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RESEARCH ARTICLE

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PHARMACY & PHARMACOLOGY

Optimization of therapeutic drug monitoring of vancomycin in newborns using “Dried Blood Spot” method

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Therapeutic drug monitoring (TDM) is used to increase the individualization of pharmacotherapy, especially in patient groups with a high interindividual variability in pharmacokinetic (PK) parameters. One of these groups of patients is newborn children, for whom drug therapy, especially drugs with a narrow therapeutic range, causes a few difficulties or cannot be used in principle.

The aim of the work was to develop and validate quantitative HPLC-MS/MS methods for the determination of vancomycin in “dried blood spot” samples using new protocols and comparison of the results obtained with the results in plasma samples using standard sample preparation methods.

Materials and methods. To prepare stock and standard solutions of vancomycin and norvancomycin as an internal standard, dry portions of the corresponding certified standards of vancomycin (Servier, France) and norvancomycin (Augsburg, Germany, purity grade >95.0%) were used. A chromatographic separation of the components was carried out on a Poroshell 120 C18 column (4.6×50 mm, 2.7 µm). When developing conditions for a mass spectrometric detection of the desired substances using the multiple reaction monitoring (MRM) method, precursor ions and their corresponding product ions were determined.

Results. A quantitative HPLC-MS/MS method for the determination of vancomycin in "dried blood spot" samples was developed and validated. A comparison was made between vancomycin concentrations in "dried blood spot" samples and plasma samples. Moreover, more than 95% of the calculated average concentrations are within the limits of d-2s and d+2s, which correspond to the values of –10.2 and 12.2. That confirms the suitability of the developed method for the analysis of patient samples.

Conclusion. The results obtained make it possible for us to recommend the “dried blood spot” method for therapeutic monitoring of vancomycin, additional studies of PK in this group of patients with subsequent use of this drug in newborns and pediatric patients.

Keywords: therapeutic drug monitoring; vancomycin; HPLC/MS; validation; bioanalytics; dried blood spot method

Abbreviations: TDM – Therapeutic Drug Monitoring; PK – pharmacokinetics; PD – pharmacodynamics; HPLC – high performance liquid chromatography; MS – mass spectrometry; LLOQ – lower limit of quantitation; QC – quality control; DBS – Dried Blood Spot.

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Оптимизация терапевтического лекарственного мониторинга ванкомицина у новорожденных с применением метода «высушенной капли крови»

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Терапевтический лекарственный мониторинг (ТЛМ) используется для повышения индивидуализации фармакотерапии, особенного у групп пациентов с высокой межиндивидуальной вариабельностью фармакокинетических (ФК) параметров. Одной из таких групп пациентов являются новорожденные дети, для которых лекарственная терапия, особенного препаратами с узким терапевтическим диапазоном, вызывает ряд трудностей или не может быть применена в принципе.

Цель. Разработка и валидация методов количественного ВЭЖХ-МС/МС определения ванкомицина в образцах «высушенной капли крови» с использованием новых протоколов и сравнение полученных данных с результатами в образцах плазмы с использованием стандартных методов пробоподготовки.

Материалы и методы. Для приготовления маточных и стандартных растворов ванкомицина и норванкомицина как внутреннего стандарта использовали сухие навески соответствующих сертифицированных стандартов ванкомицина (Servier, Франция) и норванкомицина (Augsburg, Германия, степ. чистоты >95,0%). Хроматографическое разделение компонентов проводили на колонке Poroshell 120 C18 (4,6×50 мм, 2,7 мкм). При разработке условий масс-спектрометрической детекции искомых веществ методом мониторинга множественных реакций (MRM) были определены ионы-предшественники и соответствующие им ионы-продукты.

Результаты. Разработана и валидирована методика количественного ВЭЖХ-МС/МС определения ванкомицина в образцах «высушенной капли». Провели сравнение между значениями концентраций ванкомицина в образцах «высушенной капли крови» и образцах плазмы. При этом более 95% рассчитанных средних концентраций находились в пределах d-2s и d+2s, которые соответствовали значениям –10,2 и 12,2, что подтверждало пригодность разработанного метода для анализа образцов пациентов.

Заключение. Полученные результаты позволяют рекомендовать метод «высушенной капли крови» для проведения терапевтического мониторинга ванкомицина, дополнительных исследований ФК у данной группы пациентов с последующим применением данного лекарственного препарата у новорожденных и пациентов детского возраста.

Ключевые слова: терапевтический лекарственный мониторинг; ванкомицин, ВЭЖХ/МС; валидация; биоаналитика; метод «высушенной капли крови»

Список сокращений: ТЛМ – терапевтический лекарственный мониторинг; ФК – фармакокинетика; ФД – фармакодинамика; ВЭЖХ – высокоэффективная жидкостная хроматография; МС – масс-спектрометрия; НПКО – нижний предел количественного определения; КК – контроль качества; DBS – метод «высушенной капли крови».

INTRODUCTION

Therapeutic drug monitoring (TDM) is used to increase the individualization of pharmacotherapy, especially in patient groups with high interindividual variability in pharmacokinetic (PK) parameters [1]. Such patients include newborns, for whom drug therapy, especially drugs with a narrow therapeutic range, is associated with a number of difficulties or cannot be used in principle [2, 3].

Antibiotics are especially frequently prescribed drugs for newborns. About 2.5% of full-term infants receive antibiotic therapy in the first three days of life. However, it should be taken into account that a set of various physiological and autoimmune characteristics

in this group of patients affects the PK of drugs and can lead to ineffective pharmacotherapy and the development of undesirable consequences; hidden or obvious characteristics of the body dictate the need for an individual approach with a tendency to minimize the effects (a minimum dose, a minimum number of injections). This particularly complicates the use of antibiotics, with a narrow range between minimal therapeutic and toxic concentrations. One of such drugs is vancomycin, which is prescribed to treat severe infections caused by gram-positive bacteria, such as *Staphylococcus aureus* (especially methicillin-resistant strains), coagulase-negative *Staphylococcus*, and ampicillin-resistant *Enterococcus* species [2, 4].

Some factors that determine intra- and interindividual variability, such as age, weight, and renal condition, affect the PK and, as a consequence, the pharmacodynamic (PD) parameters of vancomycin. This necessitates carrying out TDM in this group of patients, conducting a PK analysis and creating population PK models that take into account the clinically significant characteristics of newborns and their impact on the PKs of vancomycin [1, 4].

The combination of this group of patients' features does not make it possible to carry out large-scale and comprehensive studies of PK and TDM. Since standard methods for collecting biospecimens are not optimal due to objective ethical and logistical limitations in newborns and young children, there is a need to develop and test new approaches, optimize TDM, build PK models and methods for collecting biospecimens, including the ones for TDM. A promising direction in this area can be considered the "dried blood spot" method, which is widely used, for example, for screening hereditary diseases in newborns [1, 5, 6].

A method for a quantitative HPLC–MS/MS determination of vancomycin in blood plasma samples has been previously developed and validated [7], protocols for the collection, analysis and validation of "dried blood spot" samples [5] have been also prepared. In this regard, in this study, these methods have been applied.

THE AIM of the work was to develop and validate quantitative HPLC-MS/MS methods for the determination of vancomycin in "dried blood spot" samples using new protocols and comparison of the results obtained with the results in plasma samples using standard sample preparation methods.

MATERIALS AND METHODS

Ethical approval

The design and protocols of the study, samples of informed consent for approval representatives of patients were accepted and then reviewed and approved at a meeting of the Local Ethics Committee, registration number IRB00005839 IORG 0004900 (OHRP), as evidenced by an extract from protocol No. 39 dated June 28, 2022.

Chemicals and reagents

To prepare stock and standard solutions of vancomycin and norvancomycin, the following substances were used as an internal standard: highly purified water, which had been obtained in the Milli-Q system, formic acid for HPLC-MS (Scharlab, Spain), acetonitrile (Scharlab, Spain), as well as dry samples of appropriate certified vancomycin standards (Servier, France) and norvancomycin (Augsburg, Germany, purity >95.0%).

Samples of "dried blood spot"

All 15 samples of the "dried blood spot" were obtained from newborn patients who were treated with vancomycin at State Clinical Hospital No. 5 of Volgograd, the department of neontology (Russia). The samples were obtained according to standard protocols [5].

Sampling was carried out according to the following protocols:

1. The informed voluntary consent of the legal representative was formalized.
2. The card / drops with patient ID and date was/were signed.
3. The puncture site on the lateral side of the heel was chosen.
4. The foot with a warm diaper was prepared.
5. The hands were cleaned and sterile gloves were put on.
6. The heel was placed below the child's body and held without sharply bending the ankle.
7. About 5 min before the injection, a 30% glucose solution was administrated *per os* with a syringe at the dose indicated in Figure 1+non-nutritive sucking.
8. The puncture site was treated with an antiseptic solution.
9. A 30% glucose solution was re-administrated *per os* to the patient with a syringe immediately before the injection.
10. The skin was quickly pricked with a lancet and the first drop of blood was wiped off with a sterile cotton ball.
11. Immediately after the injection, a 30% glucose solution was given *per os*+nonnutritive sucking.
12. The puncture site was held down, the adjacent area was being gently pressed on and the blood was sampled a on filter paper form;
13. The map was held without touching the marked area.
14. The filter paper card was carefully touched to a drop of blood and applied to the card. The blood was let to soak in until the circle was full. The marked area was not to be touched after applying blood.
15. The blood stain was let to dry in a dark place, out of direct sunlight, for at least 4 h. The "dried blood spot" samples were not to be heated or come

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into contact with other surfaces during the drying process.

16. The card (or parts of the card) was to be sealed in a gas-tight zip-lock bag. No more than one card per package should be stored in the refrigerator at 2–8°C until being sent to the laboratory.

Equipment

The components were separated using an Agilent 1260 HPLC system (Agilent, USA). The analytes were detected using a Sciex QTRAP 5500 hybrid mass spectrometry system (AB Sciex Pte. Ltd., Singapore). To weigh dry substances, semi-microanalytical balances Ohaus Explorer EX225/AD (Ohaus, USA) and centrifuge 5427 R (Eppendorf, USA) were used. GE Whatman FTA WB129242 DMPK-B (USA) card was used for archiving "dried blood spots". While the system was on, the acquired data was sent to the Analyst Software.

Preparation of stock solution of substance and internal standard

To prepare standard solutions of vancomycin and an internal standard with a concentration of 1.00 mg/ml, dry samples (weighing 25 mg) were weighed and then placed in a 25-ml volumetric flask and diluted to the mark with ultrapure water.

Preparation of solution for extracting “dried blood spot” samples

Separately, an extraction solution was prepared; that was a mixture of water and methanol in a 1:1 ratio with 0.1% formic acid.

Preparation of working standards solutions

For each analytical series, fresh working standards solutions were prepared. The final concentrations of working solutions were: 10, 20, 50, 100, 200, 500 and 1000 µg/ml.

Preparation of calibration standards and quality control samples in “dried blood spot” samples

100 µl of the whole blood was transferred into 1.5 ml microtubes, and 10 µl of a working solution of the appropriate concentration was added to obtain calibration solutions: 1, 2, 5, 10, 20, 50 and 100 µg/ml for vancomycin. For quality control (QC) samples 1 (a lower limit of quantitation – LLOQ); 7.5 (low QC); 35 (average QC); 75 (high QC) µg/ml for vancomycin. Next, 20 µl of working solutions were applied to filter paper and dried for 3 h.

Analyzed samples preparation

To prepare the samples of “dried blood spot”, a 6 mm diameter card disk was used, onto which the samples were applied using a special device and which was subsequently analyzed.

Extraction of “dried blood spot” samples

400 µl of the extraction solution was added to the test tube with a cut out disk. The tube was stirred at 1000 rpm for 30 min at 25°C. After 10 min of centrifugation at 10 000 g, a 350 µl aliquot of the supernatant was evaporated at 45°C in a vacuum centrifuge. The dried extract was collected in 100 µl of mobile phase A, centrifuged for 10 min at 10 000 g, and 20 µl of the supernatant was injected into a high-performance liquid chromatography with a mass spectrometric detection – an HPLC-MS/MS system.

Chromatographic and mass spectrometric conditions

Thus, based on the data sources available and the authors’ research, the authors have developed and validated a bioanalytical method for vancomycin in human plasma using an HPLC-MS/MS system that meets the requirements of the research protocol and is validated in accordance with the FDA guidance for enterprises “Bioanalytical Method” Validation” and the EMEA “Guideline on bioanalytical method validation”.

When developing the conditions for a mass spectrometric detection of the sought substances, “precursor ions” (725.3 m/z) and their corresponding “product” ions (88.1 and 387.9 m/z) were used.

Statistical analyses

Validation of the bioanalytical method was carried out in accordance with the “Guide for the Evaluation of Drugs” (Vol. I), as well as with the guidelines of the FDA (U.S. Food and Drug Administration) and EMA (European Medicines Agency) for the following indicators: stability, selectivity, linearity, accuracy, precision, a lower limit of quantitation. Experimentally calculated concentrations of calibration standards should be within ±15% of the nominal values (with the exception of LLOQ, for which these values may be within ±20%) and also according to specific indicators, such as the effect of a drop volume, the effect of hematocrit, drop uniformity according to the already developed protocols [8–10]. The obtained data were processed using the statistical Software environment R 3.6.1 in the RStudio 1.2 program, as well as specialized Software Sciex Analyst 1.6.2.

When validating the homogeneity of the drop, the results of two concentration levels of QC samples at three different hematocrit levels, obtained with 2 options for cutting the drop – from the center of the drop and at its edge, were compared. The analysis was carried out in 5 replicates. In this case, when comparing concentrations from the samples obtained from the central and marginal cutouts, the relative error should not exceed 15%.

To assess the influence of the drop volume effect, it was necessary to analyze 3 different volumes (10, 40, 70 µl) at an average hematocrit level (0.4), at 2 concentration levels in 5 replicates. In this case, the relative error should not exceed 15%.

The effect of hematocrit was also assessed at 3 levels (0.3; 0.4; 0.5), at 2 concentration levels in 5 replicates. The relative error of the calculated concentrations should not exceed 15% of the obtained values at an average hematocrit level.

The level of agreement between the developed HPLC-MS/MS method for the "dried blood spot" method and the traditional HPLC-MS/MS method with a protein precipitation as a sample preparation was examined by the Bland-Altman method.

The method makes it possible to compare the results of measurements performed in two different ways. Its essence is that for each pair of measurements the difference and the average are calculated. The average difference calculated for all pairs of characteristics in the data set under study characterizes the systematic discrepancy of indicators, the presence of which indicates an incomplete correspondence of the results obtained by different methods, and the standard deviation of the differences reflects the dispersion degree of the results.

The mean values and standard deviations were calculated using Microsoft Excel 2010 (Microsoft Corporation, USA); the statistical processing of the study results was carried out using GraphPad Prism 6 (USA).

The mean (M) and standard deviation (SD) of the difference between the two matching readings were calculated to determine the equivalence of the two methods. To confirm the suitability of the DBS analytical method for the analysis of patient samples, however, more than 95% of the calculated mean concentrations between the two methods must fall within the d-2s and d+2s limits.

RESULTS AND DISCUSSION

The development of a quantitative HPLC-MS/MS method for the determination of vancomycin included the determination of optimal parameters for a chromatographic separation, as well as a subsequent mass spectrometric detection.

Using the previously accumulated experience, optimal conditions for the chromatographic separation had been selected. The separation was carried out on a Poroshell 120 C18 column (4.6×50 mm, 2.7 µm). The mobile phase was an 80/20 acetonitrile and water mix at a flow rate of 0.3 ml/min. A 0.1% solution of formic acid was added to both the aqueous and organic components of the mobile phase. When optimizing the chromatographic separation conditions, an isocratic elution mode was chosen [5, 8, 11, 12].

In the isocratic elution mode, the retention time of vancomycin was 1.63 min (Fig. 1).

The electrospray ionization (ESI) was used as the ionization method. The ion detection was carried out in a positive polarity mode.

When developing the conditions for the mass spectrometric detection of the sought substances, "precursor ions" (725.3 m/z) and their corresponding "product" ions (88.1 and 387.9 m/z) were used.

The developed method confirmed its linearity in the concentration range from 1 to 100 µg/ml (K1 – 1 µg/ml, K2 – 3 µg/ml, K3 – 5 µg/ml, K4 – 10 µg/ml, K5 – 25 µg/ml, K6 – 50 µg/ml, K7 – 80 µg/ml, K8 – 100 µg/ml) when a weighted coefficient of *1/x^2*, with R^2 >0.99 was used. The coefficient of variation (%) calculated when determining an inter- and intraday accuracy did not exceed 15% for the main concentration range.

The lower level of quantification (LLOQ) of the methods was determined based on the linearity, accuracy and precision data given in Table 1. The selectivity was validated by analyzing 6 blank samples and 6 lower level of quantification (LLOQ) samples. The peak area in the analyte retention time region did not exceed 20% of the LLOQ (Table 1).

For the "dried blood spot" method, the influence of hematocrit, drop volume and drop homogeneity was also assessed.

The effect of hematocrit was assessed at 3 hematocrit levels (0.3; 0.4; 0.5), for QCL and QCH, and the resulting concentrations ranged from 94.3 to 105.8% of nominal ones (Table 2).

To validate the volume effect, 3 volumes (10, 40, 70 µl) at an average hematocrit level (0.4), at 2 concentration levels in 5 replicates were analyzed. The relative error of the calculated concentrations did not exceed 15% of the obtained values at an average volume.

When validating the drop homogeneity, the results of QC samples with QCL and QCH, obtained with 2 options for cutting the drop – from the center of the drop and from the edge, were compared. The analysis was carried out in 5 replicates. At the same time, in the comparison of the concentrations from the samples obtained from the central and marginal cutouts, the relative error did not exceed 15% of the nominal values.

The thermal stability was assessed by storing the "dried blood spot" samples for 14 days at 22 and 45°C, as potential temperatures for storing and transporting samples. To assess the stability, ''dried blood spot'' samples were used at the QCL and QCH levels, herewith, the samples were analyzed at three time points of 1, 7 and 14 days along with freshly prepared samples in one analytical batch. The calculated concentrations of the samples after the storage were compared with the mean concentrations of freshly prepared quality control samples. After 14 days of storage, the values were in the range of 85.4–87.5%.

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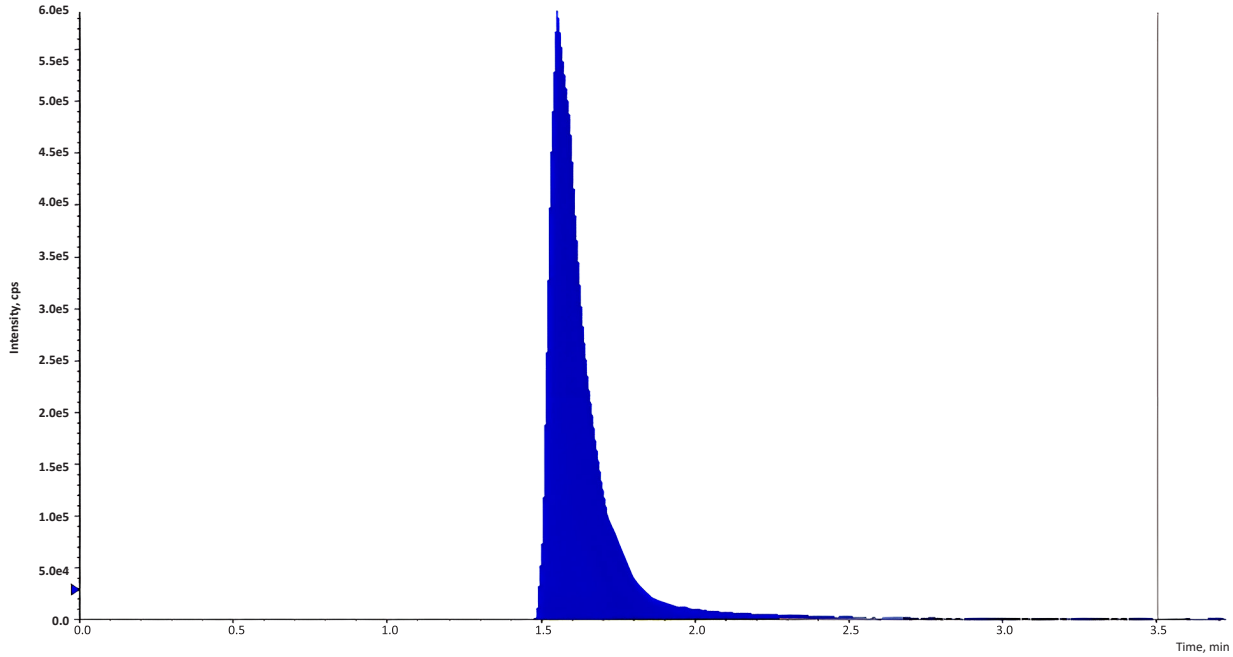

Figure 1 – Chromatography-mass spectra of vancomycin in DBS samples

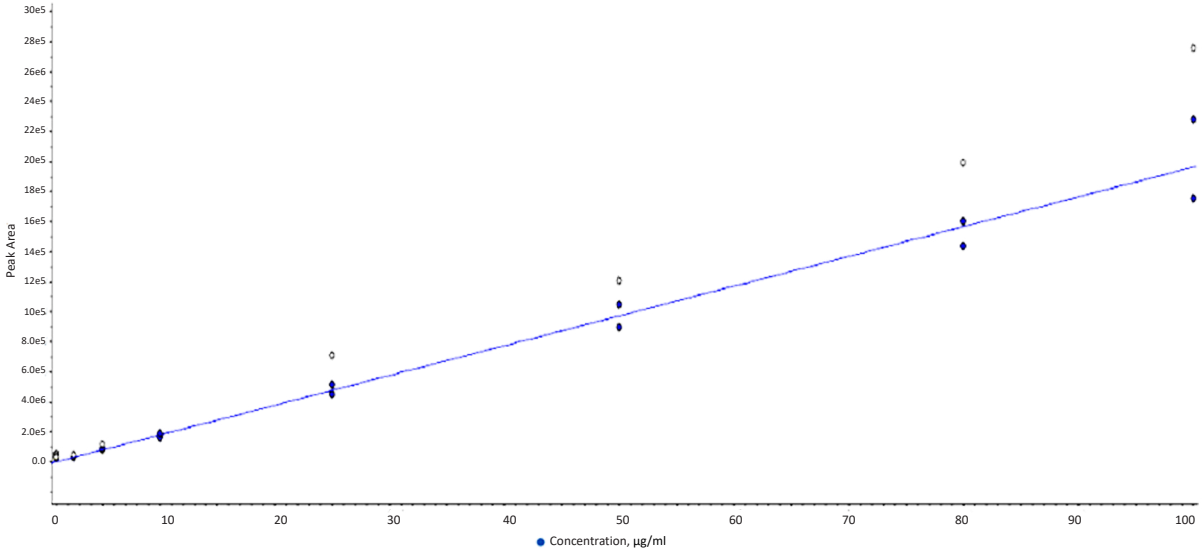

Figure 2 – Calibration curve of vancomycin in blood plasma

Table 1 – Table of validation parameters for the method using a "dried blood spot" as a sample preparation

Parameter		Value			
		LLOQ (1 µg/ml)	Lower QC (7,5 µg/ml)	Mean QC (35 µg/ml)	Top QC (75 µg/ml)
Precision (CV%)	Within a cycle	2.7	2.1	2.7	3.7
	Between cycles	7.6	4.7	6.9	7.0
Correctness (%)	Within a cycle	104.6	94.4	101.6	101.8
	Between cycles	91.3	95.1	103.2	107.6
Stability (%)		–	85.3	–	87.5
Selectivity (%)		2.7	–	–	–
Correlation coefficient			0.99		

Note: QC – quality qontrol.

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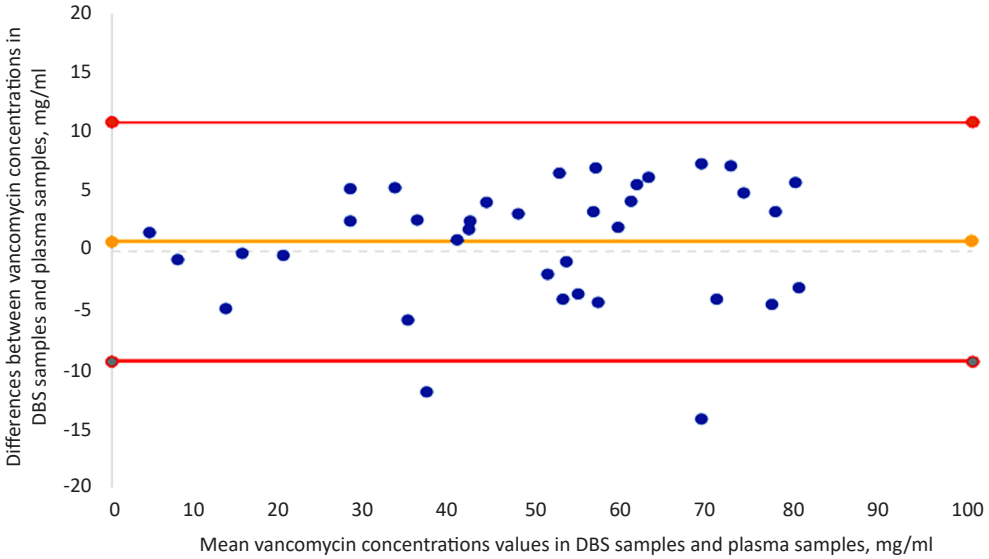

Figure 3 – Comparison of two Bland-Altman sample preparation methods for vancomycin
Note: DBS – Dried Blood Spot.

