiological signatures of the euphoric versus dysphoric actions of substances.

RU-1205 did not produce the full spectrum of effects seen with U-50488. Its impact was limited to changes in cortical connectivity and a reduction in coherence between the prefrontal cortex and the hippocampus. These findings could be related to either the analgesic action of RU-1205 or potential cognitive side effects. For example, disruption of hippocampal-prefrontal connectivity has been shown to impair working memory [33], which is consistent with a known side effect of kappa-opioid agonists [34]. Importantly, no electrophysiological signs of aversive action were found for RU-1205. The mechanism underlying the aversive effects of kappa-opioid agonists (e.g., depression, sedation, dysphoria) is linked to intracellular signaling via the β-arrestin pathway and subsequent activation of p38 MAP kinase. The activity profile of RU-1205 might be explained by ligand-biased signaling (i.e., ligand-dependent selectivity for specific intracellular signaling pathways) [35, 36] or by a dual-target mechanism (kappa-opioid receptor activation combined with p38 MAPK blockade). To test the latter hypothesis, we employed the drug discrimination paradigm.

Our results lead to the conclusion that the pharmacological effects of RU-1205 bear little resemblance to those of SB203580, while showing a high degree of similarity to U-50488. The inability of trained animals to discriminate the effects of RU-1205 in the presence of naloxone suggests that the compound does not possess subjectively perceivable off-target activity.

The electrophysiological approach is a valuable tool for understanding how opioid compounds affect receptor signaling and physiological processes such as pain, respiration, and addiction [37]. Gillis et al., in a comprehensive comparative analysis of GPCR ligands, concluded that the pharmacological properties of biased agonists are determined not only by the degree of imbalance in post-receptor cascade activation but also by other factors [38], such as the intrinsic efficacy of the ligands. Birdsong and colleagues also underscore the importance of employing diverse approaches, particularly electrophysiology, in the study of ligand-biased signaling [37]. Our research was limited to observing changes that are not direct readouts of secondary messenger activity. However, this limitation is offset by the versatility of our approach, which allows for the indirect assessment of a wide range of biochemical processes as they manifest in brain function and behavior.

Limitations of the study

This work has several methodological limitations. The experiments were conducted exclusively on adult male rats, which may limit the generalizability of the findings to females. Bioelectrical activity was recorded at a single, fixed 30-minute time point post-injection, precluding analysis of earlier or later effects. The spectral analysis was confined to the standard EEG range (up to 50 Hz), excluding higher frequency components that could be informative for assessing synaptic activity. The study focused on a limited set of cortical and limbic structures, omitting other brain regions involved in opioid signaling. Finally, while the intracerebroventricular route of administration ensures direct brain exposure and controlled experimental conditions, its clinical relevance is limited, a factor that should be considered when interpreting the results.

CONCLUSION

In summary, we have established that, compared to the selective kappa-opioid agonist U-50488, RU-1205 induces less pronounced changes in LFP activity and lacks the electrophysiological patterns associated with β-arrestin pathway activation and aversive effects. The electrophysiological effects of RU-1205 were confined to cortical regions, the hippocampus, and the prefrontal cortex. The absence of behavioral evidence for p38-inhibitory activity, as confirmed in the drug discrimination model, underscores the relevance of investigating ligand-biased signaling as the potential mechanism for its unique profile in future studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Konstantin Yu. Kalitin — statement of key objectives, analysis of scientific and methodical literature, data processing, writing, and editing of the manuscript; Olga Yu. Mukha — data collection, data processing, writing, editing, and formatting of the manuscript; Alexander A. Spasov — critical revision of draft manuscript with valuable intellectual investment, final manuscript approval. All authors confirm that their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept, conduct of the study and preparation of the article, read and approved of the final version before the publication).

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ОБЗОРЫ

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Modern Approaches to Enterovirus-Based Oncolytic Immune Virotherapy of Malignant Diseases

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The aim. The review presents the analysis of publications on modern approaches to oncolytic viral immunotherapy of malignant diseases which is predominantly based on usage of enteroviruses.

Materials and methods. Electronic data bases — PubMed, Scopus, Web of Science, Google Scholar, eLibrary, and other accessible datasets were used for gathering and analyzing appropriate publications for the following keywords: oncolytic virotherapy, oncolytic viruses, enteroviruses, *poliovirus, coxsackievirus, echovirus,* preclinical and clinical trials. The research included the time interval from 1990 till 2024.

Results. The data present the properties of wild type and gen modified viruses — the supposed basis for development of the drugs, as well as their action mechanisms. The described mechanisms include direct cytolysis caused by the intracellular reproduction of the virus, activation of antitumor immunity of the host body (viral recipient) due to presentation of the tumor-associated antigens from the damaged cells to dendritic cells for their further maturation, presentation of these antigens to T-lymphocytes and activation of cytotoxic lymphocytes, modulation of tumor microenvironment due to immunostimulation, and transition of “cold” tumor and its environment into “hot” state. It has been noticed that the most pronounced therapeutic efficacy is observed in immunosensitive tumors. This observation correlates with the action mechanism of the oncolytic viruses. Clinical trials of viral drugs still have not led to superior results in therapeutic efficacy but they have demonstrated the synergistic efficacy with other methods of conservative therapy. According to the results of preclinical and clinical trials, enteroviruses demonstrate a favorable toxic profile. Factors which reduce the efficacy of virotherapy were evaluated. They include non-targeted and non-specific absorption of viruses by tumor cells, weak endocytosis and reproduction followed by distribution in the body, preexisting immunity against the concrete viruses and induction of antiviral antibody expression during viral therapy, and lack of sensitivity of the tumor and its microenvironment to the virus.

Conclusion. Enterovirus-based oncolytic therapy is a promising therapeutic option but its efficacy needs to be enhanced using mechanisms of its therapeutic impact.

Keywords: oncolytic virotherapy; oncolytic viruses; enteroviruses; *poliovirus; coxsackievirus; echovirus;* preclinical and clinical trials

Abbreviations: APCs — antigen-presenting cells; DCs — dendritic cells; CAR-T — chimeric antigen receptor; CD — cluster of differentiation; CXADR — coxsackie-adenovirus receptor; DAMPs — damage associated molecular patterns; EM — extracellular matrix: HSV — herpes simplex virus; IFN — interferon; IL — interleukin; MDSC — myeloid-derived suppressor cells; NOAEL — no-observed-adverse-effect level; NOD-SCID — non-obese diabetic / severe combined immunodeficiency; PAMPs — pathogen associated molecular patterns; $TCID_{50}$ — Tissue Culture Infectious Dose; TME — tumor microenvironment; TNF — tumor necrosis factor; MN — malignant neoplasm; CTs — clinical trials; MHC — major histocompatibility complex; ICAM-1 — intercellular adhesion molecule 1.

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Современные подходы к онколитической иммуновиротерапии онкологических заболеваний на основе энтеровирусов

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Цель. Представить обзор современных подходов к онколитической вироиммунотерапии онкологических заболеваний с применением энтеровирусов по данным мировой научной литературы.

Материалы и методы. Для сбора и анализа сведений использованы электронные базы данных PubMed, Scopus, Web of Science, Google Scholar, библиотечная база данных (eLibrary.ru) и другие доступные ресурсы. Поиск проведён по публикациям за 1990–2024 гг. по ключевым словам: «онколитическая виротерапия», «онколитические вирусы», «энтеровирусы», «*poliovirus*», «*coxsackievirus*», «*echovirus*», «доклинические исследования», «клинические испытания».

Результаты. Представлены данные о свойствах онколитических вирусов дикого типа и геномодифицированных вирусов, на которых основан выбор вируса для разработки лекарственного препарата, и о механизмах их действия. Они включают прямое цитолитическое действие, обусловленное внутриклеточным размножением вируса; активацию противоопухолевого иммунитета организма — реципиента вируса за счет презентации опухоль-ассоциированных антигенов дендритным клеткам с последующим их созреванием, презентацией антигенов Т-лимфоцитам и активацией цитотоксических лимфоцитов; модуляцию опухолевого микроокружения окружения в результате иммуностимуляции и перехода «холодной» опухоли и окружающей ткани в «горячее» состояние. Отмечено, что наиболее выраженный терапевтический эффект наблюдается в отношении иммуночувствительных опухолей, что коррелирует с механизмом действия онколитических вирусов. Клинические испытания лекарственных препаратов пока не привели к прорывным результатам по терапевтическому действию, но показали синергизм по эффективности с другими видами консервативной терапии. По результатам доклинических и клинических исследований, энтеровирусы проявляют благоприятный профиль токсичности. Оценены факторы, снижающие эффективность виротерапии: недостаточно целенаправленное попадание вируса в опухолевые клетки, неактивный эндоцитоз и размножение с последующим распространением в организме; предсуществующий в организме иммунитет против конкретного вируса и индукция выработки антител к нему в процессе виротерапии; отсутствие чувствительности самой опухоли и ее микроокружения к вирусу.

Заключение. Онколитическая виротерапия на основе энтеровирусов перспективна, но эффективность ее необходимо повышать, опираясь на механизмы действия.

Ключевые слова: онколитическая виротерапия; онколитические вирусы; энтеровирусы; *poliovirus; coxsackievirus; echovirus;* доклинические и клинические исследования

Список сокращений: АПК — антиген-презентирующие клетки; ДК — дендритные клетки; CAR-T — химерный антигенный рецептор Т лимфоцитов; CD — кластер дифференцировки; CXADR — Коксаки-аденовирусный рецептор; DAMPs – молекулярный фрагмент, ассоциированный с повреждениями; ECM — внеклеточный матрикс; HSV — вирус простого герпеса; IFN — интерферон; IL — интерлейкин; MDSC — миелоидные супрессорные клетки; NOAEL — уровень отсутствия наблюдаемых побочных эффектов; SCID — тяжёлый комбинированный иммунодефицит; PAMPs – патоген-ассоциированные молекулярные паттерны; $TCID_{50}$ — инфекционная доза тканевой культуры; TME — опухолевое микроокружение; TNF — фактор некроза опухолей; ЗНО — злокачественное новообразование; КИ — клинические исследования; ГКГС — главный комплекс гистосовместимости; ICAM-1 — молекула межклеточной адгезии 1.

INTRODUCTION

Malignant diseases remain to be one of the major death causes over the world despite the obvious progress in the development of both surgical and conservative treatment methods [1, 2]. It is well-known that tumor cells use various methods to successfully fight with chemo- and target drugs, as well as inhibit the immunity of tumor-bearing body [3–5].

It should be admitted while evaluating modern state-of-art in oncology, that one of the major advances of the XXI century is understanding that tumor node is formed not only by malignant cells but presents a huge dynamically developing network containing both transformed and non-transformed cells tightly connected with each other, as well as soluble mediators which form tumor microenvironment (TME) [6].