Table 2 – Effect of hematocrit on analysis results

Hematocrit, %	QC	Nominal concentration, mg/ml	Accuracy, %
0,3	LQC	7.5	94.3
	HQC	75	97.6
0,4	LQC	7.5	95.8
	HQC	75	98.1
0,5	LQC	7.5	95.6
	HQC	75	105.8

Table 3 – Effect of drop volume on analysis results

Drop volume, µl	QC	Nominal concentration, mg/ml	Accuracy, %
10	LQC	7.5	105.7
	HQC	75	108.9
40	LQC	7.5	96.4
	HQC	75	104.5
70	LQC	7.5	91.5
	HQC	75	96.4

Table 4 – Influence of cut location on analysis results

Cutout location	QC	Nominal concentration, mg/ml	Accuracy, %
Center cutout	LQC	7.5	98.3
	HQC	75	103.7
Edge cutout	LQC	7.5	93.3
	HQC	75	96.5

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For a comparative study, the samples of a "dried blood spot" were used; they had been obtained from newborns undergoing vancomycin therapy at the State Healthcare Institution of Clinical Hospital No. 5. Vancomycin concentrations were compared between dried blood spot and plasma samples (Fig. 3).

M and SD of the difference between the two matching readings were calculated to determine the equivalence of the two methods. The results of the study showed that more than 95% of the calculated mean concentrations are within the limits of d–2s and d+2s, which correspond to the values of –10.2 and 12.2. This result confirms the developed method suitability for the analysis.

DISCUSSION

TDM is an algorithm for measuring the amount of specific drugs at specific intervals to maintain constant concentrations in the patient's bloodstream in order to optimize and individualize dosing regimens. This approach is used to monitor drugs with a narrow therapeutic index, significant PK variability, or those whose target concentrations are difficult to control. For drugs with a clear dose-effect relationship, TDM can significantly increase the effectiveness and safety of treatment and reduce costs in the social and medical sphere.

Currently, TDM in newborns is not a routine practice. Large clinical centers that have the capacity to conduct it to assess the target exposure, are most often limited to the determination of C_{trough} vancomycin [4].

As defined by C_{trough}, the generally accepted therapeutic aim of vancomycin monitoring is to assess a systemic exposure as the residual concentration before the next dose of the drug is administered. According to the international recommendations, the target vancomycin C_{trough} should be at a level of 10–20 mg/l and be above the MIC (minimum inhibitory concentration) [1]. The recommended C_{trough} range makes it possible to achieve the required PD parameters – AUC24 / MIC [4].

However, the main problem facing physicians is the need to assess the possibility and accuracy of the extrapolation of target PK and PD parameters (C_{trough}=10–20 mg/l and AUC24 / MIC >400) [1].

According to the international recommendations, the target C_{trough} level is currently 15-20 mg/l and the AUC24 / MIC ratio is >400. A number of studies in individual populations indicate a high variability in PK parameters, with target C_{trough} values of 15–20 mg/l and the ratio AUC24 / MIC >400 did not reach an average of 30–33% of the study population [13, 14].

Today, TDM of vancomycin involves, in addition to measuring steady-state residual concentrations (C_{trough}), calculating the area under the PK curve over 24 h (AUC24), followed by the assessment of the target PK/PD ratio, which is expressed as the ratio of AUC24 to the minimum inhibitory concentration (MIC) AUC24 / MIC >400 [13, 15, 16].

Newborns and pediatric patients are not only sensitive to certain xenobiotics, but also exposed to various risks of viral, bacterial or fungal invasions, many of which significantly alter functioning of the metabolic and excretory systems. The neonatal age may be a risk factor for the development of nephrotoxicity, the significance of which increases in the proportion to prematurity.

The previous studies with the use of mathematical modeling and TDM in patients with an impaired renal function and infectious complications have shown that their presence significantly affects PK parameters. Thus, it was demonstrated that with a standard approach to dosing, in 65% of cases it is not possible to achieve the target values for C_{trough} 15–20 μg/ml after 48 h from the start of therapy. This indicates a high variability of PK vancomycin parameters, especially in the group of patients with an impaired renal function [17].

In a study by Kim J. et al. it has been shown that in neonates with an adequate renal function, a dosing regimen of 9 to 12 mg/kg IV every 8 h is less likely to achieve the target vancomycin concentrations [18].

To evaluate all these parameters and optimize TDM, various mathematical models that make possible the prediction of unknown PK parameters, are available. There are known both simple one-compartment models – "medical calculators", and more complex ones – "dose calculators", the use of which allows you to adjust the dosage regimen to achieve C_{trough} in the range of 15–20 μg/ml [1, 19].

It is worth highlighting that international recommendations for vancomycin dosage regimens are not always optimal. Thus, in premature patients, these recommendations are insufficient to obtain minimum serum concentrations of 10 to 20 μg/ml [20].

PK / PD studies for antibiotics already used in clinical practice are necessary to optimize dosage regimens, including preventing the development of resistance. Since the results of studies conducted on adults are often not applicable to newborns, local TDM is not only a useful tool for optimizing therapy, but also a valuable source of the data that can be not only of the applied importance, but also of a fundamental nature. Separate issues that need to be addressed are methods of collecting biospecimens and optimizing their quantity, which is associated with the age and size of patients [21].

Most recommendations are developed for adult patients [1, 3, 4, 11]. As for newborns, standardization of the process is hampered by a smaller number of studies, which means a significantly smaller amount of data for the analysis, variability of PK parameters, more complex modeling with the need to take into account more covariates [4].

Modern development of guidelines, instructions and protocols requires additional PK studies, construction of mathematical models and implementation of TDM algorithms. This is what causes difficulties, since research and TDM, in addition to financing and going through a large number of bureaucratic procedures, require solving many technical problems [6]. For example, the issue of determining the optimal number of biological samples has not been resolved; the blood from which plasma or serum is subsequently obtained is taken from a vein, but its quantity and technical complexity significantly complicate the process of developing regulatory documentation [1]. TDM of antibiotics in newborns and premature infants is rarely practiced precisely because of difficulties with blood sampling [1].

The above points to the need to develop efficient and less invasive methods for obtaining biospecimens from newborns and pediatric patients. One of them is the "dried blood spot" method (DBS), which is the most convenient way to obtain biomaterial compared to the standard ones [6]. It is based on the use of a small amount of blood, which is applied to special paper and dried under certain conditions. The resulting biological samples are more stable and more convenient to transport and store [4, 22]. The indicated advantages not only reveal the feasibility of using the "dried blood spot" method for TDM in cases with newborns or pediatric patients, but also determine the need for development processes in this direction, for example, when using vancomycin in the treatment of diseases in newborns and pediatric patients [1].

Obtaining biological samples for a sample preparation and analysis using the "dried blood spot" method requires additional optimization. Currently used bioanalytical method validation protocols, which are described in manuals for traditional samples, do not take into account all the necessary aspects of the new "dried blood spot" technology and complicate the subsequent application of the method in TDM. Specific characteristics such as hematocrit, droplet homogeneity, and droplet size may influence assay results, requiring the identification of new approaches to the development and validation of qualitative and quantitative assays [4, 23].

The information content and predictive value of TDM when conducting well-founded, from a PKs point of view, individualization when selecting a dosage regimen is determined by the reliability of the analytical methods used for the quantitative determination of drugs in biological samples [24]. For these purposes, it is necessary to use selective and highly sensitive analytical methods, such as high-performance liquid chromatography with a mass spectrometric detection (HPLC-MS/MS) [25, 26].

PK studies and TDM require large numbers of biospecimens, which is difficult to implement when working with neonates [27]. As a result, there is a need to use less invasive methods of a sample preparation for their subsequent implementation in algorithms for TDM. One of the promising approaches for carrying out such monitoring in newborns may be the "dried blood spot" method. A necessary direction, without which the introduction of new methods of monitoring and optimization of pharmacotherapy in newborns will be difficult, is the development of highly selective and specific methods for the qualitative and quantitative determination of vancomycin using the "dried blood spot" method [28].

CONCLUSION

One of the important results of this study was the determination of the optimal conditions for a quantitative analysis method using high-performance liquid chromatography and mass spectrometric detection for the determination of vancomycin in "dried blood spot" samples. As a result of the study, it was possible to validate the quantitative HPLC-MS/MS method for the determination of vancomycin in "dried blood spot" samples. The identity of the results of a quantitative vancomycin determination with standard and proposed sample preparation options has been proven.

The above makes it possible to recommend the "dried blood spot" method for TDM of vancomycin and additional studies of PKs in this group of patients, which can optimize and bring the pharmacotherapy of newborns closer to the ideals of personalized medicine. The development of such methods makes it possible to solve an important practical problem in the field of studying the PKs of newborns, which is important for clinical pharmacology since conducting clinical studies in young patients is complicated, among other things, by these reasons. The development of methods for the qualitative and quantitative determination of various substances in the blood using biological samples of minimal size will significantly expand the possibilities of conducting clinical studies, which will certainly contribute to the development of not only general and clinical pharmacology, but will also significantly enhance pharmacotherapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHORS' CONTRIBUTION

Ivan S. Anikeev, Tatyana E. Zayachnikova, Yulia S. Kazmina – concept, planning of the article, review of literary sources, collection of materials, article writing and editing; Vladimir I. Petrov, Andrey V. Strygin, Denis V. Kurkin – development of the study design, editing and final approval of the article.
All authors made equivalent and equal contributions 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, conduct of the study and preparation of the article, read and approved of the final version before the publication).

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Antitumor activity of three new azoloazine derivatives in orthotopic transplantation model of human breast cancer cells into mice

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Breast cancer (BC) is one of the most common types of malignant tumors, which makes scientific research in this area extremely relevant. The difficulties of breast cancer chemotherapy stimulate the search for new drugs to treat this nosology. Derivatives of imidazotriazine and imidazotetrazine, which are analogues of the antitumor drug temozolamide, can be ones of the promising drugs in this regard.

The aim of the work was to evaluate the antitumor activity of three new azoloazine derivatives in a xenogeneic breast cancer model in mice *in vivo*.

Materials and methods. A study was conducted on a xenogeneic model of BC. After the immunosuppression with azathioprine, 48 white BALB/c mice were injected with MCF-7 cells, test derivatives, and the reference drug epirubicin at doses of 1/2 IC_{50} and 1/10 IC_{50}, into the base of the mammary gland once. The body weight of the mice was monitored; on the 15th day, at the end of the experiment, the relative volume was assessed.

Results and discussion. Among the three compounds studied, imidazotetrazine 1 showed the most encouraging results: stopping the loss of body weight in the mice caused by the administration of tumor cells, and reducing the tumor volume on the 15th day of the experiment to 50.6% of that in the control when using a dose of 1/10 IC_{50}, up to 39.2% – when using a dose of 1/2 IC_{50}, which significantly exceeded the values of the reference drug epirubicin and the values in the control group. In the histological examination, the signs of spread and preservation of tumor cells viability of the MCF-7 line after its administration were minimal, the value of the histological malignancy index decreased by 9.3% of the control value.

Conclusion. Among the tested azoloazine derivatives, 3-cyclohexyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide is the undisputed leader, causing inhibition of the tumor growth in a xenogeneic model *in vivo*.

Keywords: imidazotriazine; imidazotetrazine; breast cancer; MCF-7 cell line; mouse models

Abbreviations: BC – breast cancer; NMR – nuclear magnetic resonance; MCF-7 (Michigan Cancer Foundation-7) – human breast cancer cell line; BALB/c – genetic line of white laboratory mice; IC_{50} – concentration of half-maximal inhibition; G1-G3 – system of morphological criteria for determining the degree of malignancy.

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RESEARCH ARTICLE

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Противоопухолевая активность трех новых производных азолоазинов на модели ортотопической трансплантации клеток рака молочной железы человека мышам

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Рак молочной железы (РМЖ) относится к наиболее распространенным видам злокачественных опухолей, что делает чрезвычайно актуальными научные исследования в этой области. Трудности химиотерапии РМЖ стимулируют поиск новых препаратов для лечения этой нозологии. Одними из перспективных в этом плане могут быть производные имидазотриазина и имидазотетразина, которые являются аналогами противоопухолевого препарата темозоламида.

Цель. Оценить противоопухолевую активность трех новых производных азолоазинов в ксеногенной модели рака молочной железы на мышах *in vivo*.

Материалы и методы. Проведено исследование на ксеногенной модели РМЖ. После иммуносупрессии азатиоприном 48 белым мышам BALB/c вводили в основание соска молочной железы клетки линии MCF-7, тестируемые производные и препарат сравнения эпирубицин в дозах 1/2 IC_{50} и 1/10 IC_{50} однократно. Проводили мониторинг массы тела мышей, по завершении эксперимента на 15-е сут оценивали относительный объем опухолевой ткани и проводили гистологическое исследование.

Результаты и обсуждение. Среди трех исследуемых соединений имидазотетразин 1 продемонстрировал наиболее обнадеживающие результаты: прекращение потери массы тела мышей, вызванное введением опухолевых клеток, и сокращение объема опухоли на 15-е сут эксперимента до 50,6% от аналогичного в контроле при использовании дозы 1/10 IC_{50}, до 39,2% – при использовании дозы 1/2 IC_{50}, что значительно превышало значения препарата сравнения эпирубицина и значения в контрольной группе. При гистологическом исследовании признаки распространения и сохранения жизнеспособности опухолевых клеток линии MCF-7 после его введения были минимальными, величина гистологического индекса злокачественности снижалась на 9,3% от значения в контроле.

Заключение. Среди тестируемых производных азолоазинов, 3-циклогексил-4-оксоимидазо[5,1-*d*][1,2,3,5]тетразин-8-*N*-пиперидинилкарбоксамид является безусловным лидером, вызывающим торможение роста опухоли в ксеногенной модели *in vivo*.

Ключевые слова: имидазотриазин; имидазотетразин; рак молочной железы; клеточная линия MCF-7; мышиные модели

Список сокращений: РМЖ – рак молочной железы; ЯМР – ядерно-магнитный резонанс; MCF-7 – клеточная линия РМЖ человека; BALB/c – генетическая линия белых лабораторных мышей; IC_{50} – концентрация полумаксимального ингибирования; G1-G3 – система морфологических критериев определения степени злокачественности.

INTRODUCTION

Malignant neoplasms are currently not only one of the main causes of death in the world, but they also cause concern to specialists due to the constant increase in morbidity, extreme variability, and resistance to various types of treatment. Among all tumors, breast cancer belongs to the most common type of malignant tumors in women [1–3]. It has been detected in 1/8 of all newly diagnosed cases of tumors and determines 14% of cancer mortality in women [4]. According to global statistics, 2.3 million people were diagnosed with breast cancer in 2020, and 685 thousand died from it [5]. As a

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result, the genetic and morphological diversity of breast cancer, the difficulties of its primary diagnosis, and the high resistance of a several varieties to chemotherapy cannot satisfy clinical oncologists with a modern arsenal of chemotherapeutic agents for its treatment. The results of antitumor therapy for metastatic breast cancer are particularly pessimistic [6–9].

All these points determine the urgent need and make research into the development of new chemotherapeutic agents that would cope with breast cancer extremely relevant [10, 11].

Based on the above provisions, the authors' attention has been focused on the study of imidazotetrazine and imidazotriazine derivatives, which demonstrate a strong alkylating effect and have been used as antitumor agents, including breast cancer, for more than 40 years. Temozolomide is the most famous of them; it has proven clinical effectiveness in the treatment of lymphomas, brain tumors, and metastatic melanoma. Unfortunately, at the present stage, many tumors are resistant to this drug, or the chemoresistance develops very quickly [12, 13]. To date, more than 30 new derivatives of imidazotetrazine and imidazotriazine have been synthesized, promising to be used as antitumor agents [14–16].

The study hypothesizes a possible antitumor activity of new azoloazine derivatives in a xenogenic model of breast cancer in mice.

This *in vivo* stage is mandatory for all preclinical studies of potential antitumor compounds, since it is cancer modeling in laboratory animals that makes it possible to study the biology of malignant growth in detail and make suggestions in an evidence-based format to improve diagnostic and therapeutic strategies. Among the existing models, the orthotopic mouse is characterized by transplantation of tumor cells or fragments into the same anatomical area where the tumor usually develops. This cancer model is widely used in practice [17, 18], because it makes possible a very accurate reproduction of relationships with stromal and vascular cells, providing a microenvironment that plays a crucial role in the development of tumor cells.

THE AIM of the work was to evaluate the antitumor activity of three new azoloazine derivatives in a xenogeneic breast cancer model in mice *in vivo*.

MATERIALS AND METHODS

Test compounds

To assess the potential antitumor properties, three azoloazine derivatives: 3-cyclohexyl-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide (imidazotetrazine **1**), diethyl ether of 4-aminoimidazo[5,1-*c*][1,2,4]triazine-3,8-dicarboxylic acid (imidazotriazine **2**) and 4-amino-8-ethoxy-carbonylimidazo[5,1-*c*][1,2,4]triazine-3-N-(p-toluyl)carboxamide (imidazotriazine **3**) synthesized at Ural Federal University named after the first President of Russia B.N. Yeltsin, have been tested in accordance with the described methods [19, 20], while NMR analysis confirmed a high chemical purity of these derivatives. Epirubicin was used as a reference drug, as a remedy widely used for the treatment of breast cancer, which toxicological effects and activities in mouse models are well known [21]. Compounds **1–3** were selected for the study from 11 candidates based on the results of an earlier determination of the minimum half inhibitory concentration of IC_{50} in an *in vitro* cytotoxicity test in MCF-7 cell culture [22].

Ethical approval

The study was conducted in accordance with bioethical standards set out in the "The Code of Practice for Care and Use of Animals for Experimental Purposes" and Directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes. After the examination, the design of the experimental study was approved by the Local Ethics Committee of Volgograd State Medical University (Protocol No. 2021/049 dated May 27, 2021). All manipulations with laboratory animals were carried out exclusively by persons with higher biological or health education.

Laboratory animals and their maintenance

The work was performed on 48 female BALB/c mice obtained from the nursery of laboratory animals of Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences (Russia). Before modeling metastatic breast cancer, the animals had undergone the necessary certification procedures and introductory quarantine. The animals lived in standard conditions of the vivarium of the Center for Innovative Medicines of Volgograd State Medical University (Russia) with Resolution No. 51 of the Chief State Sanitary Doctor of the Russian Federation dated Aug 29, 2014 "On approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the device, equipment and maintenance of experimental biological clinics (vivariums)"[1].

The animals were kept in plastic cages for 5 individuals on a litter of sawdust with free access to water and feed, with the replacement of both daily. The air temperature in the vivarium was maintained in

[1] Resolution of the Chief State Sanitary Doctor of the Russian Federation dated August 29, 2014 No. 51 "On approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the design, equipment and maintenance of experimental biological clinics (vivariums)" (together with "SP 2.2.1.3218-14. Sanitary and epidemiological rules..."); registered with the Ministry of Justice of Russia on October 31, 2014 No. 34547). Available from: https://www.consultant.ru/document/cons_doc_LAW_155631/. Russian

the range of 20–26°C, the relative humidity was in the range of 30–70%, a 12-hour light cycle was realized with a combined (natural+fluorescent) type of lighting. Bactericidal air treatment was carried out continuously by stationary bactericidal plants.

Experimental study design

The main experimental model of this study is orthotopic implantation of cancer cells subcutaneously under the nipple into the fatty pad of the breast, which simulates metastatic stages and decays breast cancer in the clinic. The injection site and technique, together with the specific properties of the selected breast cancer cell line, largely determine the dynamics of the behavior of transplanted cells in mouse tissues [18].

To induce temporary immunosuppression, white BALB/c mice were injected intraperitoneally with azathioprine at the dose of 1 mg/kg body weight three times: 3 h before the injection of tumor cells, 24 and 48 h after it. At the main stage, orthotopic tumor transplantation was performed, for which 48 mice (6 animals in each group) were injected into the base of the breast nipple with 106 human breast cancer cells of the MCF-7 line in 0.2 ml of a sterile saline solution. To reduce the vaccination dose, Matrigel (Corning, USA) was used as a carrier [23]. All the animals vaccinated with MCF-7 cells were divided into 8 groups (Table 1).

Two doses of the substance (1/2 C_{50}, and 1/10 IC_{50}) were used to study the antitumor activity spectrum. The choice of doses of the tested derivatives was justified by the authors' previous studies of their cytotoxic activity and genotoxic activity. The substances were administered once intraperitoneally in 0.2 ml of a saline solution 72 h after the tumor cells injection, in the control group, only a sterile saline solution was used.

Every three days, the mice were weighed and the injection area was visually monitored.

The animals were removed from the experiment on the 15th day with an overdose of Tiletamine at a dose of 200 mg/kg of the intraperitoneal weight. The euthanasia of mice was carried out in a room where no other animals were kept, and the disposal of corpses was carried out by a responsible person.

After euthanasia, the sections of the subcutaneous tissue of the abdominal wall where tumor cells had been injected, as well as similar areas on the opposite side were taken away. When cutting out the material, the total volume of the papilla and its base (mm^3) was calculated. The difference between the described value for intact and injected mammary glands was taken as the relative volume of the tumor tissue.

The material was placed in a buffered 10% formalin solution for a subsequent histological examination. The following histological equipment was used for the study – Microm STP 420 wiring apparatus (Microm, Germany), Leica EG 1160 modular filling system (Leica, Germany), Leica RM 2255 rotary microtome (Leica, Germany), Dako coverstainer (Dako, Denmark), Dako Link 48 immunohistostainer (Dako, Denmark), microscope Leica DM 6000B with Leica $DFC_{50}C$ digital camera (Leica, Germany). The possibilities of classical light and fluorescence microscopy were used for the visualization and morphometric examination of cells and tissues. The image analysis was performed using the ImageJ program (USA).

To prove the presence of human MCF-7 cells in mouse tissues, immunohistochemical detection of marker cytokeratin 8/18, clone EP 17+EP30 using Dako Cytomation kits (Denmark), was used. Imaging was performed using an indirect immunoperoxidase method, using positive antigen controls, a negative antigen, and antibody controls[2].

The dynamics of an animal body weight, the estimated volume of tumor tissue, and histological characteristics of the injection site were selected as indicators determining the antitumor effects of the test compounds.

For a general conclusion about the tumor condition, a histological index was calculated based on a breast cancer gradation from 0 for normal non-tumor tissue to 10 for an undifferentiated tumor[3] with a slight difference – due to a relatively small amount of tumor tissue, the maximum values of the mitotic index were reduced to 4 (Table 2).

Statistical analysis

Statistical processing of experimental data was carried out using the Statistica 10.0 program (Dell USA). After checking the samples according to the Shapiro-Wilk criterion for compliance with the normal distribution and establishing its absence, the results were presented as the median, the boundaries of the first and third quartiles – Me [Q1÷Q3]. An intra-group comparative analysis was performed using the Kraskel–Wallis criterion, and a comparison between unrelated samples was performed using the Mann-Whitney criterion. The differences were considered significant at a confidence level of p <0.05.

RESULTS

Dynamics of body weight and volume of tumor tissue

The administration of tumor cells to mice was accompanied by the cessation of their growth and loss of body weight, which amounted from 6 to 11% of the initial value in all experimental groups during the period from the injection of MCF-7 to the administration of an antitumor drug (Table 3).

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Table 1 – Characteristics of experimental animals groups

Group	Compound	Dose	Number of heads
Control	Saline solution	–	6
1а	Imidazotetrazine **1**	1/10 IC_{50}	6
1б		1/2 IC_{50}	6
2а	Imidazotetrazine **2**	1/10 IC_{50}	6
2б		1/2 IC_{50}	6
3а	Imidazotetrazine **3**	1/10 IC_{50}	6
3б		1/2 IC_{50}	6
Reference	Epirubicin	1/2 IC_{50}	6

Table 2 – Histological gradation of breast cancer to determine histological index in experiments on immunodeficient mice

Criteria	Score in points
Structure formation	
Glandular/tubular structures occupy: – more than 75% of the tumor area	1
– from 10 to 75% of the tumor area	2
– less than 10% of the tumor area	3
Nuclear polymorphism	
Monomorphic small nuclei with a clear contour, uniform chromatin, similar to the nuclei of normal epithelium	1
Enlarged moderately polymorphic vesicular nuclei with prominent nucleoli	2
Polymorphic vesicular nuclei, variable in size, with noticeable nucleoli, often of atypical shape	3
Mitoses and invasions	
Single mitoses	1
Multiple mitoses	2
Multiple mitoses and invasion G1	2
Multiple mitoses and invasion G2-G3	4

Table 3 – Antitumor effects of azoloazines 1–3 and epirubicin during orthotopic transplantation of MCF-7 cells to immunodeficient mice

Group (all *n*=6)	Weight of mice, g		Tumor volume on day 15, mm^2
	Initial	Final	
Control	48.8 [46.9÷50.7]	40.7 [37.9÷43.1]	24.5 [19.9÷27.8]
Epirubicin 1/2 IC_{50}	48.3 [46.2÷50.0]	43.1 [40.5÷46.2]*	18.5 [15.6÷20.9]*
Imidazotetrazine **1**			
1/10 IC_{50}	48.1 [46.0÷49.6]	47.5 [46.1÷48.8]*	12.4 [9.9÷15.1*#
1/2 IC_{50}	48.1 [46.0÷49.6]	48.6 [46.5÷50.5]*#	9.6 [7.7÷11.8]*#
Imidazotetrazine **2**			
1/10 IC_{50}	49.0 [47.2÷51.0]	45.1 [43.4÷46.9]*	18.7 [15.1÷20.4]*
1/2 IC_{50}	49.0 [47.2÷51.0]	46.3 [44.5÷47.4]*	16.5 [13.3÷19.0]*
Imidazotetrazine **3**			
1/10 IC_{50}	48.4 [47.1÷50.2]	45.6 [43.3÷48.0]*	17.3 [14.8÷20.5]*
1/2 IC_{50}	48.4 [47.1÷50.2]	46.9 [43.7÷48.2]*	15.0 [12.9÷18.1]*#

Note: statistically significant differences * – between the use of azoloazine derivative and control; # – between the use of azoloazine derivative and epirubicin (p <0.05).

Table 4 – Histological index after administration of azoloazines 1–3 and epirubicin in orthotopic transplantation model of human breast cancer cells MCF-7 to mice

Test compound	Dose	
	1/10 IC_{50}	1/2 IC_{50}
Control	8.8 [7.7÷9.6]	
Epirubicin	–	8.5 [7.2÷9.4]
Imidazotetrazine **1**	8.9 [7.7÷9.7]	8.2 [7.3÷9.1]
Imidazotetrazine **2**	8.5 [7.4÷9.3]	9.1 [7.9÷9.8]
Imidazotetrazine **3**	8.4 [7.5÷9.2]	8.8 [7.7÷9.6]

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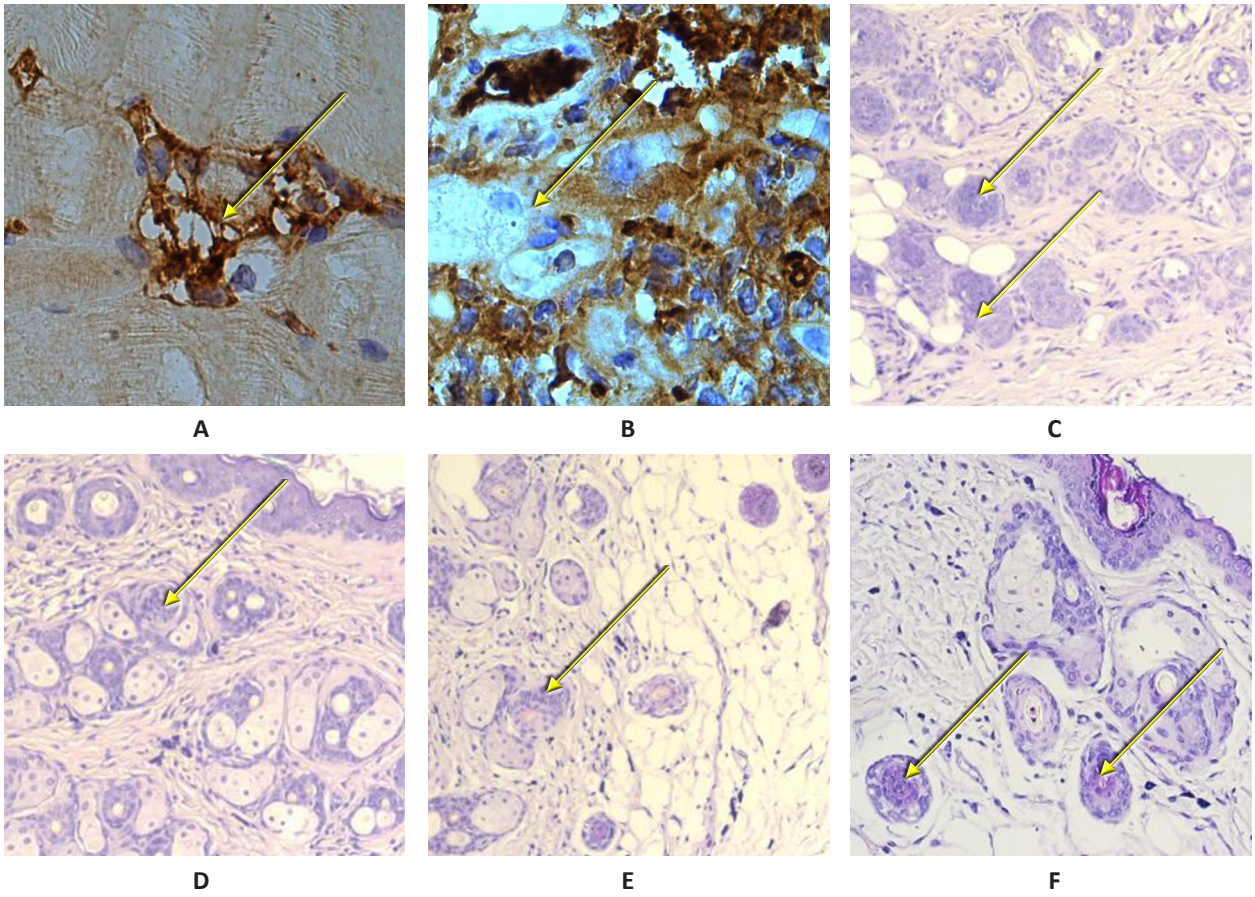

Figure 1 – Transplantation area of MCF-7 cells to mice in xenogenic model of human breast cancer

Note: A – control series without the use of antitumor drugs. Accumulation of immunopositive material in the area of the neurovascular bundle of the adjacent muscle. B – application of imidazotriazine 2. Accumulation of tumor cells with invasion into the lumen of the duct of the breast. C – application of epirubicin. Perry and intraductal clusters of tumor cells. D – use of imidazotetrazine 1. Single clusters of tumor cells around the ducts of the breast and in the adjacent fatty tissue. E – use of imidazotriazine 2. Accumulation of tumor cells with invasion into the lumen of the duct of the breast. F – use of imidazotriazine 3. The histological picture is similar to the use of imidazotriazine 3. A, B – immunohistochemical detection of human cytokeratin 8/18. C–F is stained with hematoxylin and eosin. Magnification: ×200.

During the same period, the body weight of intact mice increased by an average of 2%. The administration of the tested compounds changed the dynamics of animal body weight to a certain extent, but these changes were different depending on the compound and its dose.

The administration of the reference drug epirubicin was accompanied by a slowdown in the decrease in body weight in mice, which eventually amounted to 89.2% of the initial *vs.* 83.4% in the control. The tumor volume calculated on the 15th day of the autopsy experiment was 75.5% of the analogous one without the administration of an antitumor drug.

Imidazotetrazine **1** caused the cessation of the body weight loss in mice and its increase over 15 days led to the restoration of this indicator to the initial one (when using a dose of 1/10 IC_{50} – 98.8%, when using a dose of 1/2 IC_{50} – 101.6%). The mass recovery was especially intense in the first three days after the injection of derivative 4: during this time, the mass increased by more than 10%. The tumor volume calculated on the 15th day of the autopsy experiment was 50.6% of the same in the control when using a dose of 1/10 IC_{50}, and 39.2% when using a dose of 1/2 IC_{50} (p <0.05).

When imidazotriazine **2** was administered to the experimental mice with transplanted MCF-7 tumor cells, the body weight dynamics similar to that of epirubicin, was observed. Stabilization of the mass and its slight increase by the 15th day of the experiment led to a final mass when using a dose of 1/10 IC_{50} in 92.0% of the initial, when using a dose of 1/2 IC_{50} – 94.5%. The tumor volume calculated on the 15th day of the autopsy experiment was 76.3% of the same in the control when using a dose of 1/10 IC_{50}, and 67.3% when using a dose of 1/2 IC_{50}, which corresponded to the inhibition of tumor growth by 23.7 and 32.7%, respectively (p <0.05).

The administration of imidazotriazine **3** caused the cessation of body weight loss and its increase by 11.5% in the first three days. Subsequently, the dynamics of body weight stabilized, and by the 15th day of the experiment

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it amounted to 94.2% of the initial when using a dose of 1/10 IC_{50}, and 96.9% when using a dose of 1/2 IC_{50}. The tumor volume calculated on the 15th day of the autopsy experiment was 70.6% of the analogous one without the administration of an antitumor drug when using a dose of 1/10 IC_{50} and 61.2% when using a dose of 1/2 IC_{50}. This corresponded to the inhibition of tumor growth by 29.4 and 38.8%, respectively (p <0.05).

Results of histological examination

The histological examination revealed changes in the transplantation area, as well as in the adjacent fatty tissue and lymph nodes (Fig. 1).

Staining for human cytokeratin 8/18 revealed the presence of this protein in mouse tissues, which indicated the validity of the applied technique. In cases where MCF-7 cells were lyzed, the marker protein was detected in the intercellular substance, since it is quite difficult to proteolysis (Fig. 1A, 1B).

In all experimental groups on the 15th day of the experiment, the morphological picture at the site of xenotransplantation of MCF-1 cells was characterized by the presence of single tumor cells, as well as their clusters in the form of small clusters or trabeculae. The amount of stroma between tumor cells in the case of cluster formation was minimal. In some cases, the formation of chains and structures like a target or an “owl’s eye” was observed around medium-sized vessels and ducts of the mammary gland. A complete obstruction of the lumen by invasive MCF-7 cells was sometimes detected in the ducts, but more often such cells were isolated, and a part of the lumen was filled with a mucus-like secret. In case of death, the tumor cells lost their nucleus and were surrounded by lymphohistiocytic infiltrate. A small number of lymphocytic cells could also be found around clusters of viable tumor cells (Fig. 1C–1F).

The tumor cells themselves were extremely variable in structure. The cell nuclei were usually large, polymorphic, with a predominance of rounded shapes. They were located, as a rule, centrally and contained well-distinguishable nucleoli. The cytoplasm of the cells was predominantly extensive and pronounced eosinophilic. A mitotic activity on the preparations varied from single mitoses to 10–15 divisions in the field of vision.

In addition to the local invasion into the lumen of ducts, lymphatic and (much less often) blood vessels, tumor cells were able to spread to adjacent tissues and lymph nodes. In the fibrous-adipose tissue surrounding the mammary gland, both single MCF-7 cells and clusters of 7–12 cells were found, often with moderate lymphohistiocytic infiltration around them. In adipose tissue, the tumor clusters were larger; morphologically they looked like typical micrometastases closely adjacent to the feeding vessels. In lymph nodes, MCF-7 cells were usually located singly, and there was a pronounced tissue reaction from macrophages and lymphoid tissue around them.

The determination of the histological index made it possible to give a semi-quantitative assessment of histological changes in the zone of xenotransplantation of human breast cancer cells when testing azoloazine derivatives and a comparison drug (Table 4).

As can be seen from the presented data, the use of epirubicin at a dose of 1/2 IC_{50} practically did not change the histological index of tumors, in comparison with the value of the indicator in the control group of animals.

The values of the histological index in all groups practically did not differ from the control (the maximum decrease with the administration of imidazotetrazine **1** was 9.3%), which once again confirmed the fact that the cells insensitive to the applied chemotherapy retain a high ability to malignant progression in the body and can even increase it.

DISCUSSION

The development of virtual screening, chemical technologies for the targeted synthesis of organic compounds with predicted properties, as well as medical biotechnology for testing potential drugs in *in vitro* cultures and modified models on laboratory animals have become the basis for a new era of development and implementation of modern chemotherapeutic agents.

The modern paradigm implies that from the moment these compounds are presented to experimenters, their chemical structure evidently assumes an antitumor activity (docking or the closest analogues of available drugs). According to the results of preclinical tests, the drugs have clearly marked targets of exposure, proven their high antitumor activity *in vitro* and *in vivo*, have minimal toxic effects on healthy tissues and do not cause rapid development resistance [24–26].

With regard to the subject of this study, it should be emphasized that solving the problem of breast cancer in prevention cases, early diagnosis, treatment and rehabilitation of women is classified as a priority task of world and domestic healthcare [1, 5].

The class of compounds chosen (imidazotriazine and imidazotetrazine derivatives) is attractive in this regard due to a sufficient number of well-established representatives, including mitozolamide and temozolomide, as well as the emergence of protocols for the synthesis of new compounds with potentially promising properties [12, 19]. According to the results of evidence-based studies, compounds of this class exhibit antitumor, anti-inflammatory, antioxidant, and antibacterial activity [27, 28].

Orthotopic implantation of cancer cells into animal breast tissue allows very accurate reproduction of the tumor microenvironment and in many ways resembles the multiple stages involved in the development of breast cancer in patients [23, 29].

The study showed that in a xenogenic breast cancer model obtained by orthotopic injection of MCF-7 cells into mice, all azoloazine derivatives included in the work showed antitumor activity, which has signs of dose dependence and decreases in the series of imidazotetrazine **1** (leader) >imidazotriazine **3** > imidazotriazine **2** ≥epirubicin. The maximum inhibition of tumor growth with the introduction of the most active derivative of temozolomide 1 was 60.8%, which significantly exceeded the activity of the comparison drug epirubicin – 24.5%. The drug significantly suppressed the proliferation and spread of tumor cells in the model of xenotransplantation of human breast cancer MCF-7 cells to mice. The administration of the drug was accompanied by a decrease in the histological malignancy index for preserved cells by 9.3%.

In all series of the experiments, the histological picture indicated a decrease and disintegration of a part of the tumor structures in the tissues of mice, but the preserved cells showed pronounced atypism, a tendency to mitosis and invasion, and formed fresh accumulations in breast tissue, adjacent fatty tissue and lymph nodes.

As can be seen, all the three drugs selected based on previous cytotoxicity studies on human breast cancer cells and non-tumor cells *in vitro,* showed results exceeding those for the reference drug epirubicin. The pharmacodynamic properties of epirubicin have been well studied and are similar to those of other anthracycline antitumor antibiotics. Epirubicin is most active in the phases of the cell cycle, accompanied by the most intensive DNA synthesis. After the intercalation between DNA base pairs, it stabilizes the topoisomerase II-DNA complex, which leads to an irreversible rupture of the DNA strand. Cytotoxicity has been proven for epirubicin both *in vitro* and *in vivo* against breast cancer and a number of other human tumor cells, and cell death increases with increasing drug concentration [30]. Nevertheless, the activity of the drug is quite moderate, as demonstrated by the present study. Therefore, epirubicin can be considered a classic reference drug for new preclinical trials, which are designed to find new, more effective drugs for tumor chemotherapy.

A comparison of the antitumor activity in an *in vivo* model of three azoloazine derivatives showed that, despite the similar structure and physico-chemical properties, these compounds exhibit an unequal activity in relation to breast cancer cells. The known reasons that cause differences in the antitumor effects of homologous molecules are, first of all, differences in the nature of distribution in the body during a parenteral administration and the ability to penetrate target cells. The experimental values of tumor cell survival obtained are consistent with earlier results of studying other azoloazine derivatives [12, 31, 32].

Implantation of human breast cancer cell lines into mice is relatively simple and makes the evidence-based genetic or pharmacological manipulation of implanted cells possible. However, it is now fairly believed that xenografts of tumors in immunodeficient mice cannot fully simulate tumors in humans, including due to a significant genetic polymorphism and the fact that human cells are apparently not fully adapted to growth in mouse tissues [33]. It is no coincidence that recently the attention of researchers has been increasingly focused on creating three-dimensional models of cancerous tumors *in vitro.* Modeling 3D microenvironments that maximally simulate the microenvironment of cells in human breast cancer makes them a valuable tool as an alternative for replacing tumor models in animals and for predicting a risk assessment in humans [34].

CONCLUSION

Using a xenogenic model of human breast cancer (NCF-7 cell culture), all three new azoloazine derivatives included in the study showed an antitumor activity exceeding that of the reference drug drug epirudbicin. Among the tested new azoloazine derivatives, 3-cyclohexyl-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-N-piperidinylcarboxamide is the undisputed leader causing the inhibition of tumor growth in the xenogenic model *in vivo.*

The high sensitivity of the MCF-7 cell lines to the tested substances makes it possible to conclude that further preclinical tests of their effectiveness and safety are promising. According to the results of the study of new azoloazine derivatives, it is possible to expect the inhibition of the growth of transplanted tumors in the experimental animals in relation to a wider range of simulated tumors.

The conclusion obtained should be interpreted as preliminary, since only one approach (xenogenic transplantation in mice) and one breast cancer cell line, MCF–7, were used in the work to assess the antitumor activity. The specific mechanisms of action of azoloazine derivatives, as well as the comparison of their activity with the structural features of molecules, require a separate further study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Ahmed Hamid Al-Humairi – development of the basic concept, hypothesis and design of the study, conducting research, processing research data, writing and editing text; Dmitry L. Speransky – development of the basic concept and design of the study; Valery V. Novochadov – development of the concept and design of the study, statistical analysis; Sergey V. Poroysky – conducting research, implementation of the experimental part of the study, research data processing, text editing; Nadezhda V. Cherdyntseva – development of the research concept; Vladimir V. Udut – development of the research concept. All authors confirm that their authorship meets the international ICMJE criteria (all authors have made a significant contribution to the development of the concept, research, and preparation of the article, read and approved the final version before the publication).

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Phytochemical and pharmacological study of biologically active compounds and dry extracts of *Populus rubrinervis* Hort. Alb. buds of various polarities

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The aim of the work was a phytochemical and pharmacological study of biologically active compounds (BACs) and *Populus rubrinervis* Hort. Alb. buds preparations of various polarities.

Materials and methods. The object of the study was dry extracts of *P. rubrinervis* Hort. Alb. buds the samples of which were prepared in January–March 2023 in the Botanical Garden of Samara University (Samara, Russia). The separation of the amount of current substances was carried consecutively by the method of circulating extraction (chloroform), then, by the method of fractional percolation, a tincture was received on 70% ethyl alcohol (1:5). Pinostrobin was used as the standard sample (SS). The analysis of the substances was carried out by the TLC method. The electronic spectra registration was carried out with a spectrophotometer "Specord 40" (Analytik Jena, Germany). The study of the pharmacological (diuretic) activity of *P. rubrinervis* Hort. Alb. buds dried extracts was carried out on 60 white outbred rats of both sexes weighing 200–220 g in the experiments with aqueous diuresis.

Results. *P. rubrinervis* Hort. Alb. buds dried extracts of various polarities (extract No. 1 (chloroform) and extract No. 2 (70% ethanol) were received. By the method of thin-layer chromatography, it was determined that the dominant complexes of the lipophilic nature with pinostrobin are isolated in extract No. 1, phenolic substances of the glycoside nature prevail in extract No. 2. Despite various polarities of the extragents, spectral characteristics of extract No. 2 have significant similarities with extract No. 1. When studying the diuretic activity, it was established that when SS pinostrobin was injected at a dose of 1 mg/kg, for 4 h of the experiment, an isolated increase in diuresis was noted (from 1.72±0.11 to 1.97±0.03 ml, p <0.05); at the same time, an isolated increase in creatininuresis (from 1.50±0.29 to 2.39±0.15 mg, p <0.05) was observed during 24 h of the experiment. When extract No. 2 was injected at a dose of 10 mg/kg, there was a moderate significant increase in diuresis (from 1.82±0.02 to 2.07±0.04 ml and from 2.38±0.39 to 3.02±0.11 ml, p <0.05) and a significant increase in creatininuresis (from 0.14±0.01 to 0.19±0.03 mg and from 2.31±0.42 to 2.79±0.51 mg, p <0.05) for 4 and 24 h of the experiment, respectively.

Conclusion. The extraction separation of the amount of *P. rubrinervis* Hort. Alb. buds by the polarity degree was carried out. Pinostrobin SS at a dose of 1 mg/kg and extract No. 2 at a dose of 10 mg/kg had a diuretic activity, in connection with which they are promising in terms of the development of effective drugs.

Keywords: *Populus rubrinervis* Hort. Alb.; dry extract; pinostrobin; UV spectrophotometry; diuretic activity

Abbreviations: HM – herbal medicine; SPh RF – State Pharmacopoeia of the Russian Federation; BAC – biologically active compounds; SS – standard sample; DSA – diazobenzenesulfonic acid; PhM – pharmacopoeial monograph.

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Фитохимическое и фармакологическое исследование биологически активных веществ и сухих экстрактов почек тополя краснонервного (*Populus rubrinervis* Hort. Alb.) различной полярности

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Цель. Проведение фитохимического и фармакологического исследования биологически активных веществ (БАВ) и препаратов на основе почек тополя краснонервного (*Populus rubrinervis* Hort. Alb.) различной полярности.

Материалы и методы. Объектом исследования стали сухие экстракты почек тополя краснонервного, образцы которых были заготовлены в январе–марте 2023 года на территории Ботанического сада Самарского университета (г. Самара, Россия). Разделение суммы действующих веществ проводили последовательно методом циркуляционной экстракции (хлороформ), а затем методом дробной перколяции получали настойку на спирте этиловом 70% (1:5). В качестве стандартного образца (СО) использовали пиностробин. Анализ веществ проводили методом тонкослойной хроматографии. Регистрацию УФ-спектров осуществляли с помощью спектрофотометра «Specord®40» (Analytik Jena, Германия). Исследование фармакологической (диуретической) активности сухих экстрактов почек тополя краснонервного проводили на 60 белых аутбредных крысах обоего пола массой 200–220 г в экспериментах с водным диурезом.

Результаты. Получены сухие экстракты на основе почек тополя краснонервного различной полярности (экстракт № 1 (хлороформ) и экстракт № 2 (спирт этиловый 70%). Методом тонкослойной хроматографии определено, что доминирующие комплексы липофильной природы с пиностробином изолированы в экстракт № 1, в экстракте № 2 превалируют фенольные вещества гликозидной природы. Несмотря на разную полярность экстрагентов, спектральные характеристики экстракта № 2 имели значительное сходство с экстрактом № 1. При изучении диуретической активности определено, что при введении СО пиностробина в дозе 1 мг/кг за 4 ч опыта было отмечено изолированное повышение диуреза (с 1,72±0,11 до 1,97±0,03 мл, p <0,05), в тоже время за 24 ч опыта отмечено изолированное повышение креатининуреза (с 1,50±0,29 до 2,39±0,15 мг, p <0,05). При введении экстракта № 2 в дозе 10 мг/кг отмечено умеренное достоверное повышение диуреза (с 1,82±0,02 до 2,07±0,04 мл и с 2,38±0,39 до 3,02±0,11 мл, p <0,05) и значительное увеличение креатининуреза (с 0,14±0,01 до 0,19±0,03 мг и с 2,31±0,42 до 2,79±0,51 мг, p <0,05) за 4 и 24 ч опыта соответственно.

Заключение. Проведено экстракционное разделение суммы веществ почек тополя краснонервного по степени полярности. СО пиностробина в дозе 1 мг/кг и экстракт № 2 в дозе 10 мг/кг проявляли диуретическую активность, в связи с чем и являются перспективными в плане разработки эффективных лекарственных средств.

Ключевые слова: тополь краснонервный; *Populus rubrinervis* Hort. Alb.; почки; сухой экстракт; пиностробин; УФ-спектрофотометрия; диуретическая активность

Список сокращений: ЛРП – лекарственный растительный препарат; ГФ РФ – Государственная фармакопея Российской Федерации; БАВ – биологически активные вещества; СО – стандартный образец; ДСК – диазобензолсульфокислота; ФС – фармакопейная статья.

INTRODUCTION

At present, herbal medicines (HMs) are actively used in medical practice for the treatment and prevention of various diseases [1–4]. These drugs have a number of advantages over synthetic ones: they are relatively safe, less toxic, and have a broader spectrum of action [4, 5].

One of the interesting and perspective sources of medicines are plants of the Willow family (*Salicaceae*) of the Poplar genus (*Populus* L.), which grow widely in temperate countries: Western and Eastern Siberia, Europe, Eastern Kazakhstan, Central Asia, and Western China [5–8]. About 20 species of poplar grow in central Russia, the most common of which (including the Samara region) are: *P. nigra* L., *P. deltoides* Marsh; *P. suaveolens* Fisch; *P. laurifolia* Ledeb; *P. balsamifera* L.; *P. alba* L.; *P. tremula* L.; *P. rubrinervis* Hort. Alb. [6, 9, 10]. Now State Pharmacopoeia of the Russian Federation XV edition (SP RF XV ed.) includes a pharmacopoeial monograph – PhM.2.5.0042.15 “Poplar buds”[1]. The following species

[1] State Pharmacopoeia of the Russian Federation. XV ed. Vol. 4. Moscow; 2018. 1832 p. Available from: https://docs.rucml.ru/feml/pharma/v14/vol4/. Russian

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are pharmacopoeial: *P. nigra* L., *P. balsamifera* L., *P. deltoides* Marsh., *P. suaveolens* Fisch., *P. laurifolia* Ledeb.

Drugs based on medicinal plant raw materials of these genus have anti-inflammatory, antipyretic, analgesic, wound healing, antimicrobial, anti-ulcer, antioxidant activities and are widely used in official and folk medicine [7, 11–13]. In current scientific studies, there is also a mention of the investigation of *Populus balsamifera* L. buds dihydrochalcones as an effective agent for a topical treatment of psoriasis along with anti-inflammatory and antioxidant effects [14–17].

A chemical composition of a pharmacopoeial raw material – Poplar buds – is rich and quite diverse. The literature sources describe the *Populus* L. buds as mainly containing compounds of phenolic nature: simple phenols (salicin, salicylic alcohol); phenolic acids, phenylpropanoids; terpenoids (mono- and sesquiterpenoids); flavanones (pinocembrin, pinostrobin); flavones (apigenin, chrysin, tectochrysin); flavonols (galangin, kaempferol, quercetin); chalcones and dihydrochalcones; caffeic and ferulic acids, as well as their derivatives [6, 14, 15, 18]. It should be noted that phenolic compounds (flavonoids, phenylpropanoids and simple phenols) make a significant contribution to the manifestation of the pharmacological activity of the HMs based on poplar raw materials [6, 19–21]. So, the leading group of BACs of the pharmacopoeial Poplar species buds are flavonoids, among which flavanones pinostrobin (5-hydroxy-7-methoxyflavanone) and pinocembrin (5,7-dihydroxyflavanone) dominate; this fact determines the presence of the drugs antimicrobial activity (Fig. 1) [22–25].

However, this group has other pharmacological properties as well [19, 26–28]. For example, a recently described study compared the anti-inflammatory activity of *Populus* L. bud extracts and flavanones (pinocembrin and pinostrobin) against pro-inflammatory human gingival fibroblasts (HGF-1), which revealed the potential protective role of both BACs and *Populus* L. bud extracts and demonstrated antioxidant properties [29]. The hepatoprotective and cardiovascular activities of the alcoholic *Populus* L. bud extract were also evaluated along with antioxidant and anti-inflammatory activities. In this study, the alcoholic extract showed significant anti-inflammatory, hepatoprotective, and vasodilatory activities, the latter being endothelium-independent and believed to be mediated mainly by the ability of the individual components to exhibit antioxidant properties, probably, related to the inhibition of Ca^{2+} ion influx [30].