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TME provides the niche for tumor growth and accumulation of metastatic cells using the extracellular matrix, maintains vitality of tumor stem cells, provides functioning of the procarcinogenic mediator signal systems and forms a barrier that prevents penetration of both endogenous and exogenous anticancer agents to the malignant node. It means that antitumor therapy should be targeted not only to malignant cells but provide a complex impact on the whole TME.

Oncolytic antitumor virotherapy has been actively developed during the last decades. Its therapeutic action is based on various mechanisms which impact multiple components of malignant processes [7, 8].

The wild type viruses with oncolytic potency not only damage the tumor cells due to direct destroying their structure but also affect various TME elements inducing both cell death and prolonged activation of immune reactions. That is why treatment including viruses with oncolytic potency has been named oncolytic viral immunotherapy and oncolytic viruses have been recognized as immunotherapeutic agents [9, 10].

Initially at the beginning of virotherapy era, when only wild type viruses were used as therapeutic agents, the results were contradictory and doubtful. The development of gene engineering technology and understanding of the serious role of TME brought back the interest to oncolytic virotherapy. Nowadays we possess a great set of both wild type and gene modified viruses which form the basis for creating medicines aimed at oncolytic immune therapy. Many of them have reached various stages of clinical trials (CTs) and 4 of them have been registered as drugs for treatment of different malignant diseases [11, 12].

Their efficacy was demonstrated in the treatment of patients with melanoma, glioma, and squamous head and neck cancer. But soon it was elucidated that monotherapy with virus drugs did not cause the cure of patients or the significant therapeutic efficacy. The combined therapy including radio- or chemotherapy, as well as immune checkpoint inhibitors or CAR-T- cell therapy, proved to be more efficient. More than 100 CTs for the evaluation of this approach have been initiated, or are under study, or have been completed recently in many countries worldwide. No oncolytic viral drugs are registered for treatment of cancer patients in Russia, so the development and registration of the original drugs are important for Russian medicine.

THE AIM. The aim of this review is to present modern approaches to enterovirus-based anticancer oncolytic viral immunotherapy using the research data from worldwide publications.

MATERIALS AND METHODS

Electronic data bases (PubMed, Scopus, Web of Science, Google Scholar, library dataset (eLibrary), and other available resources were used for collection and analysis of information. The search was performed through the publications from 1990 till 2024 using the following keywords: “oncolytic viruses”, “enteroviruses”, “*poliovirus*”, “*coxsackie virus*”, “*echovirus*”, “preclinical study”, and “clinical trials”.

RESULTS AND DISCUSSION

Selection of oncolytic virus

Before selecting a virus — the future basis of the supposed antitumor oncolytic drug — it is important to study its biological properties and genetics in order to decide if it would be relevant to use it as a wild type virus or if its genome should be modified for safety and efficacy of the therapy.

The ideal oncolytic virus for antitumor viral therapy should possess the following properties:

- be able to replicate and produce active offspring after accumulation in malignant cells;
- be oncolytically active in the infected tumor cells which could excrete viral offspring into extracellular area after their damage;
- be immunogenic, that is be able to induce the immune response;
- not cause the development of chronic or infectious disease;
- not integrate into human genome;
- be safe for various cohorts of people;
- be available for genetic modification aimed to enhance immunogenicity or stimulation of targeted antitumor impact.

From the very beginning of antitumor viral immunotherapy and early CTs the development of medicines has been performed using various DNA and RNA viruses of both wild type and gene modified ones. These are: *Adenovirus, Herpes simplex virus type 1, Parvoviruses,* and *Poxviruses* (*vaccinia virus* и *myxoma virus*) of the DNA-viruses [13, 14] and *Coxsackie virus, Seneca Valley virus, Maraba virus, Measles*

virus, Newcastle disease virus, Vesicular stomatitis virus, Sindbis virus, and *Poliovirus* type 1–3 of the RNA-viruses [15].

Genome stability and the possibility to insert large transgenes into DNA-viruses without the loss of viral infectivity and the ability to replicate are undoubtedly their advantages. The advantages of the RNA-viruses are absence of integration into recipient genome and their higher immunogenicity in some cases. But smaller volume of the genome in comparison to DNA-viruses is their disadvantage because it limits the size of the inserted transgene.

A serious problem in selecting a virus for oncolytic therapy is its tropism to tumor cells. Constructing recombinant viruses is aimed to enhance their ability to interact and penetrate the tumor, but not the normal, cells. Tropism of wild type viruses to malignant cells depends on many factors, presence of the receptors on the cell surface being one of them. They provide tight connection of the virus with a tumor cell and endocytosis of the virus in some cases. So, CD46, CD155, α2β1, CD55, CXADR (coxsackie-adenovirus receptor) molecules are often overexpressed on tumor cells of various histogenesis and provide the linking of the *measles virus, poliovirus, echovirus, adenovirus, coxsackievirus* [16–19].

Other molecules promoting tumor growth and progression of malignant process may also serve as receptors for various oncolytic viruses [20, 21]. But it should be noted that not all tumor cells possess enough receptors for efficient linkage and penetration of the viruses. Moreover, the expression of viral receptors may be seen not only on the tumor cells. Normal cells sometimes also express these receptors though less intensively, so we cannot consider that virus-receptor interactions on tumor cells would be extremely selective regarding normal cells.

Metabolic cell status and the ability of the virus to overcome antitumor immune reactions and intracellular signal pathways independently or with the help of auxiliary incentives are also very important [22–24].

Cancer is a complex heterogeneous disease with many genetic mutations which induce various alterations in antiviral signal pathways thus creating excellent conditions for viral replication. For instance, cells often sacrifice some elements of their congenial antiviral defense system provided by cytokines — interferons I or II (IFNs), tumor necrosis factor (TNF), or some others [25].

Apart from the ability to infect tumor but not normal cells which is determined by the defective IFN signal pathway in malignant cells, there are some other innate antiviral defense pathways. They help normal cells to identify and block viral replication, and play a crucial role in the ability of oncolytic viruses to infect and replicate selectively in tumor cells. For instance, tumor-specific aberrations in *RAS, TP53, RB1, PTEN, EGFR, WNT, BCL-2*, and other related genes provide the predispositions of malignant cells to viral infections [21, 22, 26].

The main advantage of genetic editing or engineering is a possibility of genetic modification of natural viruses by rational elimination of viral genes responsible for virulence and by insertion of tumor-specific promotors or sequences — targets for miRNA, thus selectively enhancing viral gene expression only in tumor cells.

Vise versa it is possible to put genes which are critical to viruses under the control of miRNA weakly expressed in tumor cells. Then viruses would not be able to replicate in normal cells where ordinary expressed miRNA would prevent high production of viral genes important for replication.

Gene engineering methods give opportunity to construct viruses which are able to infect cells only with abnormal content of some genes. In order to provide the selective infection of tumor cells some proteins of viral shell may be especially modified for further viral interaction only with tumor specific receptors.

The usage of antibodies targeted to tumor antigens can also enhance the specificity of viral therapy [27]. This approach results in the viral ability to damage tumor but not normal cells providing high selectivity of antitumor viral therapy [28].

Mechanisms of antitumor oncolytic viral immunotherapy

The main components of the oncolytic virus antitumor impact are intracellular viral replication, cell destruction due to induction of various death mechanisms (apoptosis, necrosis / necroptosis, piroptosis, autophagia, and others), elimination of immunogenic components from the damaged cells, and stimulation of innate and adaptive antitumor immunity.

The death pattern of malignant cells depends on the characteristics of oncolytic virus and tumor cell type

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because some genes inhibiting/stimulating apoptosis, necrosis, or autophagia may express in these cells [12]. Gene engineering helps to obtain viruses which induce a particular kind of cell death resulting in the increase of immunogenicity of viral therapy.

It is quite obvious now that after linking with and accumulation in the tumor cells oncolytic viruses use various mechanisms for destroying these cells which may depend or not depend on viral replication in them. It is assumed that antitumor viral activity is based on the following mechanisms:

1) A virus may selectively accumulate in malignant cells inducing cytolytic effect (oncolysis). The exact mechanism of viral oncolysis is not fully studied but it is known that it may greatly vary between the viruses as well as the types of tumor target cells. 2) There may appear indirect effects of cell death (for instance, apoptosis-like and necrosis-like effects) in infected, non-infected and endothelial cells of intratumoral blood vessels which decrease angiogenesis. 3) Activation of systemic antitumor and antiviral immunity as well as recruitment of activated immunocompetent cells into TME may happen [29–31].

As it was noted above, all mechanisms greatly depend on the viral type and interactions between oncolytic virus, TME, and immune system of the recipient — tumor-bearing body [32, 33]. But commonly virus infected malignant cells die due to activation of cell death pathways or virus induced damage of their integrity.

It has been proved that TME is the pool of elements with abnormal metabolic pathways — stromal and immune cells, blood vessels, extracellular matrix, and others which actively stimulate proliferation and metastasis of malignant cells due to local cytokines, chemokines, and signal intracellular chains. At the same time tumors may be classified in most cases as immunologically “cold” structures because of low level of tumor antigens, tumor infiltrating suppressor immune cells, and signal molecules.

Oncolytic viruses become a potent immunotherapeutic weapon due to their ability to destroy immunosuppressive TME and create a “hot” environment which promotes development of prolonged tumor-specific immunity providing opportunity of control over the “surveillance against relapse” [11, 34–36].

One of the major characteristics of oncolytic therapy is its ability to induce immunogenic cell death (apoptosis, necrosis / necroptosis, pyroptosis). It results in release of molecular structures demonstrating cell damage (DAMPs — damage associated molecular patterns) — calreticulin, heat shock proteins, ATP, uric acid, and others, together with pathogen-associated molecular structures (PAMPs — pathogen associated molecular patterns) — double-stranded DNA, double- and single-stranded RNA, glycoproteins, lipoproteins, and membrane viral components, as well as cytokines (IFN-γ, IFN-α, TNFα, IL-1, IL-6, IL-8, IL-12) and tumor-associated antigens [37]. These components act as danger signals and induce immune responses to viral infection – initially local (activation of dendritic cell (DC), infiltration, and their maturation) and then systemic adaptive antitumor immunity which is the second effective impact of viral immunotherapy.