The analysis of literature sources has shown the presence of a diuretic activity, i.e., an increase in the renal excretion of water, electrolytes, creatinine preparations and individual BACs from the raw materials of the genus *Poplar* plants: decoction of the aspen bark (*Populus tremula* L.) (100 µl/kg), the infusion of aspen buds (100 µl/kg), tincture of aspen buds (100 µl/kg), tincture of the aspen bark (100 µl/kg), tincture of poplar buds (50 and 100 µl/kg), an increase in the renal excretion of water, electrolytes, creatinine [5]. Tremuloidin isolated from the aspen bark at doses of 25 and 50 mg/kg stimulated diuresis, saluresis, and creatininuresis, and at a dose of 100 mg/kg, it stimulated only the excretion of sodium, potassium, and creatinine [5]. The diuretic effect was also found for the dry extract of *P. simonii* Carrier buds [31, 32].

So, the research of the family *Salicaceae* of the genus *Populus* representatives is currently relevant for the scientists all over the world. The annually updated and summarized data on the chemical composition of buds of the genus *Poplar* plants, as well as on the pharmacological activity of the active substances and developed dosage forms based on the buds of the genus Poplar plants [19, 22–24]. Currently, it is known that *P. rubrinervis* Hort. Alb. is actively used for landscaping of populated areas [10]. It has its own differences and advantages over other species: all plants are male specimens, it does not form fluffy seeds, it is winter-hardy, gas tolerant, propagates well by unwooded stem cuttings, and it is distinguished from other species by the highest growth vigor [9, 10, 33]. The significant phytomass of the bud allows us to talk about a greater economic efficiency in the processing of this raw material relative to the classical species – black poplar [33]. However, there is very little information in the literature on the chemical composition and pharmacological activity of preparations and BACs of *P. rubrinervis* Hort. Alb. buds, which contributed to the authors' scientific interest in this object.

THE AIM of the work was a comparative phytochemical and pharmacological study of biologically active compounds (BACs) and *Populus rubrinervis* Hort. Alb. buds preparations of various polarities.

MATERIALS AND METHODS

The object of the study was dry extracts of *P. rubrinervis* Hort. Alb. buds, harvested in the period from the end of January to March 2023 before swelling. The collection plantings of the Botanical Garden of Samara University were the main base for collecting raw materials (Samara, Russia). Drying was carried out naturally without heating. The dried raw materials were subjected to a classical sample preparation in accordance with the requirements of SP RF XV edition of the pharmacopoeial monograph "Buds" (GPhM.1.5.1.0009.15)[2]. The SS of pinostrobin (5-hydroxy-7-methoxy-2-phenylchroman-4-one),

[2] GPhM.1.5.1.0009.15 "Buds". State Pharmacopoeia of the Russian Federation. XV ed. Moscow, 2023. Available from: https://pharmacopoeia.regmed.ru/pharmacopoeia/izdanie-15/1/1-5/1-5-2/pochki/. Russian

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obtained earlier from *P. balsamifera* L. buds according to the requirements of PhM 42-0073-01 "Pinostrobin as a standard sample" was also analyzed.

Preparation of work solutions for analysis by thin-layer chromatography and UV-spectrophotometry methods

In order to separate the amount of current substances contained in the buds of *P. rubrinervis* Hort. Alb., the forextraction of raw materials by the method of circulating extraction in the Soxhlet apparatus, was carried out. During the extraction, 30 complete cycles were counted, the end of the extraction was determined by the absence of staining of the extractant in the working Soxhlet flask. Chloroform was used as an extractant. The resulting lipophilic chloroform extract of *Populus rubrinervis* Hort. Alb. buds (extract No. 1) had been evaporated in a rotary evaporator under vacuum at 45°C until a dry extract was obtained. After the forextraction, the defatted raw material was dried under traction without heating. Then, a tincture on a 70% ethyl alcohol in the ratio of raw material and extractant is 1:5 was obtained on the basis of this raw material by the method of a fractional percolation. Then, to obtain a dry extract of *P. rubrinervis* Hort. Alb. buds (extract No. 2), the tincture, similarly to the lipophilic extract, was evaporated in a rotary evaporator under vacuum at 65°C t.

Sample processing for dry extract of *P. rubrinervis* Hort. Alb. buds (extract No. 1)

The aliquot of 0.3 g of *P. rubrinervis* Hort. Alb. buds dry extract (chloroform) (accurately weighed quantity) was placed in a 50 ml capacity measuring flask, dissolved in 25 ml of 96% ethyl alcohol by heating in a boiling water bath and the volume of the solution was adjusted by the same solvent (sample solution A_1). The aliquot of 1 ml of the sample solution A_1 was placed in a 50 ml capacity measuring flask, 2 ml of 3% ethanolic solutions of aluminum (III) chloride was added, and the volume of the solution was adjusted by 96% ethanolic solutions (sample solution B_1). The reference solution was prepared in the following way: 1 ml of the sample solution A was placed in a 50 ml capacity measuring flask and the volume of the solution was adjusted by 96 % ethanolic solutions.

Sample processing for dry extract of *P. rubrinervis* Hort. Alb. buds (extract No. 2)

The aliquot of 0.3 g of *P. rubrinervis* Hort. Alb. buds dry extract (ethanol) (accurately weighed quantity) was placed in a 50 ml capacity measuring flask, dissolved in 25 ml of 96% ethyl alcohol by heating in a boiling water bath and the volume of the solution was adjusted by the same solvent (sample solution A_2). The aliquot of 2 ml of the sample solution A_2 was placed in a 25 ml capacity measuring flask, 2 ml of 3% ethanolic solutions of aluminum (III) chloride was added, and the volume of the solution was adjusted by 96% ethanolic solutions (sample solution B_2). The reference solution was prepared in the following way: 2 ml of the sample solution A_2 was placed in a 25 ml capacity measuring flask and the volume of the solution was adjusted by 96% ethanolic solutions.

Methods of qualitative analysis of *P. rubrinervis* Hort. Alb. buds preparations by thin-layer chromatography and UV spectrophotometry.

To study dry extracts from *P. rubrinervis* Hort. Alb. buds in the phytochemical analysis, the thin-layer chromatography method was used.

The chromatographic plates used in the study were types «Sorbfil-PTSH-AF-A-UV» and «Sorbfil-PTSH-P-A-UV». The plates were placed in a thermostat at 100–105°C before chromatographic analysis. The solvent system tested in the study was chloroform – 96% ethanol (19:1).

The extraction samples were applied with a capillary to the start line and then the plate was immersed in a chromatography chamber pre-saturated with the vapors of the solvent system for 24 h. Chromatography was carried out in an ascending technique. The analysis was considered complete when the solvent front reached 7–8 cm.

After the removal from the system, the plates were dried and evaluated visually in daylight. Additionally, the plates were viewed in the UV light at a wavelength of 254 and 366 nm, then treated with an alkaline solution of diazobenzenesulfonic acid (DSA).

As physical and chemical methods, the method of direct and differential spectrometry was used.

The analysis was performed on a spectrophotometer "Specord 40" (Analytik Jena) at wavelengths in the range of 190–600 nm in cuvettes with a layer thickness of 10 mm. The results of the spectrophotometric determination were processed using the program "WinAspect Excel".

Methodology of BACs pharmacologic study and P. *rubrinervis* Hort. Alb. Buds preparations

The pharmacological study was conducted in accordance with the plan of research works of Samara State Medical University (Samara, Russia) on the theme: "Chemical-pharmaceutical, biotechnological, pharmacological and organizational and economic research on the development, analysis and application of pharmaceutical substances and drugs", State Registration No. AAAA-A19-119051490148-7, dated 14.05.2019. The study was approved by the Bioethics Committee of Samara State Medical University (Report No. 222 dated 07.04.2021).

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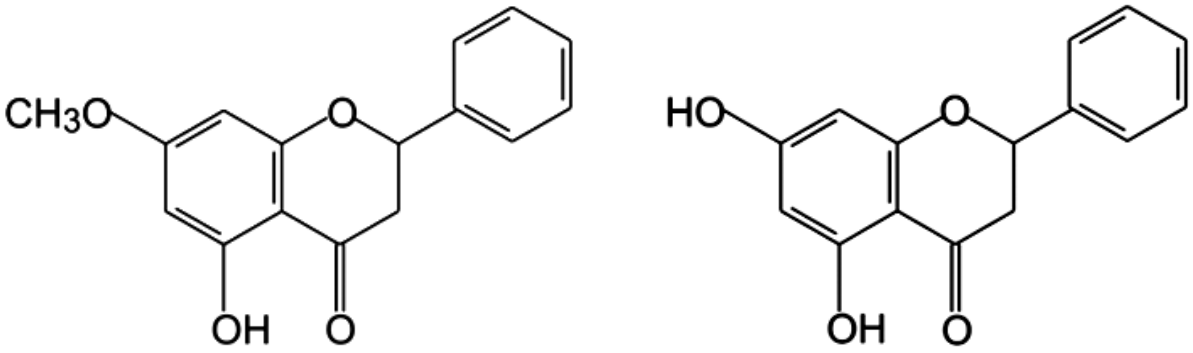
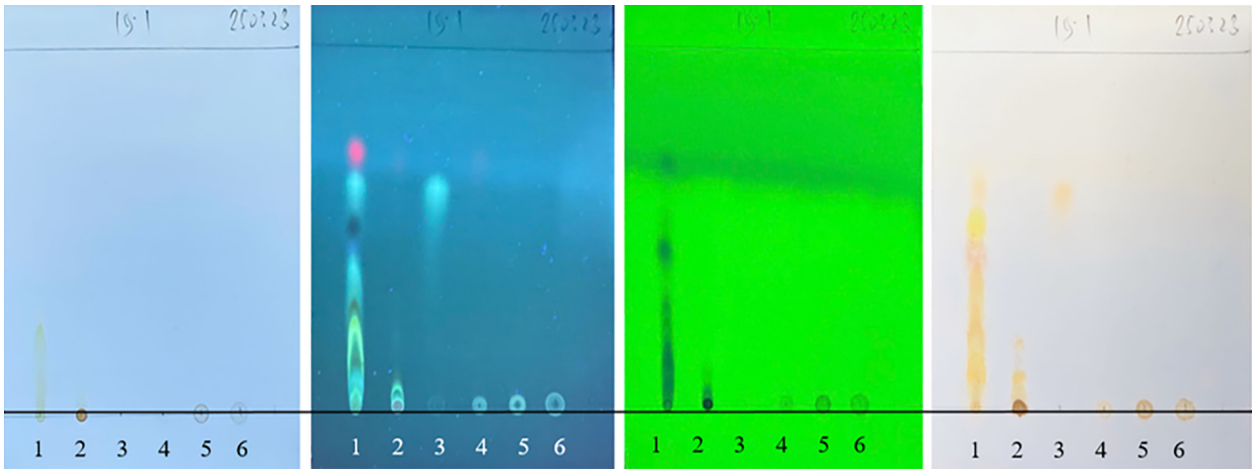


1 2

Figure 1 – Structural formulas of dominant buds flavonoids of pharmacopoeia species of *Populus* genus

Note: 1 – pinostrobin; 2 – pinocembrin.



A B C D

Figure 2 – Chromatogram analysis of extracts and meal from *P. rubrinervis* Hort. Alb. buds in chloroform: ethanol (19:1) solvent system

Note: A – in daylight; B – UV detection in UV light at a wavelength of 366 nm; C – UV detection in UV light at a wavelength of 254 nm; D – DSA treatment; 1 – dry extract No. 1 of *P. rubrinervis* Hort. Alb. buds; 2 – dry extract No. 2 of *P. rubrinervis* Hort. Alb. buds; 3 – pinostrobin; 4 – meal (ethanol 96%); 5 – meal (ethanol 70%); 6 – meal (ethanol 40%).

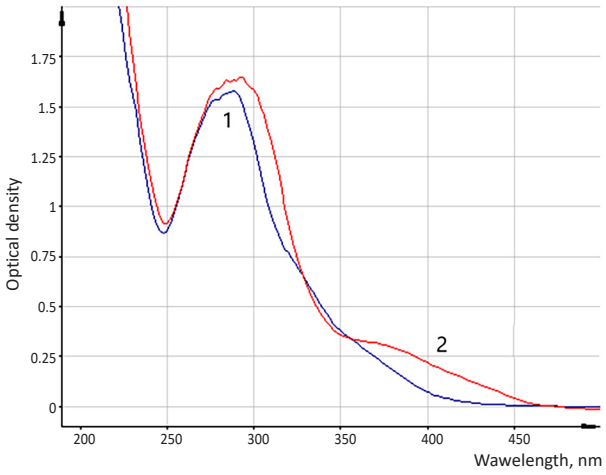


Figure 3 – Electronic spectra of *P. rubrinervis* Hort. Alb. buds chloroform extract (extract No. 1)

Note (here and for Fig. 5): 1 – electronic spectrum of the extract; 2 – electronic spectrum of the extract in the presence of aluminum (III) chloride.

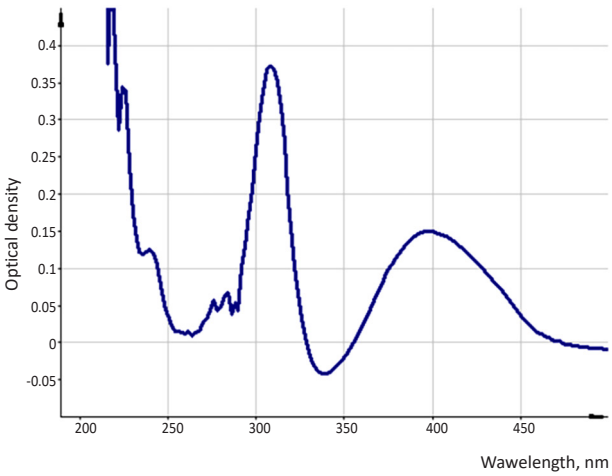


Figure 4 – Differential spectrum of *P. rubrinervis* Hort. Alb. buds chloroform extract (extract No. 1)

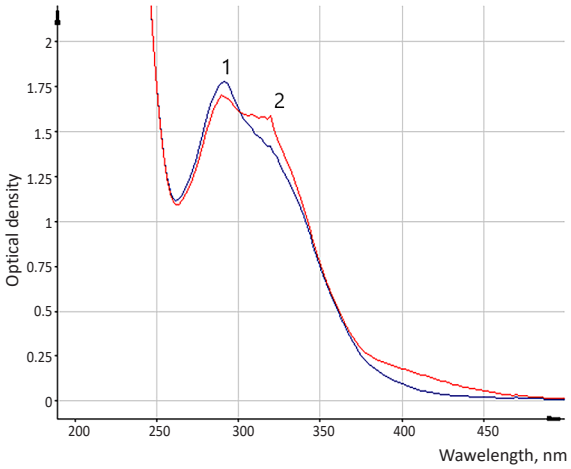


Figure 5 – Electronic spectra of 70% alcohol extract of *P. rubrinervis* Hort. Alb. buds (extract No. 2)

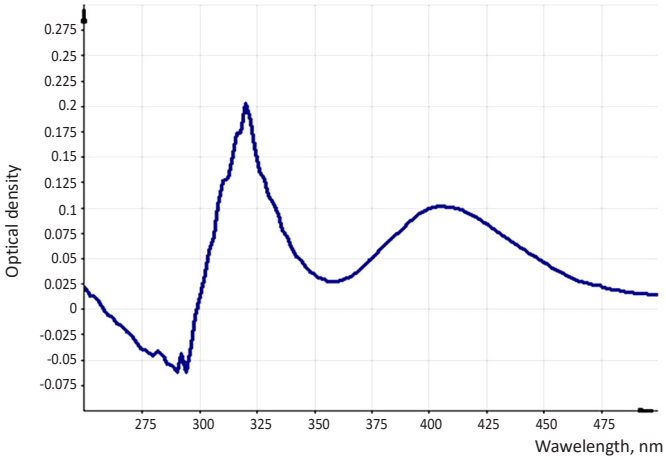


Figure 6 – Differential spectrum of 70% alcohol extract of *P. rubrinervis* Hort. Alb. buds (extract No. 2)

Table 1 – Effect of intragastric injection of SS pinostrobin at a dose of 1 mg/kg and dry extracts of *P. rubrinervis* Hort. Alb. buds No. 1 and No. 2 at a dose of 10 mg/kg on excretory function of intact rats' kidneys of (M±m, *n*=10)

Time, h	Indicators	Control	Experiment No. 1: SS of pinostrobin, 1 mg/kg	Experiment No. 2: Extract No. 1 (chloroform), 10 mg/kg	Experiment No. 3: Extract No. 2 (70% ethanol), 10 mg/kg
4	Diuresis, ml	1.72±0.11	1.97±0.03*	1.82±0.02	2.07±0.04*
	Diuresis, %	100	115	106	120
	Reliability	–	*p*=0.047	*p*=0.698	*p*=0.011
	Creatinine excretion, mg	0.10±0.02	0.13±0.01	0.14±0.01	0.19±0.03*
	Creatinine excretion, %	100	130	140	190
	Reliability	–	*p*=0.341	*p*=0.249	*p*=0.038
24	Diuresis, ml	2.58±0.10	2.52±0.25	2.38±0.39	3.02±0.11*
	Diuresis, %	100	98	92	117
	Reliability	–	*p*=0.844	*p*=0.629	*p*=0.011
	Creatinine excretion, mg	1.50±0.29	2.39±0.15 *	2.31±0.42	2.79±0.51*
	Creatinine excretion, %	100	159	147	186
	Reliability	–	*p*=0.019	*p*=0.229	*p*=0.047

Note: here and further * – $p < 0.05$ – reliability of differences between the data of the experimental group and the control group.

Table 2 – Effect of intragastric injection of comparative drug furosemide at a threshold dose of 1 mg/kg on intact rats' renal excretory function of (M±m, *n*=10)

Time, h	Indicators	Control	Furosemide 1 mg/kg
4	Diuresis, ml	1.97±0.13	3.81±0.22*
	Diuresis, %	100	193
	Reliability	–	*p*=0.001
	Creatinine excretion, mg	0.07±0.01	0.09±0.02
	Creatinine excretion, %	100	129
	Reliability	–	*p*=0.361
24	Diuresis, ml	2.98±0.22	5.42±0.34*
	Diuresis, %	100	182
	Reliability	–	*p*=0.001
	Creatinine excretion, mg	1.19±0.11	1.58±0.13
	Creatinine excretion, %	100	133
	Reliability	–	*p*=0.052

Note: here and further * – $p < 0.05$ – reliability of differences between the data of the experimental group and the control group

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The diuretic activity was studied on 60 white outbred rats of both sexes weighing 200–220 g in chronic experiments with aqueous diuresis. The animals were obtained from the vivarium at the Research Institute of Biotechnology "BioTech", Samara State Medical University of the Ministry of Health of Russia (Samara, Russia). The rats were kept in cages of 4 individuals of the same sex in the vivarium conditions on the standard food ration and with a free access to water. Four experimental and two control groups of animals participated in the experiment. The distribution of animals into groups was performed by drawing lots. Each group consisted of ten animals (n=10). Control and experimental animals received an intragastric 3% water load using a special probe. The experimental animals were additionally administered intragastrically with dried extracts of *P. rubrinervis* Hort. Alb. buds No. 1 and No. 2 at a dose of 10 mg/kg, SS pinostrobin at a dose of 1 mg/kg. Classical diuretics – furosemide at a threshold dose of 1 mg/kg (4 h experiments) and hypothiazide at an effective mean therapeutic dose of 20 mg/kg (24 h experiments) were taken as drugs. After all manipulations, the animals were placed in exchange cages for a urine collection with an access to food and water. After 4 and 24 h, the obtained urine portions were collected. Their volume (diuresis) was determined and creatininuresis was recorded by a calorimetry method on KFK-3 (Zagorsky Optical and Mechanical Plant, Russia).

Statistical processing

In accordance with the general recommendations for the preclinical study of drugs, a comprehensive statistical processing of the data obtained from the pharmacological experiments was carried out using adequate methods of the statistical analysis, the required volume of statistical samples, in the presence of reference drugs. The statistical processing of the results obtained was carried out using the Statistica 10.0 program using the Mann–Whitney test with Bonferroni and Kruskal–Wallis correction. The nonparametric test was chosen because the sample was small, and the distribution in the sample was non-normal (Shapiro-Wilk W test; if p <0.05, the analyzed distribution was considered to be different from normal). The significance level was accepted at p <0.05.

RESULTS AND DISCUSSION

The obtained dry extracts based on the buds of the *P. rubrinervis* Hort. Alb. were in the form of dry powder of golden yellow (extract No. 1) and brick red (extract No. 2) with a specific odor.

The analysis of chromatographic profiles of dry extracts from *P. rubrinervis* Hort. Alb. buds compared with SS of pinostrobin in the solvent system chloroform: ethanol (19:1) allowed us to reliably determine its presence in the studied samples and to assume the presence and dominance of phenolic compounds in the studied object. The TLC analysis of the obtained dry extracts allowed us to arrive at the conclusion about the separation of the initial amount of substances (Fig. 2).

The dominant phenolic complexes of a lipophilic nature with pinostrobin were isolated into extract No. 1 obtained on chloroform. In the hydrophilic extract (extract No. 2), phenolic substances of a glycosidic nature prevail. Specifically, catechins and a number of other phenolic compounds not identified at this stage (Fig. 2B and 2D).

After a double extraction, the chromatographic profiles of the aqueous-alcohol extractions from the raw meal on alcohols of different concentrations (40, 70, 96% ethyl alcohol) showed a maximally depleted composition (Fig. 2).

Thus, using different types of extraction and solvents of a different polarity, it was possible to separate the initial amount of metabolites into lipophilic and hydrophilic complexes. Two dry extracts based on the *P. rubrinervis* Hort. Alb. buds differing in chemical composition, were received. It should be noted that the dominant component of pharmacopoeial poplar buds, pinostrobin, is localized in the first lipophilic (hydrophobic) extract.

The dry extracts were further studied by spectrophotometry. Based on the known previously developed methods for the analysis of pharmacopoeial poplar species buds [1, 2, 4], the direct spectra of *P. rubrinervis* Hort. Alb. buds extracts were analyzed (Fig. 3 and 4).

The analysis of spectral curves showed that extract No. 1 is characterized by the presence of one pronounced maximum in the spectral curve in the area of 288±2 nm and a small "shoulder" in the area of 320±2 nm, which coincides with the absorption maximum of the extracts from the pharmacopoeia poplars buds (289±2 nm) (Fig. 3). Such coincidence can be explained by the presence of the main dominant flavonoids of the buds of pharmacopoeial poplar species, particularly pinostrobin, the content of which had been proven by TLC before.

The differential absorption curve has two expressed analytical maxima: 308±2 nm, characteristic for the phenylpropanoids, and 400±2 nm, characteristic for the amount of flavonoid substances, indicating the presence of these groups of substances in the composition of *P. rubrinervis* Hort. Alb. buds, as in pharmacopoeial species (Fig. 4).

Despite the different polarity of the extractants, the spectral characteristics of hydrophilic extract No. 2 have significant similarities with extract No. 1 obtained using chloroform (Fig. 5 and 6).

Based on the data of chromatographic studies of the extracts (Fig. 2), it can be assumed that the characteristics of the spectra of the compared extracts are largely determined by the presence of simple phenolic compounds that differ in polarity due to

the presence or absence of glycosidic bonds. For a more accurate separation in the future, the study and isolation of biologically active substances from the buds of *P. rubrinervis* Hort. Alb. by column chromatography is planned to be carried out.

The samples of the dry extracts of *P. rubrinervis* Hort. Alb. buds No. 1 and No. 2 and SS pinostrobin were used to study the excretory function of the kidneys in preclinical studies on white mongrel laboratory rats at a dose of 10 mg/kg for the extracts and 1 mg/kg for the SS pinostrobin (Table 1).

When studying the effect of SS pinostrobin on the excretory function of the kidneys it was revealed that in a 4-hour chronic experiment with a single intragastric injection of BAC at a dose of 1 mg/kg against the background of a 3% water load, there was a significant isolated increase in diuresis (by 15%). There was also a significant isolated reliable increase in creatininuresis (by 59%) during 24 h of the experiment (Table 1).

Consequently, SS pinostrobin at a dose of 1 mg/kg against the background of a 3% water load caused an accelerated diuretic response mainly due to an increase in the tubular reabsorption of water, as evidenced by the increase in diuresis in the first 4 h of the experiment, as well as by a delayed increase in glomerular filtration, which was confirmed by an increase in creatininuresis over 24 h.

At the same time, when analyzing the effect of dry extract No. 2 (70% ethanol) of *P. rubrinervis* Hort. Alb. buds it was found that in a 4-hour chronic experiment at a single intragastric injection of the dry extract at a dose of 10 mg/kg against the background of a 3% water load in the animals of the experimental group relative to the indicators of a water control, there was a significant increase in diuresis (by 20%) and a significant increase in creatininuresis (by 90%); at the same time, for 24 hours of the experiment there was a significant increase in diuresis (by 17%) and a significant increase in creatininuresis (by 86%).

Thus, dry extract No. 2 of *P. rubrinervis* Hort. Alb. buds (ethanol) at a dose of 10 mg/kg in the 4-h and daily experiments induced an accelerated and prolonged diuretic response, both by increasing a tubular reabsorption of water (an increase in the renal excretion of water) and by increasing a tubular filtration (an increase in the renal excretion of creatinine).

However, when studying the effect of dry extract No. 1 of *P. rubrinervis* Hort. Alb. buds (chloroform) on the excretory function of kidneys in 4 and 24-hour chronic experiments at a single intragastric injection at a dose of 10 mg/kg against a background of a 3% water load in the animals of the experimental group relative to the indicators of the water control, no significant differences were found.

Presumably, this is due to the fact that this extractant contributed to the release of associated lipophilic compounds from the medicinal plant raw materials, which did not have a proper stimulating effect on the tubular and tubule apparatus of the kidneys.

In its turn, the comparative drug furosemide at a threshold dose of 1 mg/kg in a 4-hour experiment against the background of a 3% water load significantly increased diuresis (by 93%) in the experimental group of animals relative to the water control (Table 2).