DCs are rarely presented in tumors but they are especially potent as antigen-presenting cells (APCs) which fulfil the linkage between the systems of innate and adaptive immunity [38]. Immature DCs are able to migrate easily whereas mature DCs activate T-lymphocytes expressing both specific linkage molecules for T-lymphocytes and co-stimulating molecules. Regulation of DC pool in TME is of great importance for obtaining efficient antitumor immunity. Oncolytic viruses are not only able to induce the outflow of new antigens into circulation and consequently their presentation to DC, but also to prepare TME for easy infiltration by DCs, their maturation, and T-lymphocyte activation. Moreover, enhancing the expression of cytokines in TME after the infection with oncolytic virus stimulates both infiltration of TME by CD4+ and CD8+ T-lymphocytes and their activation. The increase of antitumor cytokine expression in TME pronouncedly impacts malignant cells and many TME components: immune cells, blood vessels, tumor-associated fibroblasts, metabolic processes, and extracellular matrix. As a result, activation of innate and adaptive immunity and prolonged tumor suppression are observed.

Despite the proof of the dual viral impact on the development of tumor structures and total malignant process, the treatment using Oncorine (H101; (E1B/E3 deficient adenovirus), one of the registered drugs, has shown that viral therapy with a single oncolytic virus is not much efficient. This is why recent studies are

mostly concentrated on constructing oncolytic viruses with inserts of genes producing immune components — cytokines and chemokines, or supplied with monoclonal antibodies (targeting them) targeted to malignant cells [39–41].

This approach provides the delivery of cytokines and chemokines to TME with the help of an oncolytic virus, localize**s** the immune reactions near the tumor node, and decrease**s** the toxic reactions of the treatment. Moreover, the replication of oncolytic viruses in tumor cells determines constant production of the cytokines encoded in recombinant viral genome [41].

The usage of recombinant viruses with inserted cytokine genes has demonstrated that after intratumoral administration they provide a more pronounced abscopal effect (regression of distant metastases) than similar viruses of wild type without cytokine production which can only stimulate cytokine expression due to lysis of malignant cells [39–41].

A most important antitumor viral impact is their influence on M1 and M2 macrophages populations in TME. Macrophage plasticity is very high and it is realized in continuum of transfer from M1 to M2 populations in TME. It is well-known that M2 macrophages in tumor node are a prognostic factor of poor survival for various malignant processes. It has been shown that oncolytic viral therapy induces repolarization of immunosuppressive intratumoral M2 macrophages to M1 phenotype expressing anti-inflammatory cytokines and chemokines, for example, IFNγ, CXCL10, IL-6, IL-2, IL-12, and IL-21, which enhance antitumor impact of TME immune complexes [42]. Thus, oncolytic therapy is an efficient control mechanism of M1 / M2 ratio in TME [43]. Besides, in estimation of the genomodified HSV1716 mechanism it was demonstrated that the infection induced not only the stimulation of macrophage polarization into M1 phenotype. Macrophages determine viral amplification due to its accumulation, replication, and elimination of the next generation in TME, thus providing oncolytic efficacy of the treatment [44].

Natural killers (NK) play an important role in antitumor fight, realizing cytotoxic functions as well as remodeling TME. Dendritic cells produce cytokines (IFN-1, IL-12, and IL-18) which enhance the NK cytotoxicity in TME. Other signal mediators — IL-15 and IL-21 produced by myeloid cells also promote their efficacy. Oncolytic viral therapy activates NK indirectly due to stimulation of cytokine and chemokine production [45, 46]. There are data that viruses change the expression of the activating and inhibiting NK ligands including MHC-1 on the malignant cells [9].

One of the important factors in maintaining TME immunosuppressive role is the population of highly suppressive myeloid-derived suppressor cells (MDSC) which significantly increases during tumor progression. These cells not only inhibit the tumor response to the immune impact but also stimulate tumor invasion by engaging various non-immune mechanisms. Besides, it was proved that MDSC decrease the efficacy of modern antitumor therapeutic methods — chemo-, radio-, and immunotherapy [47]. Viral impact on these cells is ambiguous. It was shown that both accumulation of MDSC in TME and remodeling of this population to phenotype destroying tumor tissue took place due to the enhanced NO production after the infection [48]. The efficacy of oncolytic viruses is not yet elucidated regarding the T-regulatory cells (Treg) — another population of immunocompetent cells in TME which possesses a pronounced immunosuppressive activity [49].

Despite the discovery of numerous antitumor mechanisms of oncolytic wild type viruses by which they damage malignant cells or inhibit their growth it came out in practice that the efficacy of viral monotherapy is quite restricted due to several reasons. 1) Antiviral neutralizing antibodies may be present in blood stream either as a factor of preexisting immunity because of previous contacts with the viruses or as the newly formed due to the therapy using systemic administration of oncolytic viruses (intravenous, intraarterial). They can prevent the intracellular viral replication and consequently the tumor cells' lysis [50, 51]. 2) Mechanisms of antivirus resistance including complement activation, antiviral cytokines and macrophages may improve oncolytic virus elimination [52, 53]. These antiviral immune reactions become a serious obstacle decreasing efficacy of viral antitumor therapy. While by now the whole range of immune impacts *pro* and *contra* of oncolytic viruses is not fully known, there is a possibility that the suppressive action against viruses may be overcome due to their local and abscopal effects.

3) Extracellular matrix, fibrosis, necrosis, and interstitial hydrostatic pressure may form an insurmountable physical barrier for linking the oncolytic

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virus with cell receptors which would cause serious decrease of viral endocytosis and consequently its replication, amplification, and antitumor efficacy [50, 54].

Understanding the oncolytic viral immunotherapy peculiarities based on natural wild type viruses led to the necessity to produce the modified viruses by directed change of their properties by inserting genes responsible for synthesis of target proteins. Gene modification helps to improve various viral characteristics**:** their tropism to malignant cells, selectivity of linking with particular receptors, expression of cytokines and chemokines, ability to recruit immunocompetent cells to TME, and increase their antitumor activity. But gene modified viruses possess their own disadvantages. The expression of transgenes or modification of the virus aimed to increase its selectivity can worsen its suitability for therapy due to decrease of replication and its oncolytic activity. Expression of transgenes may prevent participation of the virus in immune reactions significant for realization of the antitumor effect [55]. Therefore, it seems justified to create complex medicines including several viruses both wild type and recombinant.

As a result, such properties of non-pathogen viruses with oncolytic potency as direct cytolysis of malignant cells,-recruitment of immunocompetent cells, activation of antitumor immune reactions, TME modulation, antiangiogenesis, and ability to use metabolic and specific signal in tumor cells have engaged the researchers in developing medicines for antitumor viral immunotherapy [10].

Preclinical study of safety and efficiency of oncolytic enteroviruses

Conservative treatment of cancer patients is often accompanied by severe adverse reactions. So, at the stage of development of new drugs great attention should be paid to evaluation of their safety and tolerability during preclinical study according to the modern guidelines which tightly restrict the study pattern for registration of original medicines. Preclinical studies of toxicity of various oncolytic viruses using experimental animals which are presented in research literature demonstrate the acceptable toxicity profile of oncolytic viruses and prospects for further promoting the antitumor viral immunotherapy [56].

As it was noted, the arsenal of oncolytic viruses promising for antitumor therapy is rather large now and includes viruses of different nature, both DNA- and RNA-viruses. Understanding viral nature and biology is crucial for planning the study of the viral therapy safety because it is well-known that DNA-viruses unlike RNA-viruses integrate into genome of recipient which may cause undesirable consequences. Therefore, only RNA-viruses may be used in their wild type for creation of a drug. DNA-viruses should be modified by exchange of the genes responsible for integration to transgenes producing “useful” proteins, for instance, cytokines or chemokines.

RNA viruses of the *Picornaviridae* family *Enterovirus* genus have attracted the researchers. So, several of them have become the basis for medicines developed in some countries which have reached the initial stages of CTs.

ECHO 7 is the active pharmaceutical substance in Rigvir (Latvia, 2004), the first official drug registered for oncolytic therapy of patients with melanoma, local skin melanoma metastasis, and for prophylaxis of melanoma recurrence after radical surgery [57]. The drug was used in 2 mL dose with the concentration $\geq 10^6 TCID_{50}$ (Tissue Culture Infectious Dose).

The cytolytic efficacy of Rigvir was initially shown using various lines of human tumor cells: FM-9 (melanoma), RD (rabdomiosarcoma), AGS (gastric adenocarcinoma), A549 (lung carcinoma), HPAF II (pancreatic carcinoma), MSC (mesenchimal stem cells from human bone marrow), and uveal melanoma lines — MP41, Mel-202, 92-1 [58, 59].

The toxicity study of Rigvir was performed according to the regulations of Good Laboratory Practice (GLP) in Han-Wistar rats at multiple administration of the drug during 4 weeks with a consequent follow up period for the next 4 weeks. The drug was administered in three doses of 2×10^6, 1×10^7, and 2×10^7 $TCID_{50}$ on the 1–3, 8–10, 15–17, and 22–24 days. Neither deaths nor adverse clinical reactions were observed. Body mass, ophtalmoscopia, clinical pathological parameters, analyses, and organ mass remained unchanged during the observation period. Only eosinophil level increased a little bit but it was normalized towards the end of observation. Rigvir has been detected both in blood samples and spleen during 48 h after the administration. But in spleen samples of 2 animals which were administered the highest dose the drug it was detected on the 29 day of the follow up period. Therefore, it may be resumed that Rigvir reaches spleen

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in intact animals. It is quite justified taking into account the immune competency of this organ and the detected increase in eosinophil content which reflects the impact of the drug on the immune system [60].

Non-intensive cell infiltration has been detected in the injection site which characterizes the development of weak inflammation confirmed by increased quantity of lymphocytes in regional lymph nodes. The majority of adverse effects were reversible and only the lymphocyte content remained increased during the whole follow up period. The highest tested dose which did not induce the detected adverse impact on animal health was 2×10^7 $TCID_{50}$ (NOAEL, no-observed-adverse-effect level) [60].

As a result, good Rigvir tolerability was observed which was a proof of the favorable toxicity profile of *ECHO* 7 in animals. Further this conclusion was confirmed by the results of the Rigvir CTs in patients with melanoma as well as by its post-registration usage [60].

Wild type enterovirus *Coxsackievirus* A21 (CVA21) is an active pharmaceutical substance of CAVATAK (Viralytics Ltd., Australia). It predominantly links with tumor cells through ICAM-1 (intercellular adhesion molecule 1) or CXADR (coxsackie-adenovirus-receptor) [61, 62]. The lytic effect of CVA21 was initially detected on human melanoma cell lines, then the study was enhanced using cell lines and xenografts of multiple melanomas, breast, pancreatic, lung, and non-muscle invasive bladder cancer [61, 62].

In vivo study using xenografts has shown the viral ability to spread in the animal body. The NOD-SCID (non-obese diabetic / severe combined immunodeficiency) mice bearing melanoma xenografts were injected one dose of CAVATAK (10^3 or 10^5 $TCID_{50}$) using various administration paths: intratumoral, intravenous, and intraperitoneal. Efficient spread of the virus and inhibition of the ME4405 melanoma xenograft growth were observed in each case. Oncolysis took place even in tumors located far away from the site of administration. Moreover, the fact that viral blood concentration got 10^5–10^6 $TCID_{50}$ titer confirmed the efficient viral replication [63].

The combination of CVA21 with DAFv and ECHO-*1* in the single doses of 7.5×10^5; 1.8×10^7, and 7.5×10^5 $TCID_{50}$ was studied in the PC-3 prostate adenocarcinoma model in SCID-BALB/c immune deficient mice. In the LNCaP human prostate adenocarcinoma model various doses of ECHO-1 (10^7, 10^5, and 10^3 $TCID_{50}$) were infused intravenously to mice. CVA21 and CVA21-DAFv in high titers were detected in mouse blood till the time of euthanasia (the 35th day after the infusion) whereas ECHO-1 titer significantly decreased in a week [56].

Serious adverse reactions — paralysis of the hind limbs and myositis of varying severity — were observed in SCID mice with breast cancer and multiple myeloma xenografts which were infected intravenously with CVA21 in 10^7 $TCID_{50}$ dose [64] but such reactions did not develop in immunocompetent patients [65]. Viral murine blood concentration varied in the range of 10^5–10^7 $TCID_{50}$ during the experiment. Viremia was observed in some mice. It is quite possible that this phenomenon was determined by the animal immune deficient status, and, respectively, inability to fight successfully the viral burdens or viral replication in muscle tissue which often occurs in immune deficient mice [64].

Recombinant *poliovirus* 3 (Sabin) attracted attention of the researchers with a prospect to use it for treatment of glioblastoma — the most malignant brain tumor [66]. But p*oliovirus* 3 (Sabin) acquires some properties that determine its neurotoxicity, and so its safety and tolerability should be evaluated in detail. Normally the effective replication of *poliovirus* is limited to two areas: gastrointestinal tract with associated lymphocytic components and motor neurons of medullar areas of spinal marrow [67]. These replication areas correspond to localization of cells with maximum expression of CD155 — the main receptor which links with *poliovirus* and provides its accumulation in the cells. *Poliovirus* replication in gastrointestinal tract does not cause enteropathology and normally is not clinically active. But viral tropism to motor neurons is the cause of paralytic poliomyelitis, it is in fact the pathognomonic signature of *poliovirus,* and as such raises serious biosafety concerns [66].

The clinically safe but replicative competent *poliovirus* should be completely deprived of its neurovirulent potency. Ideally the safety characteristics of oncolytic *poliovirus* must meet the following requirements: a virus should possess the ability to replicate in malignant cells, be devoid of neurovirulent properties inherent in it, and the viral genotype attenuated in the neurovirulent potency should be stable for a long period.

Sequencing genome of neuroattenuated poliovirus

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(Sabin) strains which were obtained by prolonged selection has elucidated that single point mutations in the IRES (internal ribosomal entry sites) critical zone are the major safety mechanism for all three strains of poliovirus (Sabin, type 1–3), whereas genetic reversal of these mutations to wild type sequence brings it back to neurovirulency [68]. This led to the creation of recombinant *poliovirus* PVSRIPO with IRES of human *rhinovirus* type 2 which is the most studied up to now [69]. It is true that substitution of IRES in *poliovirus*-1 caused significant decrease of neurovirulent viral action which excluded development of poliomyelitis, meningitis, and encephalomyelitis at intracerebral administration of attenuated recombinant virus to primates of Old World in a WHO standard model for evaluation of these infectious diseases [70]. These complications were not observed in patients in phase I of CTs [67].

High expression of CD155 in tumor cells of various histogenesis is very important for realization of viral oncolytic potency because this protein is the major *poliovirus* receptor determining endocytosis and intracellular accumulation of the virus [71, 72]. It has been found out recently that CD155 serves as an immune control point due to its ability for tight linking to T-cell immune receptor expressed in natural killers and T-cells. Though the CD155 physiological properties are not fully known they may include modulation of immune response [73, 74].

It is well-known that *enteroviruses* that include *poliovirus* induce the development of pronounced antitumor response determined by the innate interferon system. This may come to be the main factor of PVSRIPO immunogenic mechanism [75]. Besides, the viral infection causes a large spectrum of inflammatory reactions which promote significant immunocompetent cell invasion into TME. Post-infectious processes resemble the development of a classic response to the inflammation induced with a pathogen and are very important in the context of antitumor therapy [76, 77].

Intratumor pathway for PVSRIPO administration is the most efficient for realization of its impact on the antitumor immune system [76]. Systemic infusion of recombinant *poliovirus,* as well as other oncolytic viruses, leads to the need for the virus to overcome various obstacles on the way to the tumor including the blood-brain barrier, the circulating neutralizing antibodies, and the complement system. Moreover, it is quite difficult to provide targeting virus to tumors and their environment at systemic infusion and**,** consequently, accumulation of the virus in high concentration in the targeted structures [76].

An original drug has been developed in the Institute of Molecular Biology named after Engelhardt RAS together with National Medical Research Radiology Center on the basis of 4 active pharmaceutical substances — viruses of *Picornaviridae* family *Enterovirus* genus. Safety and efficacy of the drug were studied in preclinical trials using experimental animals. The obtained results bear evidence of good prospects for its further study in CTs.

Clinical trials and clinical usage of oncolytic viruses-based drugs

Only 4 drugs on the basis of various oncolytic viruses have been registered in the world, among them wild type viruses (*Enteroviruses*) and gene modified viruses (*adenovirus*, *herpes simplex virus* type I) [12].

The first drug on the basis of non-modified *Picornaviridae* (*ECHO* 7), Rigvir, has been registered for melanoma treatment in Latvia in 2004**,** and then in Georgia, Armenia, and Uzbekistan [57, 78]. But it has not spread further because of its poor therapeutic efficacy. The next drug named Oncorine (H101) was developed on the basis of gene-engineered *adenovirus* with deletion of the *E1B* gene. It was approved in China in 2005 for treatment of squamous cell head and neck and esophageal cancer [79]. But its efficacy also came to be rather poor because it was mostly provided by oncolysis, and not by active stimulation of antitumor immunity [54]. The third was the gene-engineered attenuated *herpes simplex virus type I* (HSV-1) with *GM-CSF* transgene (granulocyte-macrophage colony stimulating factor), Talimogene Laherparepvec (T-VEC, Imlygic), which was sequentially approved in 2015 in USA and Europe, and then in Australia and Israel for local treatment of the unresectable skin melanoma, as well as the subcutaneous and lymphatic nodes in patients with recurrent melanoma after surgical extinction of primary tumor [80–83]. Delytact (teserpaturev/G47Δ) is one more drug based on the modified *herpes simplex virus* type I registered in Japan in 2021 as a medicine for treatment of brain tumors including glioblastoma [84–86]. Increase of survival and favorable toxicity profile were observed in patients with residual tumor or relapse of glioblastoma against the background of viral immunotherapy. But these results are

preliminary and the registration of the drug has still a conditional status.

Therefore, only Talimogene Laherparepvec (T-VEC, Imlygic) keeps a strong position at the moment in the arsenal of therapeutic antitumor medicines.

Still, it is interesting to note that no transgenes have been included into the *HSV-1* genome of Delytact. So, a question arises: is genome modification through transgenesis necessary for improving therapeutic efficacy? Since it was shown that *GM-CSF* can stimulate MDSCs [87] and, respectively, decrease innate and adaptive antitumor responses in tumors of various genesis after administering virus with this gene, another question arises: is it expedient to insert *GM-CSF* gene in the *HSV-1* genome**,** as it was done for Talimogene Laherparepvec?

These questions together with many other concerning rational designs of oncolytic viral medicines, dosages, usage regimen both individually and in combination with other methods of conservative antitumor therapy, safety, and efficacy, would be answered by the results of CTs which grow geometrically and now exceed three hundred.

More than half of CTs now are being performed according to phase I, ¼ — according to phase II, and not more than 5% — according to phase III [54]. Undoubtedly, viral immunotherapy as an individual treatment method is more interesting than the combined methods but unfortunately its efficacy leaves much to be desired though the safety profile is favorable for most viruses.

Data on T-VEC treatment of 436 patients with melanoma were published in 2019. Patients were randomized in 2 groups: group 1 — 295 patients were treated using T-VEC and group 2 — 141 patients were treated using *GM-CSF*. The differences revealed by comparative statistical data processing were quite striking: the objective response was 31.5% (group 1) and 6.4% (group 2); complete response — 16.9% (group 1) and 0.7% (group 2); 88.5% of patients with complete response were alive at a 5-year assessment of the results [83].

The results of other T-VEC CTs in melanoma patients were also rather promising [81, 88]. The enterovirus-based oncolytic viral immunotherapy in patients with advanced melanoma was efficient too. The Phase I CTs of *coxsackievirus* V937 (CVA21) provided a 12-months survival without progression in 32.9% cases and general survival in 75.4% cases during this observation period [89]. The PVSRIPO phase I CTs in similar patients led to analogous results [90].

The fact that melanoma is one of the tumors most sensitive to immunotherapy**,** this being proved by good results of the oncolytic viral therapy, bears indirect evidence of the induction of antitumor immune processes at viral application. It is also noted in the review by Lin et al [54] that the most immunogenic tumors can be easier cured than malignancies which do not respond to immune impacts.

Some CTs concern administration of oncolytic viruses in patients with stage IV of gliomas. It seems that G47Δ and G207 (HSV-1) are the most promising viral medicines [86, 91, 92]. The Phase II CT of G47Δ has shown that 1-year survival of patients with residual or recurrent glioblastoma reaches 84.2% and general survival median — 20.2 months after the treatment initialization. It is interesting to note that G47Δ and G207 have not been modified by insertion of exogenous genes into the viral genome.

The Phase I CTs of PVSRIPO recombinant non-pathogenic virus has demonstrated that its intratumoral injection to 61 patients with IV stage of malignant glioma caused no signs of neurovirulent potency even at dose escalation from 10^8 $TCID_{50}$ till 10^{10} $TCID_{50}$: no poliomyelitis, meningitis, or encephalomyelitis were observed in the treated patients [93]. Only one dose limiting toxic effect was revealed during the treatment using PVSRIPO: at the maximum dose of 10^{10} $TCID_{50}$, immediately after removal of the catheter, hemorrhage was noted which did not cause serious complications after removal of the hematoma and the patient's life span was as long as 57 months.

Comparison of survival of patients treated by oncolytic virus and patients in the control group has demonstrated that survival in the experimental group reached a plateau of 21% by 24 months and this level was kept for 36 months, whereas in the control group it was only 14 % by the same period and decreased to 4% by 36 months.