It follows that SS pinostrobin and dry extract No. 2 moderately stimulated the renal excretion of water, significantly inferior to the comparative drug furosemide (causing maximal diuresis). It is noteworthy that SS pinostrobin and dry extract No. 2 have the ability to stimulate the tubular filtration, in contrast to furosemide, which has an exclusively tubular mechanism of a diuretic action. Based on all of the above, these drugs are promising in terms of the development of drugs with nephroprotective properties.

CONCLUSION

The presence of a promising understudied representative of the genus Poplar (*Populus* L.) – *P. rubrinervis* Hort. Alb., favorably differing from pharmacopoeial species of poplars by a much larger phytomass of buds, has been revealed. The literature review has also made it possible to confirm the existing significant habitat of this representative in the Samara region, which indicates the prospect of harvesting this type of raw materials.

The extraction separation of the amount of *P. rubrinervis* Hort. Alb. buds substances by polarity has been conducted using technological methods – a circulation extraction and percolation.

The presence of pinostrobin as the main flavonoid of pharmacopoeial poplar species was proven chromatographically in the chloroform extract of *P. rubrinervis* Hort. Alb. buds and its absence in the alcoholic extract. The similarity of spectral absorption curves of lipophilic and hydrophilic amounts of phenolic compounds of *P. rubrinervis* Hort. Alb. buds extracts of various polarities has been revealed, i.e., the presence of a pronounced absorption maximum at 289 nm±2 nm and a “shoulder” at 320±2 nm.

At a single intragastric injection of the BAC pinostrobin at a dose of 1 mg/kg against the background a 3% water load in the animals of the experimental group against the indicators of the water control, an isolated increase in diuresis was observed for 4 hours of the experiment, at the same time, for 24 hours of the experiment there was an increase in creatininuresis.

Consequently, pinostrobin at a dose of 1 mg/kg caused an accelerated diuretic response, inferior to the comparative drug furosemide at a threshold dose of 1 mg/kg. The SS Pinostrobin at the indicated dose exhibited a delayed creatininuretic response, which compares it favorably with comparative drugs.

At the same time, at a single intragastric injection of

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dried extract of *P. rubrinervis* Hort. Alb. buds No. 2 on 70% ethanol at a dose of 10 mg/kg against the background of a 3% water load in the animals of the experimental group against the indicators of a water control, a moderate reliable increase in diuresis and a significant increase in creatininuresis for 4 and 24 hours of the experiment was observed due to an increase in glomerular filtration. The action of the study drug is inferior in the strength of diuresis to furosemide at a threshold dose of 1 mg/kg (4 h experience) and hypothiazide at an effective threshold dose of 20 mg/kg (a 24 h experience), but superior to comparative drugs in stimulation of the glomerular filtration, contributing to the increase in creatininuresis. When studying the effect of *P. rubrinervis* Hort. Alb. buds dry extract on chloroform No. 1 on the excretory function of the kidneys for 4 and 24 h, no differences between the experimental group and the control have been found, therefore, further studies of the dose-dependent effect of this preparation are required.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Elena A. Urbanchik – data collection, conducting an experiment, writing a text and compiling a list of references; Vladimir A. Kurkin – concept and design of the study, editing, final approval for the publication of the manuscript; Elena N. Zaitseva – conception and design of pharmacological experiments, participation in the description and analysis of the results obtained, statistical processing of measurement results, participation in manuscript writing and final approval for publication; Vitaly M. Ryzhov – collection of plant material for analysis, participation in the study; Alexey V. Dubishchev – critical study analysis; Anastasia S. Tsybina – participation in the research, literature analysis; Anastasia I. Altareva – participation in the research; Yulia D. Sirotkina – participation in the research.
All the authors confirm that their authorship meets the ICMJE international criteria (all the authors contributed substantially to the conceptualization, research and preparation of the article, read and approved of the final version before publication).

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RESEARCH ARTICLE

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Studying the possibilities of pharmacological correction of hypoxia-induced pulmonary hypertension using a phenolic compound with a laboratory cypher KUD975

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The aim of our work was to study a pharmacological activity of a selective arginase-2 and thrombin inhibitor from a phenolic compounds group with a laboratory cypher KUD975 on a model of arterial pulmonary hypertension induced by hypoxia.

Materials and methods. To simulate pulmonary hypertension (PH), animals were placed in a normobaric hypoxic chamber and subjected to 5 weeks of hypoxia with an oxygen content of 10% in the air. After 3 weeks of hypoxia, the animals were administered with the test compound KUD975 (intragastrically, at a dose of 2 mg/kg once a day for 2 weeks). L-norvaline (intragastrically, 20 mg/kg) was used as a reference drug. To assess the development and correction of PH, measurements of cardiohemodynamics, analysis of blood gas composition, study of the number of circulating endothelial precursor cells (EPCs), quantitative PCR assessing the expression of mRNA VEGF-R2, SGF-1 (stromal growth factor-1) and MCP-1 (monocyte chemoattractant protein-1). Next, a histological examination of the lungs and heart was performed, the degree of pulmonary edema and the concentration of cardiotrophin-1 and atrial natriuretic peptide were assessed.

Results. The administration of the studied phenolic compound with laboratory cypher KUD975, as well as the reference drug L-norvaline, led to a decrease in the right ventricular systolic pressure against the background of modeling PH. The present study shows a more than twice-fold decrease in the number of circulating (EPCs) in the animals group with modeling a hypoxia-induced circulatory PH (171.3±12.1) in comparison with the group of intact animals (296.1±31.7; p=0.0018). The recovery of EPCs was noted in the animals group administered with KUD-975 and L-norvaline, up to 247.5±34.2 (p=0.0009 compared with a pulmonary arterial hypertension (PAH) and 235.6±36.4 (p=0.008 compared to PAH), respectively. The studied compounds had a protective effect by statistically significantly increasing the expression of VEGF-R2 mRNA and decreasing the expression of SGF-1 mRNA, reducing the lung moisture coefficient and the concentrations of cardiotrophin-1 and atrial natriuretic peptide and preventing vascular remodeling caused by hypoxia.

Conclusion. When studying the pharmacological activity, it was shown that the phenolic compound with the laboratory cypher KUD975 normalizes hemodynamic parameters, reduces the signs of remodeling of the heart and pulmonary vessels and has a pronounced endothelial protective effect on the model of hypoxia-induced PH, and is superior to the activity of the reference drug L-norvaline.

Keywords: pulmonary hypertension; endothelial dysfunction; nitric oxide; heterocyclic acids; endothelium; arginase-2; thrombin

Abbreviations: PH – pulmonary hypertension; PAH – pulmonary arterial hypertension; CTPH – chronic thromboembolic pulmonary hypertension; COPD – chronic obstructive pulmonary disease; RVAP – right ventricular average pressure; RVSP – right ventricular systolic pressure; HR – heart rate; EPCs – endothelial precursor cells; SDF-1 – stroma-derived growth factor; VEGF – vascular endothelial growth factor; MCP-1 – monocyte chemoattractant protein-1; RV – right ventricle; LV – left ventricle; PaO_2 – oxygen partial pressure; $PaCO_2$ – carbon dioxide partial pressure; EC – endothelial cell; MP – interventricular septum; ED – endothelial dysfunction.

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Изучение возможностей фармакологической коррекции легочной гипертензии, индуцированной гипоксией, с использованием соединения фенольной природы с лабораторным шифром КУД975

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Цель. Изучение фармакологической активности селективного ингибитора аргиназы-2 и тромбина из группы соединений фенольной природы с лабораторным шифром КУД975 на модели артериальной легочной гипертензии, индуцированной гипоксией.

Материалы и методы. Для моделирования легочной гипертензии (ЛГ) животных помещали в нормобарическую гипоксическую камеру и подвергали 5 неделям гипоксии с содержанием кислорода в воздухе 10%. После 3-х недель гипоксии животным вводили исследуемое соединение КУД975 (внутрижелудочно в дозе 2 мг/кг 1 раз в сут в течение 2 недель). В качестве препарата сравнения использовали L-норвалин (внутрижелудочно 20 мг/кг). Для оценки развития ЛГ и ее коррекции проводили измерение показателей кардиогемодинамики, анализ газового состава крови, изучение количества циркулирующих предшественников эндотелиальных клеток (ПЭК), количественную ПЦР с оценкой экспрессии мРНК VEGF-R2, SDF-1 (стромальный фактор роста-1) и MCP-1 (моноцитарный хемоаттрактантный белок-1). Далее проводили гистологическое исследование легких и сердца, оценивали степень отека легких и концентрацию кардиотрофина-1 и предсердного натрийуретического пептида.

Результаты. Введение исследуемого соединения фенольной природы с лабораторным шифром КУД975, как и препарата сравнения L-норвалина, привело к уменьшению систолического давления в полости правого желудочка сердца на фоне моделирования ЛГ. В настоящем исследовании показано снижение количества циркулирующих ПЭК более чем в 2 раза в группе животных с моделированием циркуляторной легочной гипертензии, индуцированной ЛГ (171,3±12,1), в сравнении с группой интактных животных (296,1±31,7; p=0,0018). Восстановление ПЭК было отмечено в группе животных, получавших КУД975 и L-норвалин, до 247,5±34,2 (p=0,0009 в сравнении с легочной артериальной гипертензией (ЛАГ) и 235,6±36,4 (p=0,008 в сравнении с ЛАГ) соответственно. Исследуемые соединения оказывали протективное действие, статистически значимо повышая экспрессию мРНК VEGF-R2 и снижая экспрессию мРНК SDF-1, а также снижая коэффициент влажности легких и концентрации кардиотрофина-1 и предсердного натурийуретического пептида и предотвращая сосудистое ремоделирование, вызванное гипоксией.

Заключение. При изучении фармакологической активности показано, что соединение фенольной природы с лабораторным шифром КУД975 нормализует показатели гемодинамики, уменьшает признаки ремоделирования сердца и легочных сосудов и обладает выраженным эндотелиопротективным действием на модели ЛГ, индуцированной гипоксией, и превосходит активность препарата сравнения L-норвалин.

Ключевые слова: легочная гипертензия; эндотелиальная дисфункция; оксид азота; гетероциклические кислоты; эндотелий; аргиназа-2; тромбин

Список сокращений: ЛГ – легочная гипертензия; ЛАГ – легочная артериальная гипертензия; ХТЭЛГ – хроническая тромбоэмболическая легочная гипертензия; ХОБЛ – хроническая обструктивная болезнь легких; СДПЖ – систолическое давление в полости правого желудочка; СрДПЖ – среднее давление в полости правого желудочка; ДДПЖ – диастолическое давление в полости правого желудочка; ЧСС – частота сердечных сокращений; ПЭК – предшественники эндотелиальных клеток; SDF–1 – стромальный фактор роста-1; VEGF – фактор роста эндотелия сосудов; MCP–1 – моноцитарный хемоаттрактантный белок–1; ПЖ – правый желудочек; ЛЖ – левый желудочек; PaO_2 – парциальное давление кислорода; $PaCO_2$ – парциальное давление углекислого газа; ЭК – эндотелиальная клетка; МП – межжелудочковая перегородка; ЭД – эндотелиальная дисфункция.

INTRODUCTION

Pulmonary hypertension (PH) is a progressive and often fatal cardiopulmonary disease characterized by an increased pulmonary artery pressure, structural changes in the pulmonary circulation, and the development of vascular complications [1].

PH, in general, consists of a mixed group of disorders, all of which ultimately lead to an increased pulmonary arterial pressure (AP). PH is clinically classified by the 6th World Symposium on Pulmonary Hypertension (WSPH, 2018) as Group 1 PH depending on the underlying etiology. Other clinical subgroups include

Group 2 PH, which develops due to the underlying heart failure (reduced or preserved ejection fraction), a valvular heart disease, or congenital heart defects [2]. Group 3 PH occurs due to lung diseases or hypoxia. Group 4 PH develops due to the pulmonary artery obstruction, which also includes chronic thromboembolic PH (CTEPH) [3]. CTEPH is characterized by a chronic organization of thrombi in the pulmonary arterioles, followed by fibrosis and vascular stenosis [4]. Group 5 PH is a complex cohort, often due to the multifactorial etiology [3].

Pulmonary arterial hypertension (PAH) is a particularly challenging form of PH because it involves a progressive hyperproliferative process that, if untreated, leads to the right ventricular failure and death [5, 6]. The pathophysiology of PAH is complex and variable, with multiple molecular mechanisms and underlying disorders involved in pathogenesis. However, the most common pathological features, regardless of the initial etiological factor, are a dysfunction of pulmonary artery endothelial cells, proliferation and migration of pulmonary artery smooth muscle cells, and a fibroblast activity dysregulation [5, 7].

Hypoxia-induced PH is a potentially severe and fatal lung disease. It is known that chronic hypoxia leads to a pulmonary vascular remodeling, PH and the right ventricular (RV) hypertrophy with a subsequent risk of developing the right ventricular failure. Chronic lung diseases such as a chronic obstructive pulmonary disease (COPD), cystic fibrosis and bronchopulmonary dysplasia can lead to diffuse chronic alveolar hypoxia [8]. The development of PH is associated with a significant morbidity and mortality in these patients [9, 10].

Despite this, there are currently a few treatments for PH, and prevention strategies remain largely unknown.

THE AIM of our work was to study a pharmacological activity of a selective arginase-2 and thrombin inhibitor from a phenolic compounds group with a laboratory cypher KUD975 on a model of arterial pulmonary hypertension induced by hypoxia.

MATERIALS AND METHODS

Experimental animals

All experimental studies were carried out in accordance with the Rules of Good Laboratory Practice, approved by Order No. 708n of the Ministry of Health of Russia dated August 23, 2010, in strict compliance with the European Convention for the Protection of Vertebrate Animals Used for Experiments or Other Scientific Purposes (Directive 2010/63/EU). Experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (protocol No. 11/9 dated February 12, 2022). The vivisection was carried out in accordance with the ethical principles for the treatment of laboratory animals as set out in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (CETS No. 123).

C57Bl/6J mice (*n*=40) obtained from the Experimental Biological Clinic of Belgorod State National Research University were used as the main test system. After completing the 14-day quarantine regime, the mice were stratified by weight and placed in separate conventional cages in accordance with their belonging to the experimental group. Before and during the study, the animals were kept in rooms with artificial lighting (12 h day:12 h night) at the temperature of 21–23°C, the humidity of 38–50% and had a free access to food and water.

Study design

To simulate PH, the animals were placed in a normobaric hypoxic chamber (the authors' development) with the air gas composition control. The experimental mice were subjected to 3 weeks of hypoxia with 10% oxygen in the air. The normobaric hypoxic chamber was opened for 1 h once every 3 days to replace bedding, water bottles, and food. Wild-type (C57BL/6) mice of both sexes (10–12 weeks of age) were used, and the animals were evenly randomized by sex (females and males per group in a 50/50 ratio) and weight (weight per group in the range of 24±2 g). The animals of the control group were kept in a normobaric hypoxic chamber under the normoxia conditions. After 3 weeks of keeping the animals in a hypoxic chamber, they received various medicinal compounds for another 2 weeks under the same environmental conditions. Thus, the animals of the experimental groups were kept in a hypoxic chamber for 5 weeks [11].

Compounds under study

In this work, the pharmacological activity of a phenolic compound with laboratory code KUD975 2-((1-hydroxynaphthalene-2-yl)thio)acetyl)-*D*-proline methyl ester) was studied. The compound was synthesized by a group of scientists under the leadership of Doctor of Sciences (Chemistry) Konstantin V. Kudryavtsev (Pirogov Russian National Research Medical University). The structural formula of the compound under study shown in Figure 1.

The arginase inhibitor L-norvaline ($C_5H_{11}NO_2$, Clearsynth, India) was used as a reference drug.

KUD975 was administered intragastrically at a dose of 2 mg/kg once a day for 2 weeks. L-norvaline was used as a reference drug at a dose of 20 mg/kg intragastrically.

Thus, the following experimental groups were formed:

1. Control (1% starch solution intragastrically);
2. Hypoxia-induced PAH;

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3. PAH+KUD975 at a dose of 2 mg/kg for 14 days;

4. PAH+L-norvaline at a dose of 20 mg/kg for 14 days.

The calculation of dosage and dosing regimens of the studied compounds and reference drugs are based on their effectiveness in the experimental studies conducted previously in the field of pharmacological correction of the endothelium-associated pathology [12–15].

The doses were recalculated using interspecies coefficients, and the design of the experimental studies was carried out in accordance with the recommendations for preclinical studies[1,2].

Pressure measurement in right ventricle heart cavity, analysis of blood gas composition

A pressure measurement in the RV heart cavity and a gas composition of the venous blood were measured in mice under anesthesia (2–2.5% isoflurane in 100% oxygen) after 5 weeks from the start of the experiment. To do this, a small skin incision was made on the neck of the mice and the right external jugular vein was isolated, then catheterized with a polyethylene (PE 10) catheter. Next, the catheter was passed into the right ventricle (RV) of the heart cavity. In each animal, the BR in the pancreatic cavity was recorded continuously with a sampling frequency of 1 kHz for at least 30 sec using a piezoelectric pressure sensor and an MP-150 polygraph (BIOPAC Systems, Inc. USA). The correct anatomical position of the catheter tip was monitored by continuously monitoring the pressure signal waveform. A systolic pressure in the right ventricular cavity (RVSP), a mean pressure in the right ventricular cavity (mean RVSP), a diastolic pressure in the right ventricular cavity (RVDP), a heart rate (HR), dP/dt max, dP/dt min were determined. Hemodynamic parameters were determined using the Biopac MP-150 hardware complex (BIOPAC Systems, Inc. USA) and the AcqKnowledge 3.8.1 computer program (USA). After measuring hemodynamic parameters, the animal was removed from the experiment by the ethyl ether overdose, and the blood was drawn to analyze the gas composition (partial pressure of oxygen and carbon dioxide) [11].

Study of the number of circulating endothelial progenitor cells

To measure the level of circulating endothelial progenitor cells (EPCs), the cell culture and staining method described in the study by Pan Y et al, was used [16]. Mononuclear cells were isolated from peripheral blood by centrifugation (Eppendorf 5430R centrifuge, Germany) in a Histopaque-1083 solution (solution containing polysucrose and sodium diatrizoate adjusted to the density of 1.083 g/ml) according to the manufacturer's instructions (Sigma Chemical, USA). The isolated mononuclear cells were seeded in a triplicate on 96-well plates coated with 1% gelatin in the endothelial cell basal medium (Thermo Scientific, USA) supplemented with 2% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). After 2 days of culture, the adherent cells were thoroughly washed with the medium and co-stained with DiI AcLDL (Thermo Scientific, USA).

Quantitative PCR

To investigate the studied drugs effect on the molecular mechanisms of the PH development, a real-time polymerase chain reaction to determine the expression of mRNA VEGF-R2, SDF-1 (stromal growth factor-1) and MCP-1 (monocyte chemoattractant protein-1) was performed. To carry out a quantitative real-time PCR, a part of the lung was homogenized and incubated for 10 min at 37°C in the "Extract RNA" solution. After lysing the sample in the reagent, it was subjected to the chloroform purification, the supernatant sample was collected and washed with isopropyl alcohol and 70% ethyl alcohol. The concentration of the resulting RNA was measured on an IMPLEN NanoPhotometer® NP80 Spectrophotometer (IMPLEN, Germany) and adjusted to the concentration of 300 ng/µl by adding deionized water (CJSC Evrogen, Russia). A reverse transcription was performed using the MMLVRTSK021 kit in accordance with the protocol of the manufacturer (CJSC Evrogen, Russia). The gene expression level was assessed relative to the values of the reference gene Gapdh. The expression at a specific point was calculated using the formula [8]:

Gene Expression=[(Ct(Gapdh)/Ct (Gene of Interest)]

Methods of histological examination

For a histological examination, organs (the heart and lungs) were removed and fixed in 10% neutral formalin. Then the material was poured into paraffin in a standard mode in a carousel-type machine "STP-120" (Microm International GMbH, Germany). The examination of histological preparations was performed under an Axio Scope A1 microscope (Carl Zeiss Microimaging GMbH, Germany); morphometry was performed using the Image J 1.54d program.

The thickness of the pulmonary artery wall was determined, the pulmonary vessels near the alveoli were assessed, and the diameters of 20 vessels were determined on a slide. Five sections from each animal were evaluated. At the same time, the number of

[1] Guidelines to experimental (preclinical) study of new pharmacological substances. Khabrieva RU, editor. Scientific Center for Examination of Medical Products Applications; 2nd ed., revised. Moscow: Shiko Publishing House, 2005. 826 p. Russian

[2] Guidelines for conducting preclinical studies of drugs: in 2 parts. Scientific Center for Examination of Medical Products Applications. Mironov AN et al, editors. Moscow: "Grif and K", 2012. Part 1. 940 p. Russian

thrombosed vessels in the field of view was assessed (microscope magnification ×400). Approximately 20 peribronchial pulmonary artery vessels were assessed on each hematoxylin and eosin-stained slide (microscope magnification ×400). The degree of occlusion was determined as the ratio between the outer and inner (i.e., luminal) circumferences of each vessel. The degree of cardiac RV myocardial hypertrophy was determined using the image analysis Software MCID 7.0 Image Research. For this purpose, a horizontal section was made through the mouse heart at the level of the ventricles, the resulting sections were scanned using a drawing tool; the left and right ventricles were manually divided with a thin line in the same way for all sections. Then, a pixel-by-pixel analysis of the RV and LV areas with the interventricular septum (IVS) was carried out. The results are presented as the ratio of RV/(LV+IVS).

Determination of pulmonary edema degree

When removing the animals from the experiment, the lungs were sampled and divided into separate lobes. Then the weight of the lung lobe was determined before and after drying in a thermostat at 70°C for 72 h. The results were expressed as the ratio of the lungs weight before and after drying.

Biochemical markers

Cardiotrophin-1 (CT-1) and atrial natriuretic peptide (ANP) were measured in serum using ELISA kits (ELM-Cardiotrophin-1/EIA-ANP-1, RayBiotech, Norcross, USA) according to the manufacturer's instructions.

Statistical analysis

The data were tested for a normal distribution using the Shapiro–Wilk test. The normally distributed data were compared using an ordinary one-way analysis of variance (ANOVA) with Tukey post hoc test. The non-normally distributed data were compared with the Kruskal–Wallis test and Dunn's post hoc test. The differences were determined at a significance level of p <0,05. The experimental data are presented as M±SD values. The statistical analysis was performed using GraphPad Prism 9.2.0 Software.

RESULTS

Modeling PH in a hypoxic chamber led to a statistically significant increase in RVSP by almost twice, RVDP – by more than twice, a maximum contraction speed (dP/dt max) and a minimum contraction speed (dP/dt min) and did not lead to a statistically significant heart rate change (Table 1). The administration of the test compound with a laboratory code KUD975, as well as the reference drug L-norvaline, led to a statistically significant decrease in all studied parameters, and the indicator dP/dt max in the groups of the animals with the administration of the study drugs was as close to those values in the group of the intact animals as possible (Table 1).

To characterize the state of the vascular endothelium, the number of circulating EPCs which statistically significantly decreased in the animals with circulatory PH induced by hypoxia, was assessed (Fig. 1).

This study shows a more than 2-fold decrease in the number of circulating EPCs in the group of animals with modeling of circulatory PH induced by hypoxia (171.3±12.1) in comparison with the group of intact animals (296.1±31.7; p=0.0018). In the groups of animals that had been administered the test compounds, the number of circulating EPCs increased statistically significantly (Fig. 2).

When analyzing the results of the blood gas composition study, a similar picture was found – a statistically significant decrease in the partial oxygen pressure (PaO^2) against the background of a statistically significant increase in the partial carbon dioxide pressure ($PaCO^2$) in the group of animals with PH. The compounds KUD975 and L-norvaline statistically significantly (in comparison with the PAH group) and comparable restored the values of the blood gas composition in the animals of the experimental groups (Fig. 3).

It was established that the levels of VEGF-R2 mRNA expression in the lungs were statistically significantly reduced, and the levels of SDF-1 were statistically significantly increased in PAH. When using the compound KUD975 and L-norvaline, a statistically significant increase in the expression of VEGF-R2 mRNA and a decrease in the expression of SDF-1 mRNA were established.

Herewith, the degree of increase in the VEGF-R2 mRNA expression in the group of animals receiving KUD975 was statistically significantly higher than that in the group of the animals receiving L-norvaline. At the same time, the studied compounds did not affect the expression of MCP-1 mRNA when modeling hypoxia-induced PH (Fig. 4).

The degree of pulmonary edema in the experimental groups was assessed by the ratio of the weight of fluid and dry lungs. Simulating pulmonary hypertension with hypoxia increased the fluid-to-dry lung weight ratio by 33%. The degree of pulmonary edema was statistically significantly reduced with the use of the compound KUD975 and L-norvaline. The value of this indicator in the experimental groups was lower than in the group of animals with PH. The value of the lungs humidity coefficient in the group of animals using KUD975 was as close as possible to the target values set in the group of intact animals (Fig. 5).

When studying the concentration of cytokines

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CT-1 and ANP in the blood plasma, it was found out that the level of both factors increased statistically significantly when PH was modeled using hypoxia. Thus, in the group of PH animals without treatment (PAH), the concentrations of CT-1 and ANP increased by more than 5 times (Fig. 6). The use of the compounds KUD975 and L-norvaline led to a statistically significant decrease in the concentrations of CT-1 and ANP in the blood plasma.

A histological picture of the lungs in the group of animals with circulatory PH induced by hypoxia is presented in Figure 7.

When analyzing the thickness of the pulmonary artery (PA) wall, it was found out that against the background of modeling circulatory PH using hypoxia, the studied indicator increased more than twice from 0.742±0.049 to 1.728±0.24 μm.

The administration of KUD975 and L-norvaline led to a statistically significant (compared to the PAH group) decrease in the thickness of the PA wall. There were no statistically significant differences in the effectiveness of reducing this indicator between the groups receiving KUD975 and L-norvaline. When assessing the number of thrombosed vessels in the field of view, it was found out that this indicator in the lungs of animals receiving KUD975 and L-norvaline was statistically significantly lower than in the group of animals with PH without treatment (Fig. 7).

When assessing the effectiveness of correcting the morphological manifestations of PH in the heart, it was shown that KUD975 and L-norvaline in the studied doses had a pronounced pharmacological activity, statistically significantly reducing the cross-sectional area of cardiomyocytes. The reduction in RV hypertrophy was also confirmed by a histological examination, demonstrating a decrease in the hypoxia-induced increase in the RV/(LV+S) ratio with both KUD975 and L-norvaline treatment (Fig. 8).

DISCUSSION

It is now clear that the development of new arginase inhibitors represents a promising strategy for the treatment of diseases associated with the nitroxidergic system. Given the different expression of arginase-1 and arginase-2 in tissues and their different physiological actions, a large number of specific and selective inhibitors of these two isoforms of the enzyme are available today. For example, endothelial cells express both isoforms of arginases, but it is not known exactly what the role of each of these isoforms is in the development of endothelial dysfunction (ED). A considerable controversy remains regarding the role of arginase expression in various conditions such as atherosclerosis and other forms of vascular inflammation. For example, hyperglycemia in diabetes causes ED through the activation of p38 mitogen-activated protein kinase (MAPK). That causes Arg1 upregulation in coronary arteries and increased Arg2 expression in mesenteric arteries [17, 18].

The studies have shown that arginase blockade can prevent the reduction of angiogenesis by increasing the NO-induced VEGF expression, initiate a vascular repair in the experimental ischemic retinopathy (a NOS function normalization and a reduction of a superoxide production) [19], promote wound healing in mice, and prevent morphofunctional changes in the cardiovascular system against the background of preeclampsia [20].

Thus, there is a substantial evidence for the therapeutic potential of arginase inhibition against endothelium-associated pathology interrelated with a low bioavailability of NO. Therefore, this enzyme is very attractive from the point of view of research and development of new compounds – drug candidates for the treatment of endothelium-associated pathology. On the other hand, we know the effectiveness of a multidirectional approach to the pharmacological correction of ED, when two or more compounds, different in their mechanism of action and point of application, are used for therapeutic effects [21, 22].

The combination effect of one compound on 2 different targets, representing two different parts of the ED pathogenesis, seems promising for the development of new drug candidates. Thrombin was chosen as a second target, which, in addition to arginase-2, is interesting for the inhibition in the conditions accompanied by ED. The interaction of platelets with vessel walls plays an important role in acute cardiovascular diseases [23].

Thrombin is a powerful platelet activator, having a pronounced effect on the endothelium. Endothelial cells (ECs) have an antithrombotic activity by releasing nitric oxide (NO) and prostacyclin, which are potent vasodilators and inhibitors of the platelet activity. The blood clotting enzyme thrombin, produced on the surface of damaged endothelium, induces blood clotting and has many functional effects on the endothelium itself. Thrombin acts on ECs by stimulating the synthesis and release of various agents, such as inflammatory mediators, vasoactive substances and growth factors. It causes adhesion of leukocytes to the endothelium, triggering the expression of adhesion molecules on the cell surface, and causes a disruption of the endothelial permeability. It is known that the effect of thrombin on EC is mediated by its receptor. To date, different responses of EC to thrombin have been shown. In general, capillary endothelial cells appear to be particularly sensitive to this enzyme. Thrombin-induced ED in the microvasculature can have pathological consequences and contribute to a target organ damage in the endothelium-associated pathology [24, 25].

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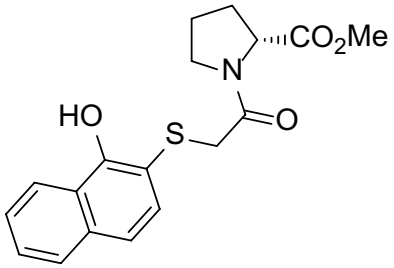

Figure 1 – Structural formula of compound under study with laboratory cypher KUD975 – 2-((1-hydroxynaphthalene-2-yl)thio)acetyl)-*D*-proline methyl ester

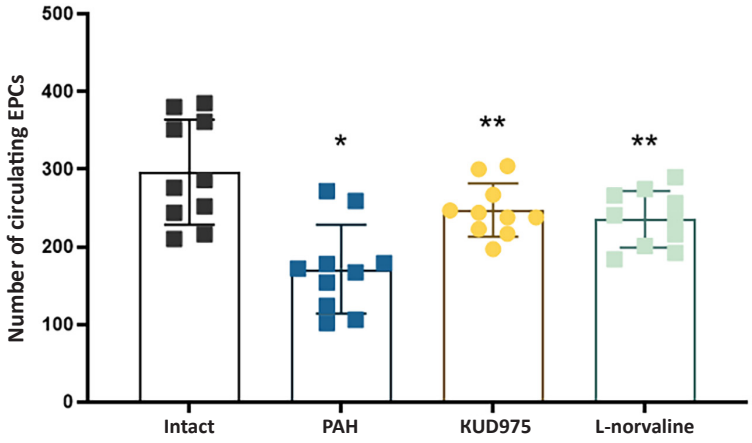

Figure 2 – Effect of compounds under study on the number of circulating endothelial cell precursors (EPCs) in animals' blood in experimental groups with pulmonary hypertension against the background of hypoxia

Note (here and in Fig. 3–6): intact – group of intact animals; PAH – hypoxia-induced pulmonary arterial hypertension; KUD975 – administration of the compound KUD975 at a dose of 2 mg/kg against the background of PH simulation; L-norvaline – administration of L-norvaline at a dose of 20 mg/kg against the background of PH modeling; * – p <0.05 compared to intact, ** – p <0.05 compared to PAH.

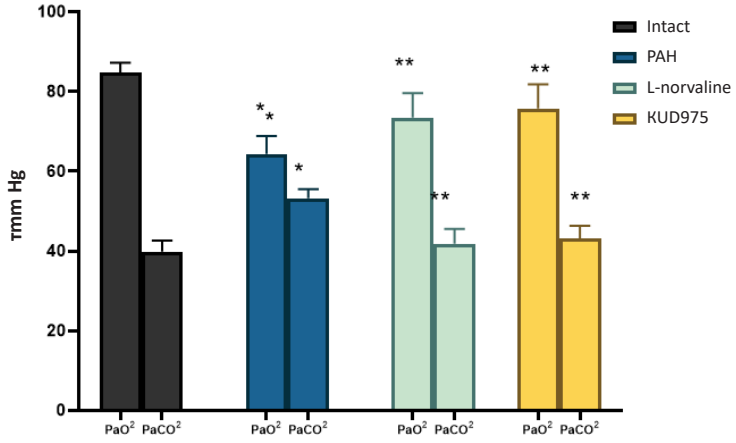

Figure 3 – Effect of KUD975 and L-norvaline on partial pressure of oxygen and carbon dioxide in experimental groups with pulmonary hypertension against the background of hypoxia

Note: PaO^2 – partial pressure of oxygen; $PaCO^2$ – partial pressure of carbon dioxide; PAH – hypoxia-induced pulmonary arterial hypertension.

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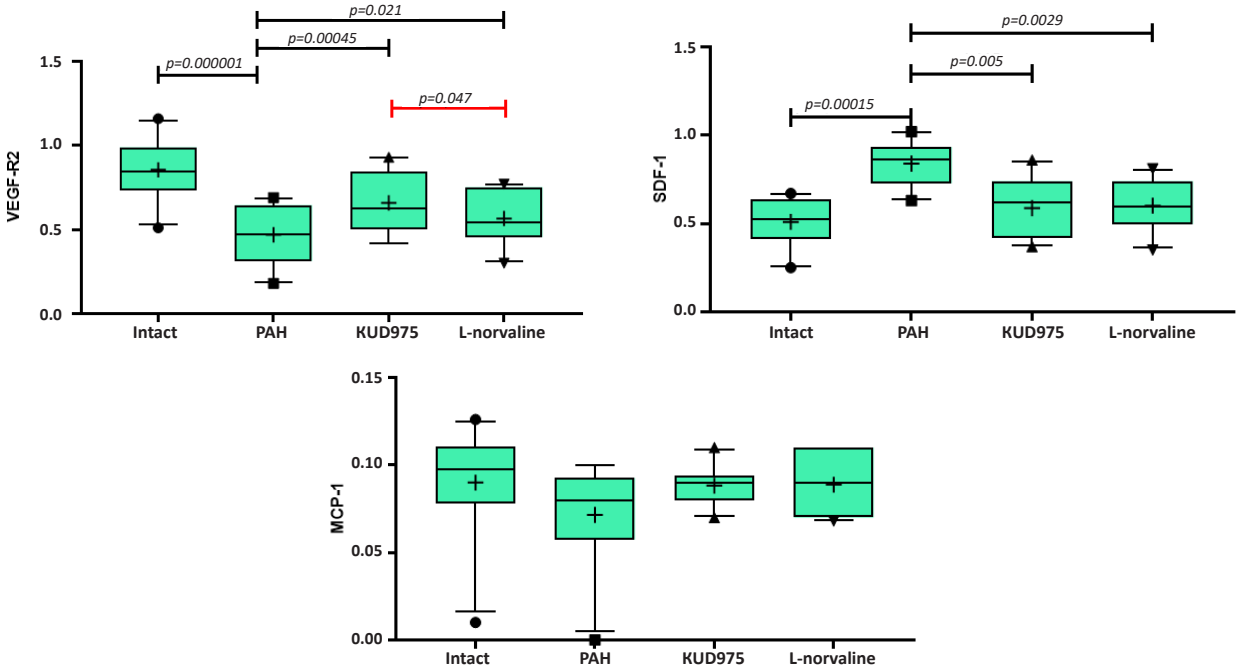

Figure 4 – Effect of KUD975 and L-norvaline on the expression of mRNA molecular targets for the development of pulmonary hypertension against the background of hypoxia

Note: VEGF-R2 – vascular endothelial growth factor receptor 2; SDF-1 – stromal cell factor 1; MCP-1 – monocyte chemoattractant protein-1.

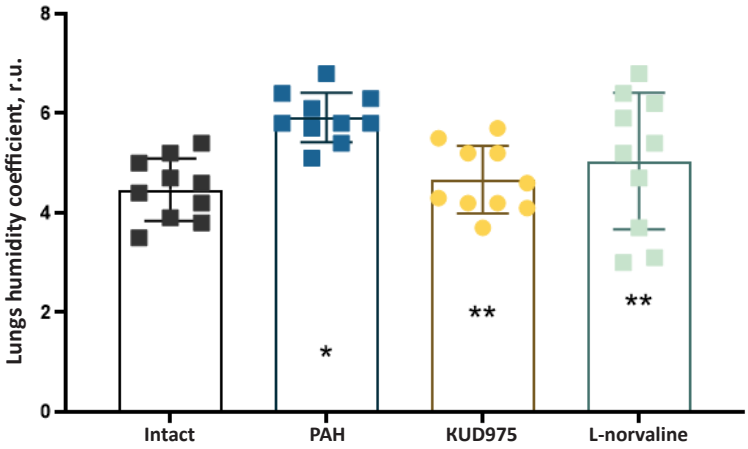

Figure 5 – Quantitative assessment of pulmonary edema formation by assessing ratio of humidity and dried lungs mass in groups of animals receiving KUD975 and L-norvaline against the background of modeling pulmonary hypertension with hypoxia

Note: * – p <0.05 compared to intact; ** – p <0.05 compared to PAH.

Table 1 – Indicators of cardiohemodynamics in animals' groups with pulmonary hypertension modeling and its correction with the help of studied compounds

Indicators	Control	PAH	PAH+KUD975	PAH+L-norvaline
RVSP	28.8±4.84	51.8±19.23*	32.5±7.51**	33.1±4.9**
RVDP	2.527±0.32	5.706±0.78*	3.918±0.5**	4.019±0.59**
dP/dt max	104.5±17.2	68.8±15.5*	88.4±10.9**	87.6±11.2**
dP/dt min	99.4±11.5	74.9±9.25*	86±7.53**	82.5±7.81**
HR	365.6±19.4	336.8±24.37	344.2±31.6	350.7±26.5

Note: PAH is a group of animals with hypoxia-induced pulmonary hypertension; RVSP – systolic pressure in the cavity of the right ventricle of the heart; RVDP – diastolic pressure in the cavity of the right ventricle of the heart; HR – heart rate; dP/dt max – maximum rate of increase in intraventricular pressure; dP/dt min – minimum rate of increase in intraventricular pressure. * – p <0.05 compared with the group of intact animals; ** – p <0.05 compared with the group of animals with experimental pulmonary arterial hypertension without treatment (PAH group).

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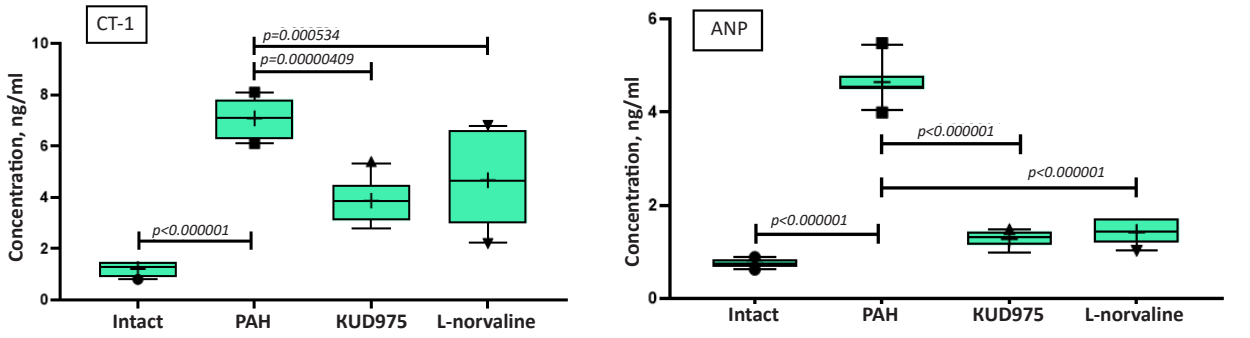

Figure 6 – Effect of KUD975 and L-norvaline on plasma concentrations of cytokines cardiotrophin-1 and atrial natriuretic peptide

Note: CT-1 – cardiotrophin-1; ANP – atrial natriuretic peptide.

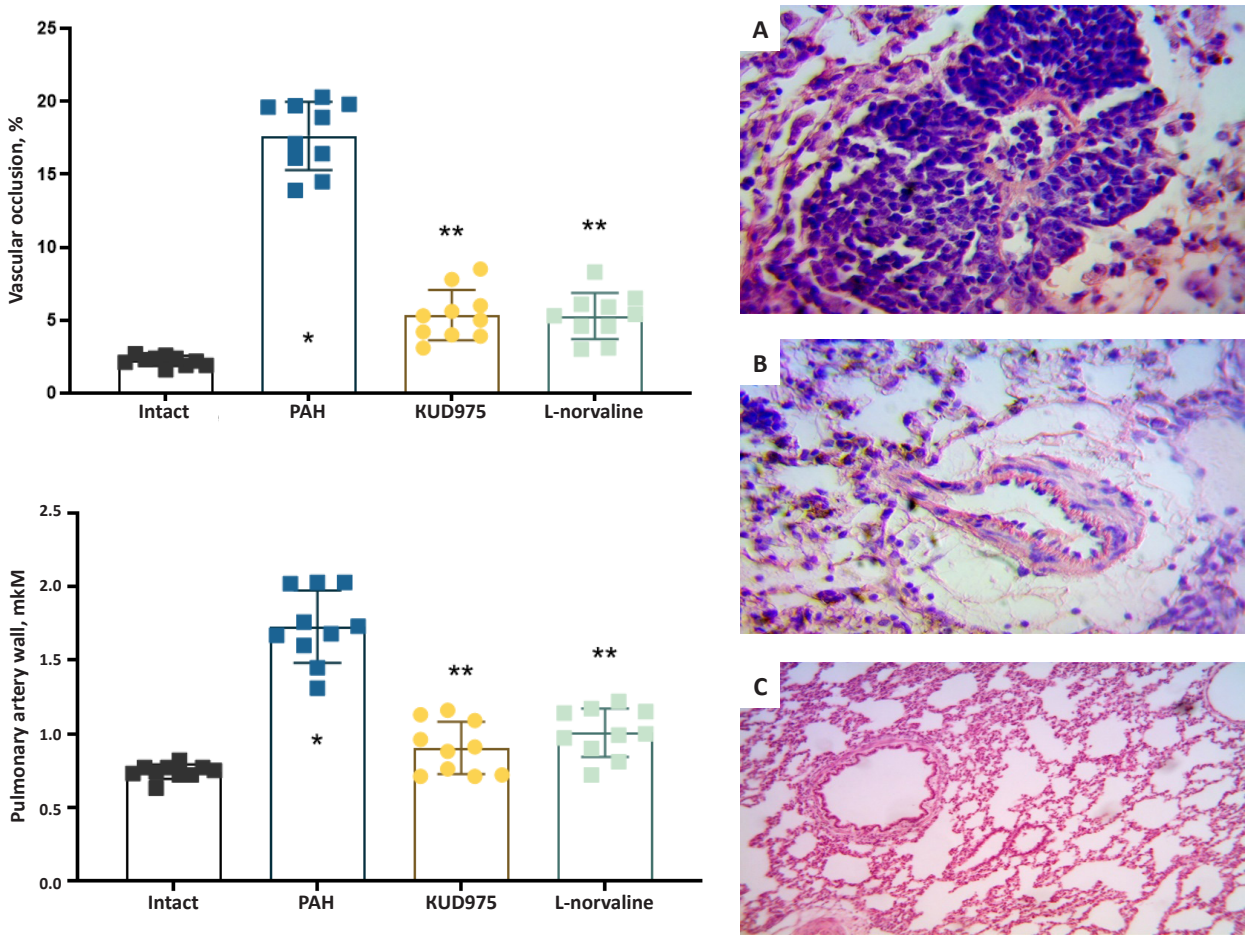

Figure 7 – Effect of KUD975 and L-norvaline on histological lungs structure when modeling circulatory pulmonary hypertension with hypoxia

Note: PA wall thickness – pulmonary artery wall thickness; A – micrograph of pulmonary artery wall (×400); B – lung microphotograph, perivascular fibrosis, stained with hematoxylin+eosin (×400); * – p <0.05 compared to intact; ** – p <0.05 compared to PAH.

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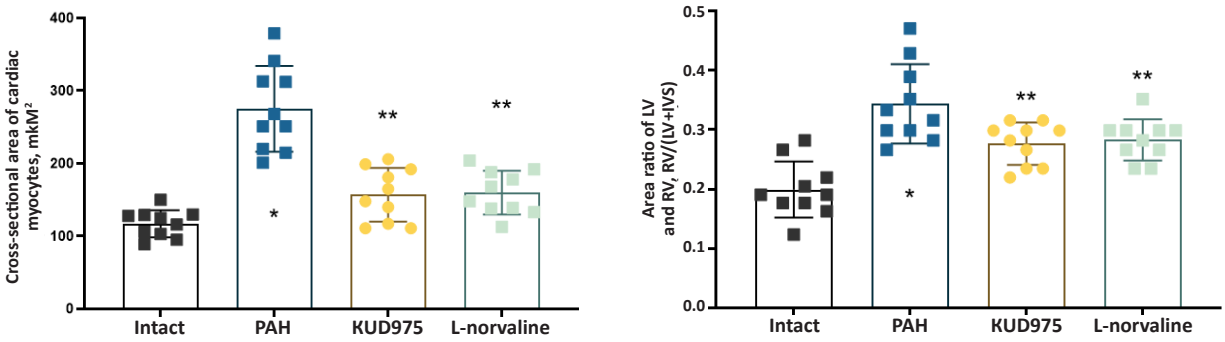

Figure 8 – Effect of KUD975 and L-norvaline on histological structure of right ventricle of animals' hearts in experimental groups

In this study, the concept of privileged structures was used to search for the compounds of a phenolic nature that have an endothelial protective effect. To conduct a virtual search for targets, several online services available on a non-commercial basis, were used. The physicochemical properties of the studied low molecular weight organic compounds were calculated using the following computational algorithms: OpenBabel, Molinspiration online service, online service of the "virtual laboratory of computational chemistry" VCCL. The phenolic compound with the laboratory code KUD975 investigated in this study is an inhibitor of arginase-2 and thrombin – the participation of these enzymes in the pathogenesis of the vascular endothelium dysfunction is currently beyond doubt. The inhibition of arginase-2, first of all, makes it possible to switch parts of the L-arginine-eNOS-NO metabolic pathway to the effective generation of NO and normalization of endothelium-dependent reactions in response to acetylcholine and vascular homeostasis in general [26, 27]. At the same time, the inhibition of thrombin leads to a slowdown in the release of thromboxane A2 by platelets, causing a powerful vasoconstriction, which is prevented by the simultaneous thrombin-induced release of prostacyclin and NO from ECs. Therefore, the inhibition of the thrombin production is an effective therapeutic strategy to correct a thrombin-induced activation of platelet-vascular wall interactions in ED [24].

It was previously shown that phenolic compounds prevent morphological changes in the cardiovascular system when modeling preeclampsia [28]. In the present study, it was shown that in a model of circulatory PH caused by hypoxia, the administration of the lead compound under study with the laboratory cypher KUD975, as well as the reference drug L-norvaline, led to a statistically significant decrease in RVSP and speed parameters of cardiohemodynamics. Against the background of PH modeling, the number of circulating EPCs in the experimental groups were studied. More and more studies demonstrate that circulating EPCs are involved in vascular homeostasis [29]. This study shows a more than 2-fold decrease in the number of circulating EPCs in the groups of animals with modeling circulatory PH and a statistically significant increase in the number of EPCs in the groups of animals that had been administered with the test compounds, which indicates the endothelial protective effect of KUD975.

To study the effect of the investigated compounds on the factors involved in the delivery of circulating EPCs to the endothelium of the affected vessels, the mRNA expression of factors necessary for the delivery of EPCs to the affected vascular walls, were studied: a vascular endothelial growth factor (VEGF), the first subtype of its receptors (VEGF-R1) and stromal cell factor-1 (SDF-1). As inflammatory processes are involved in the pathophysiology of PAH, the levels of monocyte chemoattractant protein-1 (MCP-1), a major marker of inflammation in inflammatory processes associated with PAH, were also measured [30]. It was found out that the levels of VEGF-R2 mRNA expression in the lungs were statistically significantly reduced, and the levels of SDF-1 were statistically significantly increased in PAH. When using the compounds KUD975 and L-norvaline in the model of hypoxia-induced PH, a statistically significant increase in the expression of VEGF-R2 mRNA and a decrease in the expression of SDF-1 mRNA were found out. Moreover, the degree of increase in VEGF-R2 mRNA expression in the group of animals receiving KUD975 was statistically significantly higher than that in the group of animals receiving L-norvaline.

To further assess the state of the cardiovascular system against the background of PH modeling, the content of CT-1 and ANP in the blood plasma was measured. The first cytokine is associated with myocardial hypertrophy and cardiovascular pathology, and the second is a hormone secreted by the atria in response to a high BP – its effect is to reduce preload on the heart, thereby lowering BP [31]. The decrease in the concentrations of CT-1 and ANP under the influence of the studied compounds indicates a decrease in the manifestations of vascular remodeling caused by PH and is consistent with the data obtained from the histological examination.

The development of PAH and its correction by the studied compounds was confirmed by histological studies. Thus, in the animals with a PH, a progressive pulmonary vascular remodeling, including a significant increase in wall thickness, occlusion and muscularization of intraacinar vessels, as well as an increase in wall thickness and wall / lumen ratio of preacinar pulmonary vessels compared with controls was observed. In the animals' hearts with PH, RV hypertrophy, including an increase in the cross-sectional area of cardiomyocytes and the ratio of the RV and LV areas of the heart was found out. The administration of KUD975 and L-norvaline made it possible to reduce signs of pulmonary vascular remodeling by reducing the thickness of the PA wall and the occlusion degree of intraacinar pulmonary vessels compared to the animals with PH induced and hypoxia.

CONCLUSION

Thus, when studying the pharmacological activity, it was shown that a compound of the phenolic nature with the laboratory cypher KUD975 normalizes hemodynamic parameters, reduces the signs of remodeling of the heart and pulmonary vessels and has a pronounced endothelial protective effect on the model of PH induced by hypoxia, and surpasses the activity of the reference drug L-norvaline in terms of the effectiveness of increasing the number of circulating EPCs, increasing the expression of VEGF-R2 mRNA and reducing the concentration of CT-1.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHOR'S CONTRIBUTION

The author confirms that her authorship meets the international criteria of the ICMJE.
Liliya V. Korokina – development of research design, planning and implementation of the experimental part of the study, evaluation and interpretation of results, literature analysis, preparation of graphic material, writing the text of the article.

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RESEARCH ARTICLE

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Comparative analysis of physicochemical properties, bioequivalence, safety and tolerability of the first domestic semaglutide

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Semaglutide is a representative of analogues of the incretin hormone human glucagon-like peptide-1 (GLP-1) and is currently used in Russia for the treatment of type 2 diabetes mellitus (T2DM; in monotherapy and in combination therapy), including patients with obesity and overweight.

The aim of the work was to conduct a comparative assessment of the physicochemical properties, a biological activity, bioequivalence and safety, including tolerability and immunogenicity, of the drug Quincent® (semaglutide, 1.34 mg/ml, a solution for a subcutaneous administration, Promomed Rus LLC, Russia) and the drug Ozempic® (semaglutide, 1.34 mg/ml, a solution for a subcutaneous administration, Novo Nordisk A/S, Denmark) when administered to healthy volunteers.