Many other CTs of various oncolytic viruses for treating cancer patients have been initiated recently and some of them have reached Phase III.

Table 1 presents CTs of *enteroviruses* which are performed now [94].

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Table 1 – Clinical trials of oncolytic viruses of *Picornaviridae* family Enterovirus genus

Oncolytic virus	Virus type	Phase No., NCT No., state of trials	Monotherapy/ combination	Pathways for virus administration
PVSRIPO	*Picornavirus Poliovirus* Lerapolturev	I	Monotherapy	Intratumoral
		NCT01491893 (active, not recruiting patients)		
		NCT03564782 (recruiting patients)		
		NCT03043391 (active, not recruiting patients)		
		NCT03712358 (II phase, active, not recruiting patients)		
		NCT02986178 (active, not recruiting patients)		
		NCT04479241 (recruiting patients)		
			Pembrolizumab	Intratumoral
CVA21	*Picornavirus Coxsackievirus* CAVATAK® Gebasaxturev	I/II	Monotherapy/ combination	Intravenous Intratumoral Intravesical
		NCT04303169 (recruiting patients)	Pembrolizumab	Intratumoral
		NCT04521621 (recruiting patients)	Pembrolizumab	Intratumoral
		NCT04152863 (active, not recruiting patients)	Pembrolizumab	Intratumoral
		NCT02824965 (active, not recruiting patients)	–	Intravenous
		NCT00235482 (completed)	–	Intratumoral
		NCT00438009 (completed)	–	Intratumoral
		NCT01227551 (completed)	–	Intratumoral
		NCT01636882 (completed)	–	Intratumoral
		NCT02307149 (completed)	Ipilimumab	Intratumoral
		NCT02565992 (completed)	Pembrolizumab	Intratumoral
		NCT02043665 (completed)	–	Intravenous
		NCT03408587 (completed)	Ipilimumab	Intravenous
		NCT00636558 (completed)	–	Intravenous
		NCT02316171 (completed)	Mitomycin C	Intravesical

The results obtained up to now are presented in scientific medical literature only for 5 CTs of enteroviruses: NCT01491893 (PVSRIPO) [94], NCT03712358 (PVSRIPO) [90], NCT01636882 (*coxsackievirus* A-21, previous name CAVATAK®, modern name Gebasaxturev) [89], NCT03408587 (*coxsackievirus* A-21) [95, 96], and NCT02316171 (*coxsackievirus* A-21) [61].

All of these CTs were performed according to Phase I, so, the primary control point was evaluation of safety of the oncolytic viruses-based medicines.

Almost all researchers mention that the most frequent adverse reactions of the applied oncolytic viruses of *Picornaviridae* family *Enteroviridae* genus, as well as other, were chills, fever, nausea, flu-like syndrome, weakness, and pain at the injection site. Still, it is noted that overall, the safety profile of these viruses is more favorable than that of other immunotherapeutic medicines.

To sum up, the oncolytic viruses demonstrate a better toxicity profile not only in preclinical studies but also in CTs completed up to now.

CONCLUSION

Oncolytic viruses have recently attracted a close attention of the oncologists because of the positive results of preclinical evaluation using cultured malignant cells demonstrating their high cytotoxic activity. Further elucidation of antitumor impact of the most active viruses using models of human tumors in immune deficient animals has proved that there are good prospects for developing medicines on the basis of selected viruses.

Four preparations for oncolytic immunotherapy

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have been registered up to now in several countries, and some more medicines have reached Phase III CTs. The results of CTs were rather promising because they demonstrated the efficacy of oncolytic viral therapy in patients with several types of malignant processes which were resistant to common antitumor treatment as well as favorable toxicity profile of the studied medicines.

Evaluation of oncolytic virus impact mechanisms characterized some major aspects in their therapeutic action: direct cytolitic impact, determined by intracellular viral replication; activation of antitumor immunity of the viral recipient due to presentation of tumor--associated antigens from the damaged cells to antigen presenting dendritic cells with their further maturation and consequent presentation of the antigens to T-lymphocytes with activation of cytotoxic lymphocytes; modulation of the tumor microenvironment as a result of immunostimulation and transfer of the "cold" tumor and TME into the "hot" state; antiangiogenic viral impact; changing both signal pathways and metabolic processes in virus infected tumor cells.

The ability to modify viral genome appears to be a great advantage of the oncolytic viruses. It gives us an opportunity to change their properties through eliminating genes responsible for virulence and inserting genes coding the functionally active proteins which are able in their turn to enhance targeting viruses to malignant cells, stimulate immunity, and modify TME.

But despite the obvious progress in viral immunotherapy, there are many aspects in design, creation, and application of viral medicines that still do not fully satisfy the specialists of various profiles. There are some barriers that significantly decrease the efficacy of viral therapy.

First of all, this is defected targeting of the virus to malignant cells, endocytosis, accumulation, and replication in them with further spread in the body. Preexisting immunity against the particular virus and induced production of antibodies during the viral therapy also decrease the tumor damage. Presence or absence of the sensitivity of the tumor and its TME to the virus play, of course, the crucial role in the response and are determined by the concrete properties of the tumor and recipient body. It was observed that immune sensitive tumors showed the most pronounced sensitivity to oncolytic viruses which well correlated with viral action mechanism.

It is obvious that viral immunotherapy efficacy directly depends on the balance between the antitumor immunity induced in the tumor-bearing body by the oncolytic virus and the antiviral immunity of this body. But the biologic mechanisms influencing this balance are not yet revealed and, respectively, the pathways for improving it are not clear.

As it is, the researchers worldwide are developing methods aimed to enhance antitumor efficacy of viral oncolytic therapy, are creating various medicines aimed to improve their therapeutic properties, and are studying various schemes of combined application of viruses and other conservative therapeutic methods.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Elena R. Nemtsova — development of the concept, search and analysis of sources, interpretation of the data obtained, writing the final version of the article, final approval of the version for publication;
Ekaterina A. Plotnikova — search and analysis of sources, interpretation of the data obtained, writing the layout of the article. All authors made an equivalent and equal contribution to the preparation of the publication.
All authors confirm that their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept and preparation of the article, read and approved the final version before publication).

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REVIEWS

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Problems and Solutions of Pharmaceutical Packaging in Bulk

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To date, pharmaceutical activities in the manufacture of medicines in a pharmacy, intra-pharmacy packaging of registered medicines in Russia and the countries of the Eurasian Economic Union (EAEU), is one of the priorities of regulatory authorities in order to improve drug supply.

The aim. To study the current state of legal regulation of the circulation of medicines "in bulk" and the possibility of their packaging in a pharmacy.

Materials and methods. To solve the tasks set, the methodology of system analysis in the field of drug provision, content analysis of documents regulating the sphere of circulation of drugs was used. The regulatory framework of the study was the legislative and by-laws regulating the sphere of circulation of medicines of the Russian Federation and the common market of the EAEU. The search for regulatory legal acts was carried out in the Directory of the legal system "ConsultantPlus". In the course of the research, a set of scientific methods was used such a systematic, logical, structural and comparative.

Results. The article consider the issues of legal regulation of the circulation of medicines "in bulk" in the pharmaceutical market. The current state of the legislative and regulatory framework has been studied and the problems of legal regulation of packaging of medicicnes "in bulk" in pharmacies have been identified. The article also focuses on the general problem of compliance with modern packaging requirements for medicines compounded in pharmacy. The possibility of introducing a new concept of "Pharmacy packaging" into Federal Law No. 61-FZ dated April 12, 2010 "On the circulation of medicines" is considered. The approaches developed in world practice to the regulation of issues related to the packaging of drugs "in bulk" in pharmacy are considered. Based on the results of the study, the possibilities have been identified and ways to improve the mechanisms of state regulation have been proposed.

Conclusion. Packaging of medicines "in bulk" will increase the effectiveness of individual drug therapy, optimize and reduce the cost of circulation of budget funds, pharmacies and citizens.

Keywords: drugs manufacturing; extemporaneous drugs; "in bulk" medicines; packaging; pharmacy; drug supply; drug packaging.

Abbreviations: EAEU — Eurasian Economic Union; SRMs — State Register of Medicines; GM — general monograph; SPh — State Pharmacopoeia; EEC — Eurasian Economic Commission; IS MMM — Information System for Monitoring the Movement of Medicines; EDs — extemporaneous drugs; PPD — prescription and production department; SEZ — special economic zone.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Расфасовка лекарственных препаратов «in bulk» в аптечной организации: проблемы и решения

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На сегодняшний день фармацевтическая деятельность в части изготовления лекарственных препаратов (ЛП) в аптечной организации (АО), внутриаптечной фасовки зарегистрированных ЛП в России и странах Евразийского экономического союза (ЕАЭС), является одной из приоритетных задач регуляторных органов в целях улучшения лекарственного обеспечения.

Цель. Изучить современное состояние правового регулирования обращения лекарственных препаратов «in bulk» и возможность их расфасовки в аптечной организации.

Материалы и методы. Для решения поставленных задач использована методология системного анализа в области лекарственного обеспечения, контент-анализ нормативных документов, регулирующих сферу обращения лекарственных средств (ЛС). Нормативной базой исследования выступали законодательные и подзаконные акты, регулирующие сферу обращения ЛС Российской Федерации и общего рынка ЛС ЕАЭС. Поиск нормативных правовых актов осуществлялся в Справочнике правовой системы «КонсультантПлюс». В ходе исследования применялся комплекс научных методов: системный, логический, структурный и сравнительный.

Результаты. В статье рассматриваются вопросы правового регулирования обращения ЛП «in bulk» на фармацевтическом рынке. Изучено современное состояние законодательной нормативной базы и определены проблемы правового регулирования расфасовки ЛП «in bulk» в АО. Также в работе уделено внимание общей проблеме соответствия современным требованиям к упаковке для ЛП, изготовленных в АО. Рассмотрена возможность введения нового понятия «Аптечная упаковка» в Федеральный закон от 12.04.2010 г. № 61-ФЗ «Об обращении лекарственных средств». Рассмотрены сложившиеся в мировой практике подходы к регулированию вопросов, связанных с расфасовкой ЛП «in bulk» в АО. По результатам исследования определены возможности и предложены пути совершенствования механизмов государственного регулирования.

Заключение. Расфасовка ЛП «in bulk» позволит повысить эффективность индивидуальной лекарственной терапии, оптимизировать и снизить издержки обращения средств бюджета, АО и граждан.

Ключевые слова: изготовление лекарственных препаратов; экстемпорльные лекарственные препараты; лекарственные препараты «in bulk»; расфасовка; аптечная организация; лекарственное обеспечение; упаковка лекарственных препаратов.

Список сокращений: ЛП — лекарственный препарат; АО — аптечная организация; ЕАЭС — Евразийский экономический союз; ЛС — лекарственное средство; ГРЛС — Государственный реестр лекарственных средств; ОФС — общая фармакопейная статья; ГФ — Государственная фармакопея; ЕЭК — Евразийская экономическая комиссия; ИС МДЛП — информационная система мониторинга движения лекарственных препаратов; ЭЛП — экстемпоральные лекарственные препараты; РПО — рецептурно-производственный отдел; ОЭЗ — особая экономическая зона.

INTRODUCTION

Improving the accessibility of medicines is a key task for regulatory bodies in fulfilling constitutional obligations for providing medicines to the population[1] [1–3].

Since September 2023, changes enshrined in Federal Law No. 502-FZ[2] of December 5, 2022 in Article 56 of Federal Law No. 61-FZ[3] of April 12, 2010 regarding the compounding and dispensing of medicines in a pharmacy have come into force in the Russian Federation. A significant legislative addition has been made to Part 2 of this article, allowing the use of medicines included in the State Register of Medicines for Medical Use (SRMs) when compounding medicines for medical use in a pharmacy. Subsequent improvement of this legislative norm is defined by the amendments introduced by Federal Law No. 1-FZ[4] of January 30, 2024, establishing the possibility of using both medicines registered under the national system and those registered in the common market of medicines of the EAEU and, accordingly, included in the unified EAEU[5] during manufacturing. This innovation significantly expands the possibilities of using medicines in circulation in the common EAEU market, which determines the particular relevance of sanctions restrictions in modern conditions.

General Pharmacopoeia Monograph (GM) GM.1.1.0004. Sampling[6] of the State Pharmacopoeia (SPh) of the Russian Federation XV edition defines the concept of "unpackaged products (bulk)" as a medicine in large packaging, including in an established dosage form, that has passed all stages of the technological process, except for packaging, and is intended for subsequent dispensing or production of medicines.

Therefore, in accordance with the new version of Article 56 of Federal Law No. 61-FZ of April 12, 2010, pharmacies have the right to use medicines "in bulk" and engage in their dispensing when compounding medicines.

Based on the results of the study, we proposed the introduction of a new concept of "Pharmacy dispensing and Packaging" into the legislative framework of the sphere of circulation of medicines, and considered solutions to emerging topical issues [4–6].

Timely organisation of medicine provision requires a comprehensive systematic approach to legal regulation, ensuring the procedure for its provision [7–9]. Dispensing of medicines "in bulk" will be of great importance for increasing personalized medicine and value-based healthcare provision to the country's population [10–12].

THE AIM. To study the current state of legal regulation of medicines "in bulk" and the possibility of their dispensing in a pharmacy.

MATERIALS AND METHODS

The legal basis of the study included legislative and regulatory acts of the Russian Federation in the field of healthcare and circulation of medicines, the common market of medicines of the EAEU, the methodology of system analysis in the field of medicine provision, and the organization of procurement of medicines using empirical, theoretical, and quantitative methods. The empirical base was collected from the official website of the Ministry of Health of Russia, the Federal Center for Pricing and Regulation of Medicines, and the ConsultantPlus legal (reference system on a contractual basis with Sechenov University).

The information base of the study for data collection and analysis used the results of scientific articles was carried out by the authors in the scientific electronic library elibrary.ru and Google Scholar. The search was conducted using the following keywords in Russian and English: "аптечная организация", "компаудинг", "расфасовка лекарственных препаратов", "экстемпоральное производство", "in bulk", "регулирование обращения лекарственных средств", "pharmacy", "compounding", "drugs packaging", "extemporaneous compounding", "regulation of the circulation of medicines". The analysis period was from January to December 2024.

RESULTS AND DISCUSSION

An analysis of foreign practice of "in bulk" packaging revealed that in many countries, medicines "in bulk" (mainly tablets and capsules) are supplied to pharmacies in large containers in quantities of up to 1000 pieces. Upon prescription dispensing, the pharmaceutical worker places the measured number of tablets or capsules in an individual container, provides the buyer with the necessary information about the medicine, and certifies it with a personal signature [13–15].

In the USA, medicines "in bulk" are used in the manufacture of dosage forms, according to the 503B

[1] Article 41 of the Constitution of the Russian Federation (accepted by popular vote dated December 12, 1993 with amendments approved during the nationwide vote dated July 1, 2020). Russian

[2] Federal Law No. 502-FZ dated December 5, 2022 "On Amendments to art. 56 of the Federal Law "On the Circulation of Medicines". Russian

[3] Federal Law No. 61-FZ dated April 10, 2010 "On the Circulation of Medicines". Russian

[4] Federal Law No. 1-FZ dated January 30, 2024 "On Amendments to the Federal Law "On the Circulation of Medicines" and Articles 1 and [4] of the Federal Law "On Amendments to the Federal Law "On the Circulation of Medicines" and the Federal Law "On Amendments to the Federal Law "On the Circulation of Medicines". Russian

[5] The Unified Register of Registered Medicines of the Eurasian Economic Union. Available from: https://portal.eaeunion.org/sites/commonprocesses/ru-ru/Pages/DrugRegistrationDetails.aspx

[6] GM.1.1.0004. Sampling. The State Pharmacopoeia of the Russian Federation XV edition. Available from: https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/1/1-1/otbor-prob/. Russian

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Bulk Drug Substances List[7], approved by the U.S. Food and Drug Administration (FDA) [16].

In European countries and in Russia, at present, the procedure for dispensing medicines in secondary or primary packaging is provided[8] [17–19].

Regulation of the packaging of medicines "in bulk" is a topical issue and requires a serious study of the existing legal framework in the field of circulation of extemporaneous drugs (EDs) [20–22], as well as the introduction of possible additions and developments in regulatory legal documents. Table 1 shows the terms and definitions relating to medicines "in bulk" and unpackaged products, prescribed in the Decision of the Eurasian Economic Commission (EEC) No. 100 of August 11, 2020[9], at the national level in GM.1.1.0004, approved by Order of the Ministry of Health of Russia No. 377 of July 20, 2023[10] and Order of the Ministry of Industry and Trade of Russia No. 916 of June 14, 2013[11].

Consequently, the current terminology indicates the harmonization of the national definition of "in bulk" products in accordance with the pharmacopoeial requirements of the common market of medicines of the EAEU.

Based on these definitions, the production of medicines "in bulk" can be carried out by one entity in the sphere of circulation of medicines — in unpackaged form, it can transfer it to another entity, including a pharmacy.

In accordance with the Regulations on the import of drugs into the customs territory of the EAEU, approved by the Decision of board of the EEC No. 30 of April 21, 2015[12] and the On the Rules for registration and examination of medicines for medical use, approved by the Decision of council of the EEC No. 78 dated November 3, 2016[13], the import of drugs is carried out on the basis of information provided in the Union Register of medicinal products. By the decision of the EEC, amendments have been made to the form of the registration certificate allowing manufacturers of drugs to indicate the form "in bulk" among the packaging options. The change allows pharmaceutical companies operating in the Union territory on an incomplete production cycle to import drugs "in bulk" for their subsequent packaging. The innovation allows confirming the drug status "in bulk" at customs and maintaining the preferential 10% VAT rate, which positively affects the pricing process of medicines compounded in pharmacies.

Information about drugs registered in "in bulk" packaging is available in the SRMs in open access on the website of the Ministry of Health of Russia[14].

In pursuance of Article 67 of Federal Law No. 61-FZ of April 12, 2010 and Decree of the Government of the Russian Federation No. 1556 of December 14, 2018[15], marking of medicines and submission of information to the Unified Federal Information System for Monitoring the Movement of Medicinal Products (MDLP) from the manufacturer to the end consumer is mandatory for all entities in the circulation of medicines [23–25]. Due to the absence of consumer dispensing on medicines "in bulk", identification means are not applied when they are imported into Russia. Marking is carried out at the company's production site during the packaging stage. Also, an exception for handling with the corresponding identification marks, in accordance with Part 1 of Article 46 "Marking of medicinal products", are medicines compounded in pharmacies.

A pharmacy that has a license for pharmaceutical activity, in accordance with Part 2 of Article 56 of Federal Law No. 61-FZ of April 12, 2010 and on the basis of Order of the Ministry of Health of Russia No. 249n of May 22, 2023, has the right to use registered finished medicines "in bulk" when manufacturing medicines. Consequently, in accordance with paragraph 54 of Chapter IV "Features of manufacturing medicines from finished medicinal products" of Order of the Ministry of Health of the Russian Federation No. 249n of May 22, 2023, it is allowed to manufacture powders from medicines "in bulk" according to the composition of the manufactured medicines and excipient (if necessary) indicated by the doctor in the prescription or requirement of the medical organization. It is not allowed to use dosage forms (tablets, capsules) of prolonged action and coated with an enteric coating when manufacturing powders. It is also not allowed to use drugs in the preparation of solutions for parenteral administration.

[7] U.S. Food and Drug Administration. 503B Bulk Drug Substances List. Available from: https://www.fda.gov/ drugs/human-drug-compounding/503b-bulk-drug-substances-list

[8] Order of the Ministry of Health of the Russian Federation dated November 24, 2021 No. 1093n "On Approval of the Rules for the Release of Medicines for Medical Use by Pharmacy Organizations, individual Entrepreneurs Licensed to carry out Pharmaceutical Activities, medical Organizations Licensed to Carry out Pharmaceutical Activities, and their Separate Units (outpatient clinics, paramedic and midwifery centers, centers (departments) of general medical (family) practice), located in rural settlements where there are no pharmacy organizations, as well as the Rules for the release of narcotic drugs and psychotropic substances registered in...". Russian

[9] Decision of the Eurasian Economic Commission dated August 11, 2020 No. 100 "On the Pharmacopoeia of the Eurasian Economic Union".

[10] Order of the Ministry of Health of the Russian Federation dated July 20, 2023 No. 377 "On Approval of General Pharmacopoeial Articles and Pharmacopoeial Articles". Russian

[11] Order of the Ministry of Industry and Trade of the Russian Federation dated June 14, 2013 No. 916 "On Approval of the Rules of Good Manufacturing Practice". Russian

[12] Decision of board of the Eurasian Economic Commission of April 21, 2015 No. 30 "About measures of non-tariff regulation".

[13] Decision of Council of the Eurasian Economic Commission No. 78 dated November 3, 2016 "On the Rules for registration and examination of medicines for medical use".