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Materials and methods. To assess the degree of similarity of the study drug Quincenta® (semaglutide, 1.34 mg/ml, a solution for a subcutaneous administration, Promomed Rus LLC, Russia) with a chemically synthesized active substance to the original (reference) drug Ozempic® (semaglutide, 1.34 mg/ml, a solution for a subcutaneous administration, Novo Nordisk A/S, Denmark), a comparative study of physicochemical properties and a biological activity was carried out. To assess the bioequivalence of the study drug and the reference drug, an open randomized parallel comparative study with the participation of healthy volunteers (*n*=54), 54 participants of which had been included in the population, was conducted. The volunteers were randomized into 2 groups in a 1:1 ratio, and received a single dose subcutaneously either of the study drug (domestic semaglutide at a dose of 0.5 mg) or the reference drug (foreign semaglutide at a dose of 0.5 mg). The mode of administration was in the morning on an empty stomach. A semaglutide concentration was determined in serum samples using a previously validated enzyme-linked immunosorbent assay (ELISA) method. A quantitative determination of antibodies to semaglutide in the human serum by ELISA was carried out with a microplate photometer using ready-made kits pre-validated by the manufacturer. The conclusion about the bioequivalence of the compared drugs was made using an approach based on the assessment of 90% confidence intervals for the ratios of the geometric mean values of the parameters C_{max}, $AUC_{(0-t)}$ of semaglutide in the measurement original units.

Results. The results of the comparative analysis of the study drug and the reference drug demonstrate the comparability of their physicochemical properties and biological activity. The results of the clinical study demonstrated the bioequivalence of the test drug and the reference drug. Thus, the pharmacokinetic parameters of the drugs were comparable to each other: the C_{max} value for the study drug was 42.088±8.827 ng/ml, for the reference drug Ozempic® it was 42.2556±7.84. Herewith, the half-life for the study drug and the reference drug was 168.39±39.47 and 157.99±28.57 hours, respectively. The resulting 90% confidence intervals for the ratio of the C_{max} and AUC_{0-t} values of the study drug and the reference drug were 90.89–109.15 and 91.66–111.27%, respectively. The tolerability of the drugs in the volunteers was notified as good. No adverse events were recorded during the study. No serious adverse events were reported throughout the study. According to the results of the immunogenicity analysis, no antibodies to Russian-made semaglutide were detected in the blood serum of the volunteers, which indicated the lack of Results. The results of a comparative analysis of the study drug and the reference drug demonstrate the comparability of physicochemical properties and biological activity. The results of the clinical study demonstrated the bioequivalence of the study drug and the reference drug. Thus, the pharmacokinetic parameters of the drugs were comparable to each other: the C_{max} value for the study drug was 42.088±8.827 ng/ml, for the reference drug Ozempic® this figure was 42.2556±7.84. At the same time, the half-life for the study drug and the reference drug was 168.39±39.47 and 157.99±28.57 hours, respectively. The resulting 90% confidence intervals for the ratio of the C_{max} and AUC_{0-t} values of the study drug and the reference drug were 90.89–109.15 and 91.66–111.27%, respectively. Tolerability of the drugs in volunteers was noted as good. No adverse events were recorded during the study. No serious adverse events were reported throughout the study. According to the results of the immunogenicity analysis, no antibodies to Russian-made semaglutide were detected in the blood serum of the volunteers, which indicated the lack of the drug immunogenicity.

Conclusion. In the course of the study, the comparability of the physicochemical properties and biological activity of the studied Russian drug with the chemically synthesized active substance Quincenta® to the reference drug Ozempic® was confirmed: the activity range of the studied drugs was within 80–120% in relation to the standard sample of semaglutide. The bioequivalence and a similar safety profile, including the immunogenicity and tolerability of the Russian drug Quincenta® (semaglutide 1.34 mg/ml, Promomed Rus LLC, Russia) were shown in comparison with the foreign drug Ozempic® (semaglutide 1.34 mg/ml, Novo Nordisk A/C, Denmark).

Keywords: glucagon-like peptide-1; GLP-1; bioequivalence; pharmacokinetics; semaglutide; type 2 diabetes mellitus; physicochemical properties; safety profile; biological activity

Abbreviations: T2DM – Type 2 diabetes mellitus; CVDs – cardiovascular diseases; GK – glycemic control; ASCVDs – atherosclerotic cardiovascular diseases; HbAlc – glycated hemoglobin; GLP-1 – glucagon-like peptide-1; CHF – chronic heart failure; iNGLT-2 – inhibitors of sodium-glucose cotransporter-2; CKD – chronic kidney disease; BMI – body mass index; ARVI – acute respiratory viral infection; AP – arterial pressure; HR – heart rate; RR – respiratory rate; ECG – electrocardiography; AE – adverse event; SAE – serious adverse event; CI – confidence interval.

Сравнительный анализ физико-химических свойств, биоэквивалентности, безопасности и переносимости отечественного семаглутида

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Семаглутид является представителем аналогов инкретинового гормона человеческого глюкагоноподобного пептида-1 (ГПП-1) и в настоящее время в России используется для лечения сахарного диабета 2 типа (СД 2; в монотерапии и в комбинированной терапии), в том числе, у пациентов с ожирением и избыточной массой тела.

Цель. Провести сравнительную оценку физико-химических свойств, биологической активности, биоэквивалентности и безопасности, включая переносимость и иммуногенность, лекарственного препарата Квинсента® (семаглутид, 1,34 мг/мл, раствор для подкожного введения, ООО «Промомед», Россия) и препарата Оземпик® (семаглутид, 1,34 мг/мл, раствор для подкожного введения, Ново Нордиск А/С, Дания) при введении здоровыми добровольцами.

Материалы и методы. Для оценки степени подобия исследуемого препарата Квинсента® (семаглутид, 1,34 мг/мл, раствор для подкожного введения, ООО «Промомед», Россия) с химически синтезированным активным веществом оригинальному (референтному) препарату Оземпик® (семаглутид, 1,34 мг/мл, раствор для подкожного введения, Ново Нордиск А/С, Дания) было проведено сравнительное изучение физико-химических свойств и биологической активности. Для оценки биоэквивалентности исследуемого и референтного препарата было проведено открытое рандомизированное параллельное сравнительное исследование с участием здоровых добровольцев (*n*=54), из них в популяцию для оценки биоэквивалентности вошли 54 участника. Добровольцы были рандомизированы в 2 группы в соотношении 1:1 и получали однократно подкожно утром натощак, либо исследуемый препарат (отечественный семаглутид в дозе 0,5 мг), либо референтный препарат (зарубежный семаглутид в дозе 0,5 мг). Концентрацию семаглутида определяли в образцах сыворотки крови с помощью предварительно валидированного метода иммуноферментного анализа (ИФА). Количественное определение антител к семаглутиду в сыворотке крови человека методом ИФА было проведено с помощью фотометра для микропланшетов с использованием готовых предварительно валидированных производителем наборов. Вывод о биоэквивалентности сравниваемых препаратов делали с использованием подхода, основанного на оценке 90% доверительных интервалов для отношений средних геометрических значений параметров C_{max}, $AUC_{(0-t)}$ семаглутида в исходных единицах измерения.

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Результаты. Результаты сравнительного анализа исследуемого и референтного препарата демонстрируют сопоставимость физико-химических свойств и биологической активности. Результаты клинического исследования продемонстрировали биоэквивалентность исследуемого препарата и препарата сравнения. Так, фармакокинетические параметры препаратов были сопоставимы между собой: величина C_{max} для исследуемого препарата составила 42,088±8,827 нг/мл, для препарата сравнения Оземпик® данный показатель составил 42,2556±7,84. При этом период полувыведения для исследуемого препарата и препарата сравнения составил 168,39±39,47 и 157,99±28,57 ч, соответственно. Полученные 90%-ные доверительные интервалы для отношения значений C_{max} и AUC_{0-t} исследуемого препарата и референтного препарата составили 90,89–109,15 и 91,66–111,27%, соответственно. Переносимость препаратов у добровольцев была отмечена как хорошая. При проведении исследования не было зафиксировано нежелательных явлений. В течение всего исследования не было зарегистрировано ни одного серьёзного нежелательного явления. По результатам анализа иммуногенности у добровольцев не были выявлены антитела к семаглутиду российского производства в сыворотке крови, что свидетельствовало об отсутствии иммуногенности препарата.

Заключение. В ходе проведенного исследования была подтверждена сопоставимость физико-химических свойств и биологической активности исследуемого российского препарата с химически синтезированным активным веществом Квинсента® препарату сравнения Оземпик®: диапазон активности исследуемых препаратов находился в пределах 80–120% по отношению к стандартному образцу семаглутида. Показана биоэквивалентность и сходный профиль безопасности, включая иммуногенность и переносимость российского препарата Квинсента® (семаглутид 1,34 мг/мл, ООО «Промомед», Россия) в сравнении с зарубежным препаратом Оземпик® (семаглутид 1,34 мг/мл, Ново Нордиск А/С, Дания).

Ключевые слова: глюкагоноподобный пептид-1; ГПП-1; биоэквивалентность; фармакокинетика; семаглутид; сахарный диабет 2-го типа, физико-химические свойства, профиль безопасности, биологическая активность

Список сокращений: СД 2 – сахарный диабет 2 типа; ССЗ – сердечно-сосудистые заболевания; ГК – гликемический контроль; АССЗ – атеросклеротические сердечно-сосудистые заболевания; HbAlc – гликированный гемоглобин; ГПП-1 – глюкагоноподобный пептид-1; ХСН – хроническая сердечная недостаточность; иНГЛТ-2 – ингибиторы натрий-глюкозного котранспортера-2; ХБП – хроническая болезнь почек; ИМТ – индекс массы тела; ОРВИ – острая респираторная вирусная инфекция; АД – артериальное давление; ЧСС – частота сердечных сокращений; ЧДД – частота дыхательных движений; ЭКГ – электрокардиография; НЯ – нежелательное явление; СНЯ – серьёзное нежелательное явление; ДИ – доверительный интервал.

INTRODUCTION

Given the increasing prevalence of type 2 diabetes mellitus (T2DM) and its associated complications[1] [1], the need to develop highly effective treatment strategies for this serious disease has never been greater. The focus of treatment for type 2 diabetes has traditionally been a glycemic control[2], but in recent years, the standards of care have emphasized the importance of a multifactorial approach that includes the correction of cardiovascular disease (CVD) risk factors such as hyperglycemia, as well as overweight/obesity, hypertension, and dyslipidemia[3] [1, 2].

T2DM people are known to have a 2- to 4-fold higher risk of developing CVDs than people without diabetes, and cardiovascular events are more likely to occur at an earlier age. CVDs are the main cause of death in T2DM[4] patients [3, 4]. It has been proven that influencing cardiovascular risk factors as a part of the T2DM treatment can reduce mortality from the disease itself and its complications. Thus, the Steno-2 study [5] showed that a comprehensive control of diabetes risk factors can reduce the incidence of cardiovascular events by more than 50%.

But although a harder glycemic control (GC) has been shown to be effective against microvascular complications [6] and recent observational studies [7, 8] have demonstrated that increased HbA1c levels are associated with a greater risk of cardiovascular events, the precise role of the GC in reducing CVDs risk remains to be detected [9].

In the treatment of T2DM patients, it is necessary to personalize the choice of glucose-lowering therapy, taking into account the individual characteristics of the patient (especially indications of a high risk of atherosclerotic CVDs (ASCVDs) or existing ASCVDs, a chronic heart failure (CHF), chronic kidney diseases (CKD), obesity, high-risk hypoglycemia) and a dominant clinical problem[5]. Thus, in patients with T2DM and existing cardiovascular diseases (CVDs), the preference should be given to glucose-lowering drugs[6] with a proven cardiovascular safety[7] [1, 2, 4, 10].

[1] International Diabetes Federation. IDF Diabetes Atlas (2021). Available from: https https://www.diabetesatlas.org/data/en/region/3/eur.html
[2] Clinical guidelines Type 2 diabetes mellitus in adults, 2022. Available from: https://cr.minzdrav.gov.ru/schema/290_2
[3] Ibid.
[4] International Diabetes Federation. IDF Diabetes Atlas, 2021.
[5] Ibid.
[6] Ibid.
[7] Ibid.

The key element regulating an insulin production in the body is glucagon-like peptide-1 (GLP-1). Glucagon-like peptide-1 receptor (GLP-1) agonists act similarly to the incretin hormone GLP-1 and mediate their effects through its receptors, which are expressed in the pancreas, gastrointestinal tract, heart, lungs, kidneys and brain. GLP-1 receptors in the pancreas and brain have been shown to be responsible for the corresponding improvements in GC and weight loss. Functional effects in the pancreas include a glucose-dependent insulin release as well as an up-regulation of insulin biosynthesis and glucokinase and glucose transporters. The impact on GLP-1 receptors also causes a glucose-dependent decrease in the glucagon secretion, which, in turn, reduces a hepatic glucose output. In the pancreas, GLP-1 receptors are predominantly localized to insulin-producing beta cells, with a markedly weaker expression on the acinar cells of the exocrine pancreas. Most of GLP-1 is produced in the gastrointestinal tract. In the brain, GLP-1 is produced in neurons and is likely a neuropeptide with physiologically and pharmacologically significant effects on the food intake and body weight, a potential neuromodulatory role, and possible effects in a number of other neuropathological conditions, including neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease), brain injuries and strokes, as well as depression, anxiety and addiction [11].

The main advantage of a number of GLP-1 drugs, including semaglutide, is the ability to reduce the risks of cardiovascular events and improve renal outcomes in T2DM patients [12].

The first drug of this kind from the arGLP-1 class was exenatide, which had been approved for use by the U.S. Food and Drug Administration (FDA). Since 2005, it has been successfully used in clinical practice in the USA, European countries, and since 2007 – in Russia. Currently, six arGLP-1 drugs, each with a unique drug delivery strategy, have been approved by the FDA, and several more are being developed. Considering a rapid elimination as the main problem for the clinical use of arGLP-1, researchers have successfully developed and implemented various strategies to increase the half-life of these drugs, including a sequential modification and an increase in the duration of their action [13].

However, in general, arGLP-1, including semaglutide, is the ability to reduce overall mortality and the risk of major cardiovascular events (Major Adverse Cardiovascular Event, MACE), such as non-fatal heart attack, non-fatal stroke or death from CVD and improve renal outcomes in patients with T2DM [14].

Semaglutide is one of the latest drugs from the arGLP-1 group approved both in Russia[8] and in many foreign countries. This drug, semaglutide, was developed established on a large body of research based on liraglutide [15].

The semaglutide molecule has a 94% homology with human GLP-1 and 3 main structural modifications compared to human GLP-1: 1) a substitution of an amino acid at position C_8 (alanine to α-aminoisobutyric acid), which prevents the peptide destruction by the dipeptidyl peptidase-4 (DPP-4) enzyme; 2) acylation of lysine in the main part of the peptide and the attachment of a C_{18} fatty acid at position C_{26} to ensure strong and specific binding to albumin; 3) the substitution of an amino acid at position C_{34} (lysine to arginine) – this prevents the addition of a C_{18}-dibasic fatty acid at the wrong site of the semaglutide molecule [16].

In preclinical and pharmacokinetic studies in T2DM adults, it was shown that, compared with liraglutide, which is administered once a day, semaglutide has an even longer half-life (from 7 days), which allows its use once a week [16–19]. The effect of semaglutide did not change in patients with an impaired renal or hepatic function, with the exception of a terminal renal and hepatic failure [20, 21]. Semaglutide is excreted mainly in urine (approximately 3% unchanged) and also in feces [22].

At the doses of 0.5 and 1.0 mg, semaglutide was approved for use in type 2 diabetes in the USA in 2017[9] and by the European Medicines Agency in 2018[10].

Semaglutide is registered in the Russian Federation and is currently included in the clinical recommendations of the Ministry of Health of the Russian Federation[11] for the treatment of T2DM patients, as well as in the Algorithms for a specialized care for T2DM patients [2]. According to these documents, semaglutide is recommended for use in T2DM patients with an indication of a high risk of ASCVDs, as well as with an already established diagnosis of ASCVDs as priority

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[11] Clinical guidelines Type 2 diabetes mellitus in adults, 2022.

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therapy, as well as in patients with CKD in stages 1–4 for nephroprotection in the presence of contraindications or intolerance to sodium-glucose cotransporter inhibitors-2 (iNGLT-2) in the presence of a concomitant diagnosis of obesity, which determines its relevance for Russian patients. Herewith, semaglutide was presented on the Russian pharmaceutical market only in the form of a foreign drug, which is currently unavailable. In this regard, it seems relevant to develop and localize the production of a full cycle from the substance to the finished dosage form and the subsequent study of the bioequivalence of the domestic analogue of semaglutide to ensure the country's medicinal independence and increase an access for our fellow citizens to a modern high-quality drug.

According to the FDA guidelines[12], alpha-amino acid polymers, such as glucagon, semaglutide, etc., containing up to 40 amino acid residues, are considered not protein molecules, but peptides. According to the FDA, to confirm the equivalence of a synthetic peptide and a biotechnologically derived semaglutide contained in a precursor drug, it is sufficient to prove the structural identity of the active pharmaceutical substance (APS) using modern analytical methods.

The Promomed Rus LLC company has developed its own technology for the production of API using methods of chemical synthesis and isolation of semaglutide into a finished dosage form for the treatment of type 2 diabetes. The Quincenta® (solution for subcutaneous administration, 0.25/0.5 mg/dose, 1 mg/dose) has passed the entire cycle of necessary studies and registered in the Russian Federation (LP-008828 dated 17 October 2023). The full production cycle from the substance to the finished dosage form on the territory of the Russian Federation allows, on the one hand, to ensure maximum control over the quality of the product, and on the other side to guarantee the uninterrupted supply of the population with a vital drug.

To further assess the quality and safety of the developed drugs, their registration in the Russian Federation in accordance with Russian regulatory requirements, in addition to physicochemical methods of analysis and preclinical studies, it is necessary to conduct a study of the pharmacokinetics, safety and immunogenicity of the drug Quicenta® (semaglutide 1.34 mg/ml, Promomed Rus LLC, Russia) in comparison with the foreign predecessor drug Ozempic® (semaglutide 1.34 mg/ml, Novo Nordisk A/S, Denmark).

THE AIM of the work was to conduct a comparative assessment of the physicochemical properties, a biological activity, bioequivalence and safety, including tolerability and immunogenicity, of the drug Quincenta® (semaglutide, 1.34 mg/ml, a solution for a subcutaneous administration, Promomed Rus LLC, Russia) and the drug Ozempic® (semaglutide, 1.34 mg/ml, a solution for a subcutaneous administration, Novo Nordisk A/S, Denmark) when administered to healthy volunteers.

MATERIALS AND METHODS

Physicochemical properties and biological activity

In order to assess the degree of comparability (similarity) of the test drug with the chemically synthesized active substance Quicenta® (semaglutide 1.34 mg/ml, Promomed RUS LLC, Russia) to the original (reference) drug Ozempic® (semaglutide 1.34 mg/ml, Novo Nordisk A/S, Denmark), a comparative study of their physicochemical properties and biological activity was carried out. In order to form a representative quality profile and obtain reliable data on comparability in accordance with Decision No. 89 "On approval of the Rules for conducting research on biological medicinal products of the Eurasian Economic Union", three series (samples) of the original (reference) drug and three series (samples) of its synthetic analogue (test drug) were used in the research.

Spectrophotometry in the ultraviolet light (200–400 nm)

The absorption spectra in the ultraviolet region for domestic / test and foreign / reference drugs were obtained using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Japan) in the spectral wave range of 190–1100 nm. To carry out the analysis, working solutions of each of the drugs (3 series) were prepared by diluting with water for the injection to a concentration of semaglutide in a solution of 0.025 mg/ml.

Mass spectrophotometry (MALDI-TOF MS)

The confirmation of the semaglutide peptide presence in the domestic drug under study was carried out using a mass spectrophotometry. The mass spectra were recorded on an Axima Confidence time-

[12] U.S. Food and Drug Administration. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin Guidance for Industry, 2021. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/andas-certain-highly-purified-synthetic-peptide-drug-products-refer-listed-drugs-rdna-origin

of-flight spectrometer (Shimadzu Biotech, Japan) in a high-resolution reflectron mode with a nitrogen laser (λ=337 nm). M/z scanning was carried out in the range from 500 to 5000 Da, using 2.5-dihydroxybenzoic acid (DHB) and sinapic acid (SA) as the matrix.

To prepare the target, the drug was adjusted to the concentration of 1 mg/ml with deionized water, then 20 µl of the resulting solution was mixed with 20 µl of the matrix solution (10 mg/ml in 50% acetonitrile/0.1% aqueous trifluoroacetic acid). The resulting mixtures were applied to a stainless-steel target and dried in air.

Relative molar ratio of amino acids

The relative molecular ratio of amino acids in the drugs was determined by HPLC. The analysis was carried out on a high-pressure liquid chromatograph equipped with an Agilent 1260 Infinity LC UV detector (Agilent Technologies, USA), using a stainless steel AccQ Tag Amino Acid Analysis Column (150×3.9 mm, 4 µm) at the wavelength of 254 nm.

Solutions of the drugs were hydrolyzed by adding a 6 M solution of hydrochloric acid containing 0.1% phenol and 1% thioglycolic acid. The hydrolysis process was carried out at the temperature of 110°C for 24 h. The derivatization of hydrolysates of the test solutions and a solution of standard amino acid samples was carried out using a derivatizing reagent by heating the solutions to 55°C for 10 min.

Size exclusion-high-performance liquid chromatography

The size exclusion chromatography method was used to determine the quantitative content of high molecular weight impurities in the preparations. The analysis was carried out on a Tosoh TSK-gel G 2000 SWXL column (7.8×300 mm, 5 µm) using an Agilent 1260 Infinity LC liquid chromatograph with UV detection (Agilent Technologies, USA).

Reversed-phase high-performance liquid chromatography

The reverse phase chromatography method was used to determine the quantitative content of semaglutide and related impurities in the test and reference drugs. The analysis was carried out using a liquid chromatograph with UV detection Prominence (Shimadzu, Japan), using a Jupiter 4u Proteo 90A column (4.6×250 mm, 4 µm; Phenomenex, USA).

Comparative studies of drugs biological activity *in vitro*

A comparative biological activity of the test and reference drugs was assessed *in vitro* on the CHO-K1/GLP-1R cell culture (GenScript, USA). This cell line has GLP-1 receptors, to which the active substance, semaglutide, binds. The cultivation of the cell line was carried out using the RPMI culture medium (PanEco, Russia) with the addition of a penicillin / streptomycin solution (1%) and fetal bovine serum (10%), under standard conditions (the temperature – 37±1°C, the CO_2 content – 5±1%), for 2 days. The results were assessed using the cAMP-GloTM Assay kit (Promega, USA) in accordance with the instructions.

Bioequivalence and comparability of safety, tolerability profile and immunogenicity

An open randomized parallel comparative study of the domestic drug Quincenta® (semaglutide 1.34 mg/ml, Promomed Rus LLC, Russia), produced on the basis of synthetic semaglutide (hereinafter referred to as the test drug), and the foreign drug Ozempic® (semaglutide 1, 34 mg/ml, Novo Nordisk A/S, Denmark), which contains a molecule obtained by biotechnological means (hereinafter referred to as the reference drug), was conducted. The test and reference drugs were comparable in composition.

Study design

This research was a single-center, open-label, randomized, parallel bioequivalence study of a single subcutaneous fasting dose of the test / reference drugs in healthy volunteers.

A flowchart of the study design is presented in Fig. 1.

Objects of study and eligibility criteria

A total of 54 healthy volunteers, male and female, aged 18 to 45 years (34.20±6.25 years) were included in the study. All participants signed an informed consent form and expressed their ability and willingness to comply with all requirements of the Study Protocol.

The main inclusion criteria were: a body weight > 50 kg; BMI 18.5–26 kg/m^2 inclusive; a verified diagnosis "healthy" according to standard clinical, laboratory and instrumental examination methods; negative results of tests for the use of alcohol, psychotropic and narcotic substances and willingness to stop drinking alcohol during the participation in the study. The participants had

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been warned to use reliable methods of contraception and to abstain from a sperm donation throughout the study and for 2 months after the end of the study.

The main criteria for non-inclusion included were: the presence of known allergies, drug intolerance, chronic diseases of various organ systems; mental illness; hypersensitivity to study drugs; history of semaglutide or other analogues of human GLP-1 use (for less than 6 months before screening), taking medications that have a pronounced effect on hemodynamics and/or a liver function for less than 2 months before screening; taking other medications, including herbal and homeopathic medications, vitamins and/or dietary supplements (biologically active supplements), for less than 4 weeks before screening; inability to perform subcutaneous injections; any history of difficulty with blood collection or any vasovagal seizures during blood collection; history of surgical interventions on the gastrointestinal tract (except appendectomy). The participants were not considered for inclusion in the study if they had the following diseases and conditions either: a history of medullary thyroid cancer, including a family history; a history of type 2 multiple endocrine neoplasia; a severe depression; suicidal thoughts or behavior, including a history; acute infectious diseases or ARVI symptoms for less than 4 weeks before screening.

The volunteers were excluded from the study if they had refused to participate in the clinical trial, if they were taking drugs for prohibited therapy and if they had been tested positive for the use of alcohol, psychotropic and/or narcotic substances, if there were gross violations of the requirements and procedures of the Study Protocol, if adverse events occurred, as well as if during the study, the volunteer had any diseases or conditions that made his further participation in the study impossible. The study physician may have decided to exclude a volunteer in the best interests of the volunteer.

Concomitant medications and exclusion criteria were assessed throughout the volunteer's participation in the study. The total duration of the study for each volunteer was no more than 35 days (including the screening period).

Randomization procedure

Each volunteer who had met all the inclusion criteria and had not met any of the non-inclusion criteria was assigned a randomization number in accordance with the randomization plan prepared for this study in the WinPepi 11.65 program (ETCETERA 3.26 module) using the random number generation method. The randomization number of the volunteer was entered by the research physician into the Register of Clinical Study Participants in Screening/Randomization. If a volunteer had left the study prematurely, their randomization number was not reused and the volunteer could not subsequently return to the study.

Study conditions and duration

The study was conducted from July 10 to October 2, 2023, at the research center of the Yaroslavl Region Clinical Hospital No. 3 (Yaroslavl, Russia).

Description of manipulations and methodology

Administration of drugs

The volunteers who had met the inclusion criteria and those who had not met the non-inclusion criteria were randomized into 2 groups in a 1:1 ratio. Group I (*n*=27) received Russian semaglutide (studied drug), group II (*n*=27) received reference drug. The reference / test drug was administered by the medical personnel in the morning on an empty stomach at a single subcutaneous dose of 0.5 mg in the abdomen.