[14] State Register of Medicines of Russian Federation. Available from: https://grls.minzdrav.gov.ru/Default.aspx

[15] Order of the Ministry of Industry and Trade of the Russian Federation dated June 14, 2013 No. 916 "On Approval of the Rules of Good Manufacturing Practice". Russian

Table 1 – Terms and definitions of "medicines "in bulk"

Term and definition	Regulatory legal act
Unpackaged products ("angro", "in bulk") — a medicine in large packaging, including in a specific dosage form, that has passed all stages of the technological process, except for dispensing, and is intended for subsequent packaging or production of medicines.	Decision of the Board of the Eurasian Economic Commission No. 100 of August 11, 2020 (as amended on October 25, 2022) «On the Pharmacopoeia of the Eurasian Economic Union».
Unpackaged products ("angro") — a medicine in large packaging, including in a specific dosage form, that has passed all stages of the technological process, except for dispensing, and is intended for subsequent packaging or production of medicines.	GM.1.1.0004. Sampling of the State Pharmacopoeia of the Russian Federation XV edition.
Unpackaged products — any product that has passed all stages of the technological process except for consumer dispensing.	Order of the Ministry of Industry and Trade of the Russian Federation No. 916 of June 14, 2013 (as amended on December 18, 2015) "On approval of the Rules of Good Manufacturing Practice".

Since March 1, 2022, on the basis of Order of the Ministry of Health of Russia No. 1093n of November 24, 2021[16], it is allowed to violate (divide) the secondary (consumer) packaging when dispensing medicines, and the Ministry of Health of Russia in the corresponding letter No. 25-4/I/2-2643 of February 18, 2022[17] provides the necessary clarifications on emerging issues. In this regard, it is possible to amend paragraph 54 of Chapter IV «Specifics of manufacturing medicines from already produced ones" of the Order of the Ministry of Health of Russia No. 249n dated May 22, 2023, as follows: "...it is allowed to dispense medicines in the form of "in bulk", including according to prescriptions from doctors and requirements of medical organizations."

Thus, the introduced amendment updates the solution of regulatory issues and requirements for the corresponding consumer packaging to maintain the effectiveness and safety of dispensed and opened medicines "in bulk".

The revival and the development of medicine manufacturing in pharmaceutical organizations solve the problem of packaging according to modern trends [26–28]. It should be noted that "in bulk" medicines and medicines compounded in pharmacies are an exception to the inclusion of their data in the MDLP, which deprives the end consumer of the opportunity to verify the authenticity of the medicine online using the "Honest Sign" state labeling application. In this regard, along with quality control of compounded medications [29–31], approved by Order of the Ministry of Health of Russia No. 249n dated May 22, 2023 (section V), the most important factor in their effectiveness and safety is solving the issue of "correct" dispensing of all medicines compounded in pharmacies. It is also necessary to note that the primary packaging determines the route of administration of EDs into the patient's body, thereby ensuring the individual variability of the provided drug therapy.

In this regard, as we consider, it is possible to introduce a new concept in Article 4 of the Federal Law No. 61-FZ of April 12, 2010 "Pharmaceutical dispensing": "Pharmaceutical dispensing is a means or complex of means that ensure the quality of medicines compounded in a pharmacy, as well as ensure the process of transportation, storage, dispensing, and ergonomic use of these medicines by the end consumer."

In pursuance of the legislative innovation, it is possible to develop and approve an order of the Ministry of Health of Russia "On approval of requirements for packaging and completeness of medicines for medical use manufactured in a pharmacy". The document should take into account the requirements of section 2.4.2. "Packaging" of the Eurasian Pharmacopoeia[18], GM.1.1.0035. Packaging of Medicinal Products of the State Pharmacopoeia of the Russian Federation XV edition[19].

The new GM.1.1.0035, approved to replace GM.1.1.0025.18[20], regarding the dispensing of pharmaceutical medicines, contains information only about its compliance with the rules for the manufacture and dispensing of, approved by the authorized federal executive body.

The rules for the production of medicines in pharmacy determine the physical and chemical

[16] Order of the Ministry of Health of the Russian Federation dated November 24, 2021 No. 1093n "On Approval of the Rules for the Release of Medicines for Medical Use by Pharmacy Organizations, individual Entrepreneurs Licensed to carry out Pharmaceutical Activities, medical Organizations Licensed to Carry out Pharmaceutical Activities, and their Separate Units (outpatient clinics, paramedic and midwifery centers, centers (departments) of general medical (family) practice), located in rural settlements where there are no pharmacy organizations, as well as the Rules for the release of narcotic drugs and psychotropic substances registered in...". Russian

[17] Letter of the Ministry of Health of the Russian Federation dated February 18, 2022 No. 25-4/I/2-2643 "On the sale of medicines". Russian

[18] Decision No. 100 dated August 11, 2020 of the Board of the Eurasian Economic Commission, "On the Pharmacopoeia of the Eurasian Economic Union".

[19] GM.1.1.0035. Packaging of medicines. The State Pharmacopoeia of the Russian Federation XV edition. Available from: https://pharmacopoeia.regmed.ru/ pharmacopoeia/izdanie-15/1/1-1/upakovka-lekarstvennykh-sredstv/. Russian

[20] GM.1.1.0025.18. Packaging, labeling and transportation of medicines. The State Pharmacopoeia of the Russian Federation of the XIV edition. Available from: https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-14/1/1-1/ upakovka-markirovka-i-transportirovanie-lekarstvennykh-sredstv/. Russian

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properties of the active and auxiliary substances of EDs in accordance with the provisions of the Order of the Ministry of Health of the Russian Federation No. 249n dated May 22, 2023, and a number of new GPMs put into effect from September 1, 2023, approved by Orders of the Ministry of Health No. 377 dated July 20, 2023, and No. 448 dated August 25, 2023[21]. Order of the Ministry of Health of Russia No. 249n dated May 22, 2023, also defines the rules for labeling pharmaceutical medicines.

For scaling up the technological process of manufacturing medicines, the primary task is to approve the formulations of medicines compounded in pharmacies. Currently, there is a collection of unified drug formulations approved by Order of the Ministry of Health of the USSR No. 223 dated August 12, 1991[22]. Consequently, the analysis and updating of extemporaneous prescriptions have not been carried out for more than 30 years. Unification and approval of modern drug formulations will serve as an important basis for resolving the issue of appropriate consumer dispensing for pharmaceutical medicines and providing the quality of the therapy.

For the proper selection of packaging materials, dispensing / closure system, including closures and other packaging elements for a specific compounded medication, one should consider the GM put into effect on May 20, 2024, by Order of the Ministry of Health of Russia No. 223 dated May 6, 2024[23], GM.1.8.0008. Stability and expiration date of Pharmacy-Made Medicinal Products[24]. In addition to the packaging requirements, the use of modern dispensing materials should also consider the GM section "Packaging, Packaging Materials and Methods of Their Analysis (subsection 1.1.2)", approved by Order of the Ministry of Health of Russia No. 377 dated July 20, 2023.

Today, one of the weak points of the pharmaceutical industry and compounding pharmacies is the dependence on imported and manufactured in Russia dispensing materials [32–34]. The state provides customs preferences, tax benefits, including effective administration, to support companies located in a special economic zone (SEZ), which certainly allows manufacturers to reduce the cost of production and significantly reduce the time to resolve organizational and administrative issues. Thus, the company "Technopolis "Moscow"" (resident (SEZ) until the end of 2024 plans to open a modern production complex for the manufacture of dispensing materials. The company's management promises to provide its products to up to 50% of Russian pharmaceutical manufacturers and access to the pharmaceutical markets of the EAEU countries. The new product line includes tubes for gels and ointments, foil for blisters, instructions for medicines, which is especially relevant for pharmacy manufacturing[25]. It should be noted that medicines compounded in pharmacies are often dispensed in glass jars, and the capacity of the packaging can be many times greater than the weight of the dosage form itself. A logical consequence in the context of political and economic sanctions [35–37] would be to consider the possibility of including the production of packaging materials for medicines in the action plan for the implementation of the program "Strategy for the Development of the Pharmaceutical Industry of the Russian Federation for the period up to 2030" ("Pharma – 2030")[26].

Regarding the dispensing of packaged "in bulk" medicines, in our opinion, it would be the right professional decision to pack tablets and capsules in blisters, and then in secondary dispensing in accordance with the requirements of Chapter VII "Rules for dispensing and labeling medicines" of the Order of the Ministry of Health of Russia No. 249n dated May 22, 2023. There are a sufficient number of offers on the market for presses for welding blisters of domestic production, which have a low cost and quick changeability in the process of reconfiguring to another type of blister[27].

So, appropriate quality packaging can also solve the following important issue of increasing the expiration date of dispensed medicines from "in bulk" medicinal products. In accordance with the Order of the Ministry of Health of the Russian Federation No. 249n dated May 22, 2023, the new dosage form will have a very short expiration date — no more than 14 days, while non-sterile hard gelatin capsules manufactured in pharmacies have an expiration date of 45 days. Paragraph 84 of this order allows pharmacies to establish other expiration dates for Eds they comply with the GM or it will be necessary to amend paragraph 83 of Chapter VI "Expiration date of compounded medicines". In accordance with GM.1.8.0008, the expiration date of medicines compounded in pharmacies cannot exceed 90 days.

[21] Order of the Ministry of Health of the Russian Federation No. 448 dated August 25,2023 "On Approval of General Pharmacopoeial Articles and Pharmacopoeial Articles and Amendments to Order of the Ministry of Health of the Russian Federation No. 377 dated July 20, 2023 "On Approval of General Pharmacopoeial Articles and Pharmacopoeial Articles". Russian

[22] Order of the Ministry of Health of the USSR dated August 12, 1991 No. 223 "On approval of the Collection of Unified Medicinal Prescriptions". Russian

[23] Order of the Ministry of Health of the Russian Federation dated 05/06/2024 No. 223 "On Approval of the General Pharmacopoeial Article and Amendments to the Order of the Ministry of Health of the Russian Federation dated 03/13/2024 No. 120 "On Approval of General Pharmacopoeial Articles and Pharmacopoeial Articles". Russian

[24] Ibid.

[25] By 2024, Moscow will be able to provide half of Russia with packaging for medicines. PHARMEDPROM. Available from: https://pharmmedprom.ru/news/v-2024-godu-moskva-smozhet-obespechit-upakovkoi-dlya-lekarstv-pol-rossii/. Russian

[26] Decree of the Government of the Russian Federation dated June 07, 2023 No. 1495-r "Strategy for the development of the pharmaceutical industry of the Russian Federation for the period up to 2030". Russian

[27] The device for welding the Upress Adapt blister. Unique technologies. Available from: https://upack.pro/main-categories/packing-equipment/privarochniy-pressupress-adapt/. Russian

Maintaining the stability and expiration date of medicines, the crushing strength of tablets in an opened package in accordance with GM.1.1.0009[28] and GPM.1.1.1.0017 continue to be the most pressing issues[29].

The following question also requires a solution — will all pharmacies be allowed to work with "in bulk" medicines or only if they have a license for pharmaceutical activities — the right to production medicines for medical use. Of course, just as the preparation of powders and other dosage forms from "in bulk" medicines, their dispensing according to prescriptions and requirements of medical organizations should be carried out by compounding pharmacies or pharmacies that have prescription and production departments (PPD) in their structure. It is necessary to determine management and logistics approaches aimed to optimize the production and economic processes of a pharmacy engaged in dispensing "in bulk" medicines.

The solution to this issue is:

1. It is necessary to determine the main compounding pharmacy with geographical mapping (city, district, region);
2. Allocate budget funds for the material and technical equipment of the compounding pharmacy or the prescription and production department of the pharmacy.

The pharmaceutical organization engaged in the manufacture of extemporaneous medicines introduces into its functionality the corresponding delivery to pharmaceutical organizations, pharmacy points engaged in preferential dispensing of medicines, and structural pharmaceutical organizations of hospital institutions. The delivery of extemporaneous medicines is organized taking into account the requirements of GM.1.1.0037 Transportation of Medicines[30] using equipment and vehicles that ensure compliance with storage conditions and safety measures.

This solution will be a guarantee of ensuring the quality, effectiveness and safety of EDs, motivating pharmacies to revive PPDs, and increase their competitiveness.

A pharmacy engaged in the compounding of medicines, including the dispensing of "in bulk" medicines, must comply with all licensing requirements; rules for the manufacture, storage and dispensing of medicines for medical use; sanitary and hygienic norms and requirements; rules of good pharmacy practice. Pharmaceutical specialists must meet the qualification requirements, have a specialist certificate or accreditation certificate [38–40]. The list of the main regulatory legal documents governing this type of pharmaceutical activity is presented below.

1. Federal Law No. 99-FZ of May 4, 2011 "On Licensing Certain Types of Activities";
2. Federal Law No. 61-FZ of April 12, 2010 "On Circulation of Medicines";
3. Decree of the Government of the Russian Federation No. 547 of March 31, 2022 "On Approval of the Regulations on Licensing Pharmaceutical Activities";
4. Order of the Ministry of Health of the Russian Federation No. 249n of May 22, 2023 "Rules for the Manufacture and Dispensing of Medicines for Medical Use by Pharmacy Organizations Licensed for Pharmaceutical Activities";
5. Decree of the Chief State Sanitary Doctor of the Russian Federation No. 44 of December 24, 2020 "On approval of sanitary rules SP 2.1.3678-20 "Sanitary and epidemiological requirements for the operation of premises, buildings, structures, equipment and transport, as well as the conditions of activity of economic entities engaged in the sale of goods, performance of work or provision of services";
6. Order of the Ministry of Health of the Russian Federation No. 647n dated August 31, 2016 "On approval of the rules of good pharmacy practice for medicines for medical use";
7. Order of the Ministry of Health and Social Development of the Russian Federation dated August 23, 2010;
8. No. 706n "On approval of the Rules for the storage of medicines";
9. Order of the Ministry of Health and Social Development of Russia No. 541n dated July 23, 2010 "On approval of the Unified Qualification Directory of positions of managers, specialists and employees, section "Qualification characteristics of positions of workers in the field of healthcare";
10. Order of the Ministry of Health of the Russian Federation No. 709n dated October 28, 2022 "On approval of the Regulations on the accreditation of specialists."

Generalized labor functions and labor functions for the manufacture of EDs are prescribed in the professional standards "Specialist in the field of pharmaceutical activity management", "Pharmacist", "Pharmacy analyst", "Pharmacy technician ".

To systematize the entire process of personalized

[28] GM.1.1.0009. Stability and shelf life of medicines. The State Pharmacopoeia of the Russian Federation XV edition. Available from: https:// pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/1/1-1/ stabilnost-i-sroki-godnosti-lekarstvennykh-sredstv/. Russian

[29] GM.1.1.1.0017. Crushing strength of tablets. The State Pharmacopoeia of the Russian Federation XV edition. Available from: https:// pharmacopoeia. regmed.ru/pharmacopoeia/izdanie-15/1/1-1/1-1-2/ prochnost-tabletok-na-razdavlivanie/. Russian

[30] GM.1.1.0037. Transportation of medicines. The State Pharmacopoeia of the Russian Federation XV edition. Available from: https:// pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/1/1-1/ perevozka-lekarstvennykh-sredstv/. Russian

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drug assistance with EDs, including those made from medicines "in bulk", it is necessary to include unified and approved EDs prescriptions in clinical guidelines, incorporating them into state guarantee programs.

To analyze the relevance of EDs prescriptions and forecast needs, it is necessary to use the data from the federal register of citizens entitled to drug provision at the expense of budgetary allocations from the federal budget and the budgets of the constituent entities of the Russian Federation, approved by Decree of the Government of the Russian Federation No. 1656[31] dated October 12, 2020. The Ministry of Industry and Trade should study the suggestions about medicines "in bulk" on the pharmaceutical market. Applications for compounded medications will need to be handled at the regional and federal levels. The Federal Center for Planning and Supply of Medicines of the Ministry of Health of Russia [41] is engaged in the centralized procurement of medicines for orphan patients, and accordingly, medicines "in bulk" for the treatment of orphan diseases will be under its jurisdiction.

Regarding the current state of regulatory features in the field of medicine manufacturing, it seems appropriate to create an updated List of unified medical prescriptions for additional professional education of medical and pharmaceutical workers. In accordance with section II "Quality system for the manufacture of medicines" of Order of the Ministry of Health of Russia No. 249n dated May 22, 2023, it is recommended to approve the local standard operating procedure (SOP) "Packaging of medicines 'in bulk'". Special attention should be paid to the legal issues of prescribing and using medicines "off-label", including "in bulk" products [42–44].

Improving state regulation in the field of EDs circulation, conducting a flexible intra-organizational policy taking into account current needs for effective and safe pharmaceutical medicines [45–47] will contribute to the sustainable development and increased competitiveness of compounding pharmacies [48–50].

CONCLUSION

Dispensing of medicines is a promising area of pharmaceutical activity for a pharmacy. Ensuring the manufacture and dispensing of medicines should be based on scientifically proved methods and tools of the legislative framework, lead to a reduction in budgetary allocations for drug assistance, and be economically beneficial for the end consumer and the compounding pharmacy. The inclusion of packaged medicines "in bulk" in state guarantee programs will improve personalized drug provision, primarily for patients with rare orphan diseases, in the pediatric population, and in geriatric practice. Packaging of medicines "in bulk" will serve as one of the factors for the sustainability of the circulation of pharmaceutical medicines and the adaptation of drug provision in the face of new challenges and threats.

[31] Decree of the Government of the Russian Federation dated 12.10.2020 No. 1656 "On Approval of the Rules for Maintaining the Federal Register of Citizens Entitled to Provide Medicines, Medical Devices and Specialized Medical Nutrition Products at the expense of Budgetary Allocations from the Federal Budget and the Budgets of the Constituent Entities of the Russian Federation". Russian

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Rimma Yu. Garankina —objectives, conception, collection and critical analysis of scientific and regulatory legal documents, comments on the content, writing the text, editing and final approval of the manuscript; Vasily V. Ryazhenov — collection and analysis of scientific and methodological literature, critical analysis of scientific and methodological literature, comments on the content, editing the article; Elena A. Maksimkina — collection and analysis of scientific and methodological literature, critical analysis of scientific and methodological literature, comments on the content, editing the article; Victor S. Fisenko — collection of scientific and methodological literature, critical analysis of scientific and regulatory legal documents, methodological literature, comments on the content, editing the article; Aleksey V. Alekhin — collection of scientific and methodological literature, critical analysis of scientific and methodological literature, comments on the content, editing the article; Vadim V. Tarasov — collection of scientific and methodological literature, critical analysis of scientific and methodological literature, comments on the content, editing the article; Kirill A. Chizhov — data collection and analysis, editing the article; Irina F. Samoshchenkova — collection and analysis of scientific and methodological literature, comments on the content, editing the article; Nana Yu. Behorashvili — collection and analysis of scientific and methodological literature, comments on the content, editing the article; Elena R. Zakharochkina — collection and critical analysis of scientific literature and regulatory legal documents, comments on the content, editing the article, final approval of the manuscript. All authors confirm that their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept and preparation of the article, read and approved the final version before publication).

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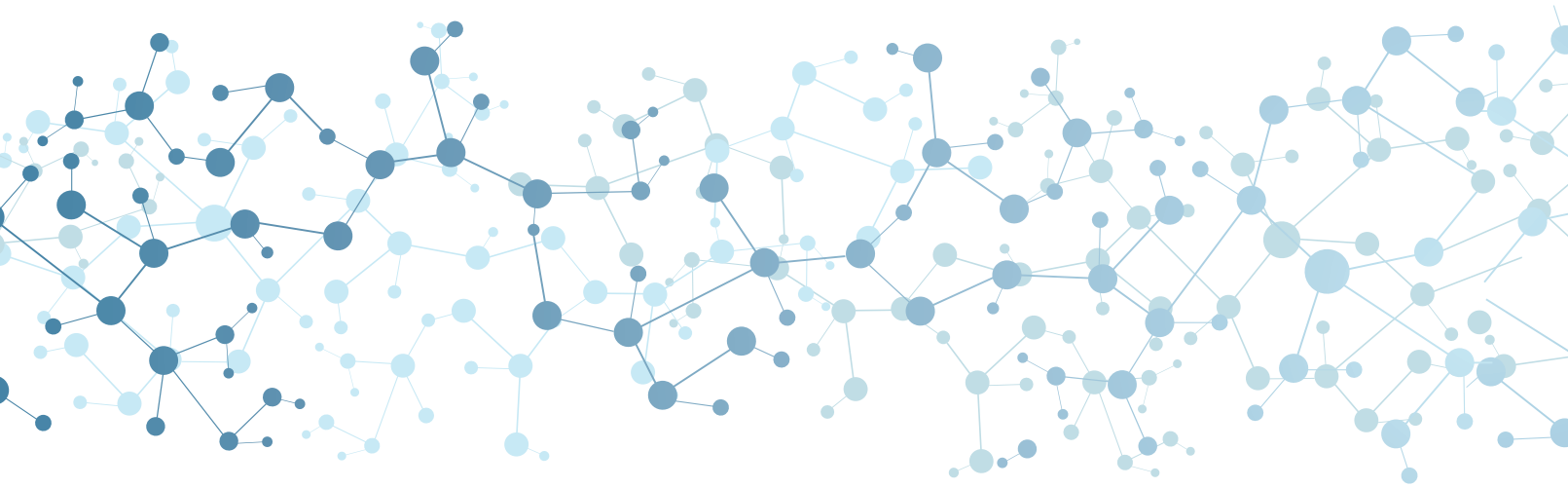


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