The choice of doses for this study was based on the information provided in the current instructions for medical use of the reference drug and the articles devoted to the study of semaglutide preparations. In contrast to the starting dose of 0.25 mg, the dose of 0.5 mg was minimally therapeutic. The selection of this dose was based on the safety of use in healthy volunteers, since when using semaglutide in a dose of more than 0.5 mg, there was a risk of developing side effects from the gastrointestinal tract. In addition, there was experience with the use of semaglutide at a dose of 0.5 mg in healthy volunteers, which showed its good tolerability[13] [23–26]. The mode of administration was consistent with the method of use of the reference drug[14] and the planned method of use of the test drug in clinical practice[15].

To administer the test / reference drugs, the volunteers were admitted to the hospital the

[13] NCT02060266 Trial Investigating the Absorption, Metabolism and Excretion After a Single Subcutaneous Dose of [3H]-Semaglutide in Healthy Male Subjects. Available from: https://clinicaltrials.gov/study/NCT02060266

[14] State register of medicines of the Russian Federation. Ozempic®. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=9859f6af-8ad6-4704-9d20-1bcc87b7dafc

[15] Ibid.

evening before and at least 10 hours before the drug administration. During the period of their stay in the hospital, the volunteers complied with the rules of their stay. The duration of hospitalization was no more than 3 days. Throughout the study, from the start of the screening examination until the completion of the final examination, the volunteers abstained from eating for at least 10 h before the administration of the test / reference drug.

Preparation and sampling

After randomization and before the baseline blood samples had been collected to assess pharmacokinetic and immunogenicity parameters, the volunteers were placed with a heparinized cubital catheter, which was removed after blood sampling at 12 h (day 1). After the catheter removal, the blood was collected from volunteers by venipuncture.

The blood samples were taken to determine pharmacokinetic parameters at the following time points: 1, 0.5, 0 h (day 1) before the administration of the test / reference drug and then after 2, 8, 12 (day 1), 24 (day 2) , 36 (day 2), 48 (day 3), 72 (day 4), 96 (day 5), 144 (day 7), 192 (day 9), 240 (day 11), 360 (day 16) and 480 h (day 21) after the administration of the test / reference drug.

The blood samples were taken to study immunogenicity no more than 15 min before the administration of the test / reference drug (the initial (0) sample) and 480 h (day 21) after their administration. The blood samples for the immunogenicity parameters analysis were collected separately from the blood samples for the evaluation of pharmacokinetic parameters.

Therefore, the study collected 16 blood samples per volunteer (6 ml each) for pharmacokinetic studies and 2 blood samples per volunteer (6 ml each) for immunogenicity studies.

At screening, at the stage of hospitalization (morning before randomization), upon discharge from the hospital and on Days 11 and 21 of the study, the blood samples were taken for clinical, biochemical tests and/or determination of blood glucose levels using a glucometer, the total volume of which in each specified per day was no more than 15 ml.

The blood samples were collected in test tubes to obtain serum with a coagulation activator. After the clot formation, the tubes were centrifuged, the resulting serum was carefully transferred into pre-labeled cryovials, dividing the serum into three 500 µl aliquots: two for the main analysis (aliquots A and B), the third for repeat analyzes (aliquot C). The serum samples were frozen immediately after the receipt, transferred into cryovials and stored at the temperature not exceeding –70°C.

Analytical method

Pharmacokinetics was assessed by the concentration of semaglutide in the blood plasma, and antibodies to it – in the blood serum of each volunteer after a subcutaneous administration of the test / reference drug. In order to comprehensively characterize the pharmacokinetic properties of the test / reference drug, the study included blood sampling to cover the entire pharmacokinetic profile, including the elimination phase. The quantitative determination of semaglutide in the serum samples by ELISA was carried out using a microplate photometer, HiPo MPP-96 (Biosan, Latvia). Semaglutide concentrations were calculated using GraphPad Prism 8.4.3 software. The determination of semaglutide in the serum samples was carried out using a previously validated enzyme-linked immunosorbent assay (ELISA) method using commercially available “KRIBIOLISA™ Semaglutide (Ozempic™) ELISA kits”; antibodies to semaglutide using the KRIBIOLISATM Anti-Liraglutide ELISA kit. The analytical range was 50–4000 pg/ml. The preparation of calibration samples from the kit was carried out by diluting the standard sample. The analytical range was selected in accordance with the instructions for the kit.

Safety and tolerability assessment

The conclusion about the bioequivalence of the compared drugs was made using an approach based on the assessment of 90% confidence intervals (CI) for the ratios of the geometric mean values of the parameters C_{max}, $AUC_{(0–t)}$ of semaglutide in the original units of measurement. The drugs were considered bioequivalent if the boundaries of the estimated CI for C_{max}, $AUC_{(0–t)}$ of semaglutide were within the range of 80.00–125.00%.

In the course of the study, a clinical observation of volunteers was carried out with the assessment of physical examination data, including a survey about the volunteers’ complaints, basic vital signs (blood pressure, heart rate, respiratory rate, body temperature), 12-lead ECG, laboratory parameters of clinical, biochemical blood tests, a general urine analysis, determining glucose levels using a glucometer.

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The safety and tolerability criteria included the frequency and severity of adverse events (AEs) as measured by abnormal laboratory tests, physical examination, vital signs, and ECG; the number of cases of early termination of participation in the study due to the development of AEs and/or serious adverse events (SAEs), including those related to the test / reference drug; the frequency of volunteers with detected antibodies to semaglutide; the assessment of the overall tolerability of the test / reference drug on a Likert scale. The safety and tolerability of liraglutide were assessed for all the volunteers. The identification of AEs occurred from the moment of administration of the study drugs until the end of the volunteers' participation in the study.

The pharmacokinetic parameters[16] were as follows: a maximum concentration of the substance in the blood serum (C_{max}); the time to reach C_{max} (T_{max}); area under the concentration-time curve from the moment of drug administration to the last detectable concentration at time point t ($AUC_{(0–t)}$); the area under the pharmacokinetic curve from the zero time to infinity ($AUC_{(0–\infty)}$); the ratio of the area under the concentration-time curve from the moment of the drug administration to the last detectable concentration at the time point to the area under the pharmacokinetic curve, starting from the zero time value to infinity ($AUC_{(0–t)}$ / $AUC_{(0–\infty)}$); the terminal elimination rate constant (K_{el}); half-life ($T_{1/2}$); the volume of distribution (Vd); the residual (extrapolated) area under the curve, determined by the formula: $V_d = AUC_{(0–\infty)} – AUC_{(0–t)}$ / $AUC_{(0–\infty)}$ ($AUC_{(t–\infty)}$).

Ethical approval

The study complied with the ethical principles set forth in the most recent revision of the Declaration of Helsinki, the rules of Good Clinical Practice of the Eurasian Economic Union, the Rules of Good Clinical Practice of the International Council for Harmonization (ICH E6 GCP R2), as well as other legislation applicable to this study. The clinical trial protocol was approved by the Ministry of Health of Russia (Extract from Protocol No. 347 dated July 5, 2023) and the Ethics Council of the Ministry of Health of Russia (Extract from Protocol No. 337 of the meeting dated June 27, 2023), as well as the Local Independent Ethics Committee at the research center of the state budgetary healthcare institution Yaroslavl region "Clinical Hospital No. 3" (Extract from Protocol No. 186 dated July 7, 2023).

[16] If necessary, additional pharmacokinetic calculations could be performed.

Statistical analysis

To calculate the number of participants, the data on the coefficients of intra-individual variability (CV_{inter}) of the parameters C_{max}, AUC_{0-t} of semaglutide, presented in the Assessment Report Ozempic[17], were used. According to the results of the study, after a single subcutaneous administration to the healthy volunteers, CV_{inter} semaglutide for the main pharmacokinetic parameters C_{max} and AUC did not exceed 24%[18]. The calculation of the required number was carried out using the PASS 11.4.12 program.

For the standard two-parallel group design conditiosn, assuming a 90% CI of 80.00–125.00%, CV_{inter}=24%, α=0.05, the power of 80%, the group ratio of 0.95, it was necessary to include at least 50 healthy volunteers who would have completed the study and would be included in the statistical analysis. Taking into account the possible dropout during the study, the randomization of 54 healthy volunteers (27 volunteers in each study group) was planned.

For pharmacokinetic calculations, the actual time of blood sampling was used. The calculation of pharmacokinetic parameters, statistical analysis of safety indicators and presentation of results were carried out using statistical packages (StatSoft Statistica version 13.3 and the R Project program (version 3.5.1, GPL-2/GPL-3 license) with the *bear* extension, version 2.8.3-2. No interim analysis was performed.

For all pharmacokinetic parameters, the following statistical parameters were calculated: arithmetic mean, geometric mean, standard deviation of the mean, coefficient of variation, median, minimum and maximum values, and variability.

A statistical analysis was carried out based on the assumption of a log-normal distribution of $AUC_{(0–t)}$, $AUC_{(0–\infty)}$, C_{max} and a normal distribution of other pharmacokinetic parameters, with the exception of T_{max}. After the log transformation, these scores were analyzed using the analysis of variance (ANOVA). The statistical analysis of the study was carried out at a standard significance level of α=0.05.

[17] Assessment report Ozempic EMA/21773/2022. 11 November 2021. Committee for Medicinal Products for Human Use (CHMP). Available from: https://www.ema.europa.eu/en/documents/variation-report/ozempic-h-c-004174-x-0021-epar-assessment-report-variation_en.pdf

[18] Assessment report Ozempic, semaglutide, Procedure No. EMEA/H/C/004174/0000, 14 December 2017, Committee for Medicinal Products for Human Use (CHMP). Available from: https://www.ema.europa.eu/en/documents/assessment-report/ozempic-epar-public-assessment-report_en.pdf

For the randomized parallel groups comparative study, the ANOVA statistical model included the following factor contributing to the observed variation in the data: a drug. The analysis of variance was used to test a hypothesis about the statistical significance of the contribution of the specified factor to the observed variability.

The conclusion about the bioequivalence of the compared drugs was made using an approach based on the assessment of 90% confidence intervals for the ratios of the geometric mean values of the semaglutide parameters C_{max}, $AUC_{(0-t)}$ in the original units of measurement.

The descriptive statistics is presented for all safety and tolerability indicators collected during the study. To analyze frequencies, the proportions were compared using a two-sided version of the Fisher's exact test or the χ^2 test. To compare quantitative continuous indicators, the Student's *t*-test (in the case of a normal distribution) or the Mann-Whitney *U*-test (in the case of a non-normal distribution) were used. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Physicochemical properties and biological activity.

Spectrophotometry in the ultraviolet area (200–400 nm)

The results of the spectrophotometric determination of the test / reference drugs are presented in Table 1 and Fig. 1.

Based on the data obtained, it can be concluded that the absorption spectra of the foreign reference drug Ozempic® and the domestic test drug Quincenta® corresponded to each other in terms of the positions of absorption maxima and minima in the region of 200–400 nm, which, in turn, indicates the identity of the studied drugs.

Mass spectrophotometry (MALDI-TOF MS)

The results of the MALDI-TOF MS analysis confirm the presence of kDa[19], in the study domestic peptide drug with a mass of 4.1, corresponding to the mass of the reference drug semaglutide (Fig. 3).

Relative molar ratio of amino acids

In the course of the study, comparable molar ratios of amino acids were obtained in the samples of the study drugs Ozempic® and Quincenta® (Table 2).

[19] Assessment report Ozempic EMA/21773/2022, 2021.

Size exclusion-high-performance liquid chromatography

The results obtained in the study demonstrated the comparable retention time of high molecular weight protein impurities. Moreover, at the time of the analysis, the domestic drug of semaglutide contained on average 1.6 times less high-molecular impurities than the foreign drug.

Typical chromatograms obtained when determining the content of high-molecular impurities in the test and reference preparations are presented in Fig. 4 and 5. The content analysis results of the of high-molecular impurities are presented in Fig. 6.

Reversed-phase high-performance liquid chromatography

The results of determining the quantitative content of semaglutide and related impurities in the test and the reference drugs are presented in Table 3.

The data obtained demonstrate that the quantitative content of related impurities and the active substance semaglutide in the samples of the studied drugs are comparable. It should be also noted that at the time of the analysis, the content of impurities in the domestic drug was 2.5 times lower compared to the foreign drug. At the same time, the foreign drug contained on average 5.6 times more hydrophilic impurities, and almost twice as many hydrophobic impurities in comparison with the domestic drug.

Comparative studies of biological activity

The obtained data from a comparative analysis of the biological activity of the test and reference drugs *in vitro* are presented in Table 4.

The results demonstrate the presence of a comparable biological activity of the study domestic drug with a chemically synthesized active substance, to a foreign drug: the range of activity of the studied drugs was in the range of 80–120% in relation to the standard sample of semaglutide.

Bioequivalence and comparability of safety and tolerability profile

Population

All volunteers were included in the population to assess safety, tolerability and immunogenicity, for the pharmacokinetic analysis and bioequivalence assessment. The average age of the volunteers in the population was 34.20±6.25 years, the average body weight was 70.41±9.42 kg, the average height was 172.61±7.38 cm, the average BMI was 23.51±1.

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39 kg/m^2. The demographic and baseline anthropometric characteristics of the volunteers did not differ between the groups (Tables 5 and 6).

Pharmacokinetics and bioequivalence

After the use of the Russian and reference drugs, their average values of the main and additional pharmacokinetic parameters are presented in Table 7.

As follows from the presented data, the average values of both main and additional pharmacokinetic parameters obtained after the use of the test and reference drugs were comparable to each other. It can be concluded that the pharmacokinetic profiles of the test and reference drugs are similar.

The results of assessing the ratio of geometric mean pharmacokinetic parameters AUC_{0-t}, C_{max} of the studied semaglutide drugs and 90% CIs for these ratios demonstrate the equivalence of the main pharmacokinetic parameters (AUC_{0-t}, C_{max}) (Table 8). According to the results of the statistical analysis, the obtained 90% CI for the ratio of C_{max}, AUC_{0-t} values of the studied Russian and foreign drugs were 90.89–109.15 and 91.66–111.27%, respectively. The coefficients of the intra-individual variation, calculated on the basis of the analysis of variance, were 20.29% for the C_{max} value and 21.50% for the AUC_{0-t} value.

Thus, the intervals obtained during the study were fully consistent with the equivalence limit of 80.00–125.00% for C_{max} and AUC_{0-t}, clearly demonstrating the bioequivalence of the study drug.

Safety

All the volunteers completed the study entirely in accordance with the approved study protocol. During the study, no AEs were recorded. In 100% (54) cases, the volunteers' tolerability was rated as "good." No SAEs were identified in the volunteers during the study or after its completion. No deaths were observed. There were no cases of pregnancy of the sexual partner of a study participant during the study or after its completion. No abnormalities were found in the results of clinical and biochemical blood tests, in the determination of blood glucose levels, general urinalysis, vital signs, physical examination and ECG.

Immunogenicity assessment

According to the results of the immunogenicity parameters analysis, no antibodies to semaglutide were detected in the blood serum of the volunteers, which indicated the absence of the drug's immunogenicity. No unexpected results were notified during the study.

Thus, the study drug semaglutide and the reference drug had a similar safety profile. At the same time, no cases of immunogenicity were observed for the Russian drug, which confirms a high safety profile and reduced the risk of ineffective therapy.

DISCUSSION

The efficacy and safety of semaglutide compared with placebo or active reference drugs (sitagliptin, exenatide, insulin glargine, dulaglutide, liraglutide) in adult patients with T2DM were consistently studied in a series of phase III clinical trials combined by a large SUSTAIN research program, which included a total of more than 10 thousand patients [27–33]. Semaglutide therapy was significantly better than placebo and comparator and reduced HbA1c by 1.2–1.5% when using a dose of 0.5 mg and by 1.5–1.8% when using a dose of 1.0 mg, compared with baseline values, and resulted in achieving target values of HbA1c <7.0% in 78.7% of patients, and HbA1c <6.5% in 66.7% of patients [27–32]. It is worth noting that 74.3% of the subjects receiving semaglutide achieved the composite endpoint of HbA1c <7.0% without severe or symptomatic hypoglycemia or weight gain. In contrast, 65.7% of patients receiving semaglutide achieved weight loss of 5% or more, and 26.7% – 10% or more, which was significantly better compared to the placebo group and the comparison group [27, 31, 32].

A retrospective observational study also showed that at a dose of 0.5–1.0 mg after 32 weeks of treatment, semaglutide reduced HbA1c by an average of 1.38%, weight by 6.03 kg, and significantly improved BP and lipid levels, reduced the number of glucose-lowering and lipid-lowering drugs taken, leading to better a patient satisfaction with the results of diabetes control and eating behavior [34].

Additionally, semaglutide, compared with lixisenatide, exenatide, liraglutide, albiglutide, and dulaglutide, demonstrated the highest rates of BG improvement and weight loss [35]. A similar benefit was also shown in two meta-analyses comparing the results of semaglutide studies with the study results of sodium-glucose cotransporter-2 inhibitors (SGLT-2) empagliflozin, canagliflozin and dapagliflozin in people with inadequate control of type 2 diabetes with one or two oral antidiabetic agents [36] or metformin monotherapy [37] found out that semaglutide was superior to iSGLT in reducing HbA1c levels and weight loss.

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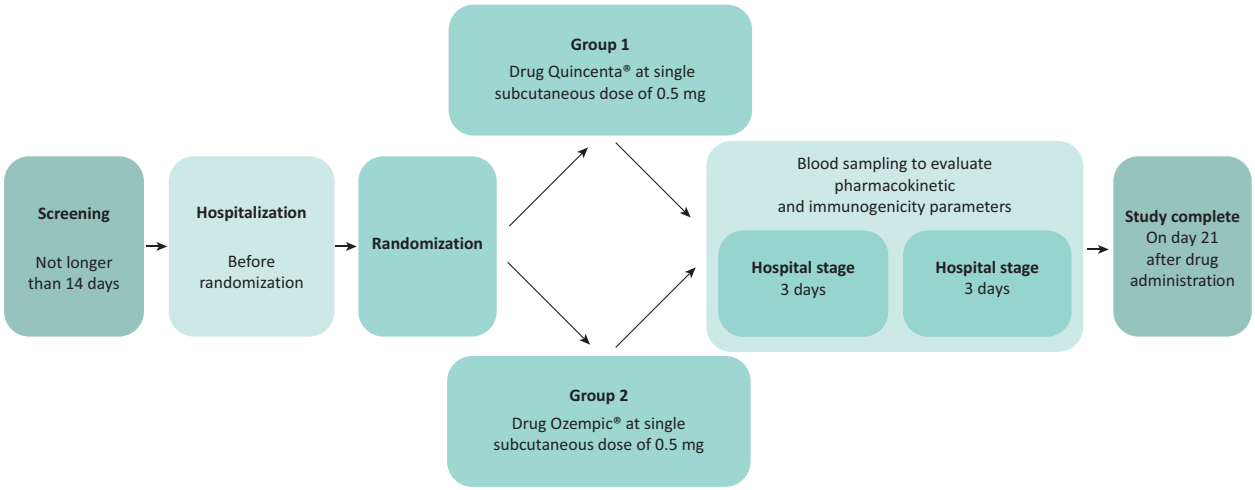

Figure 1 – Study design

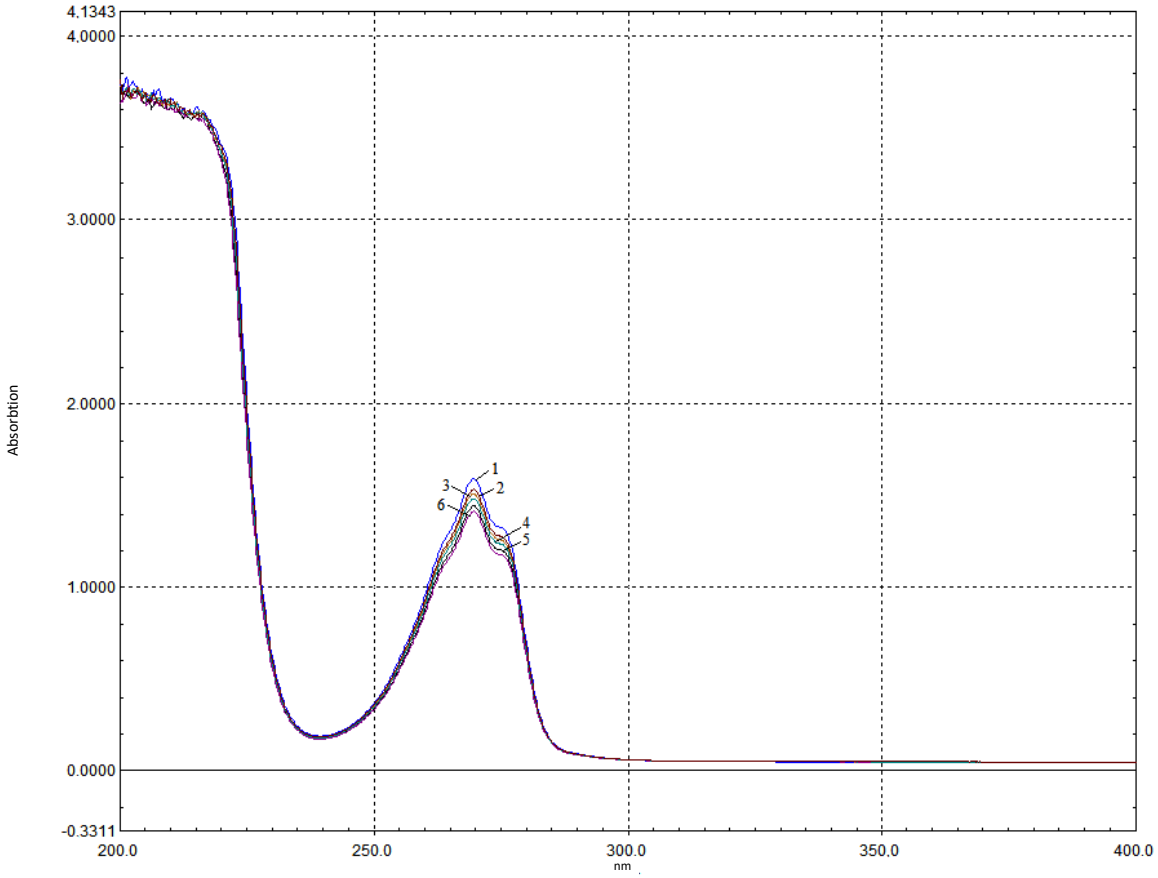

Figure 2 – Absorption spectra overlay of test and reference drugs

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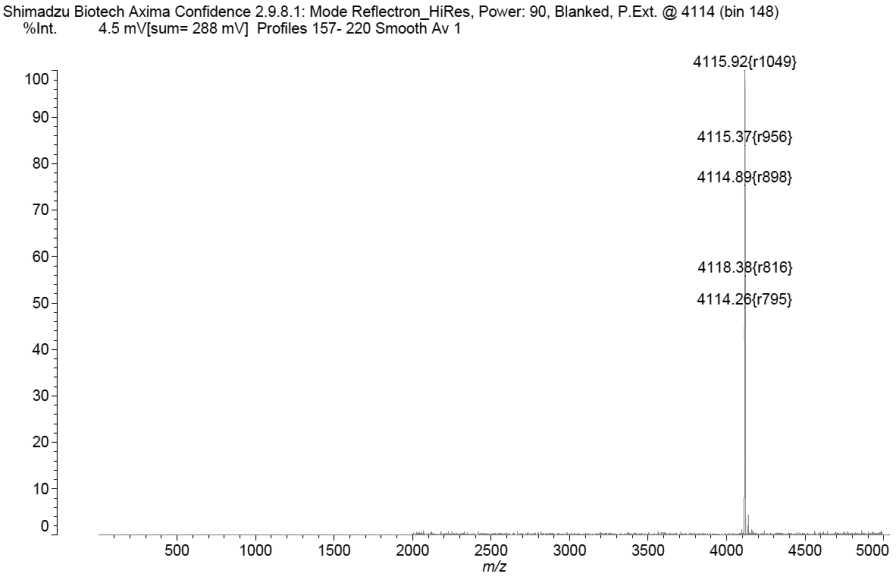
Series 1



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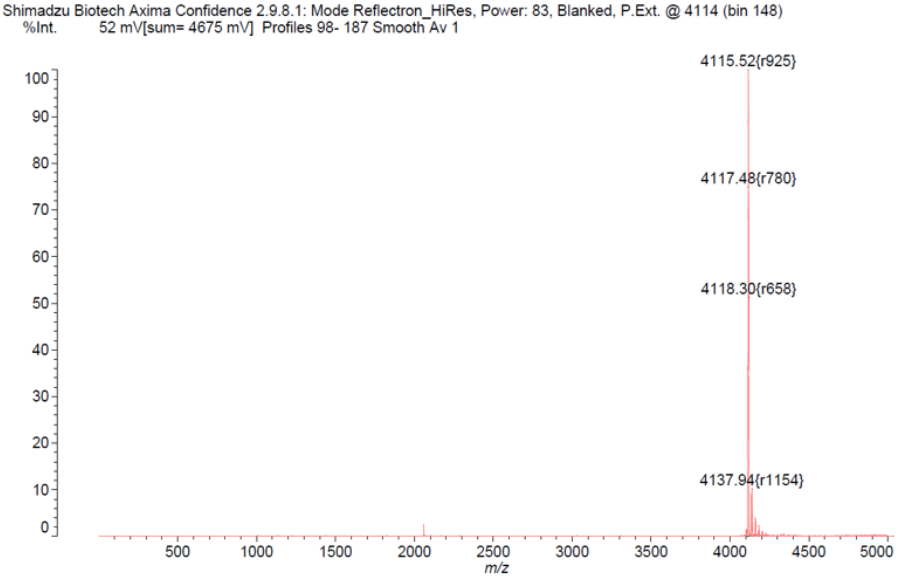

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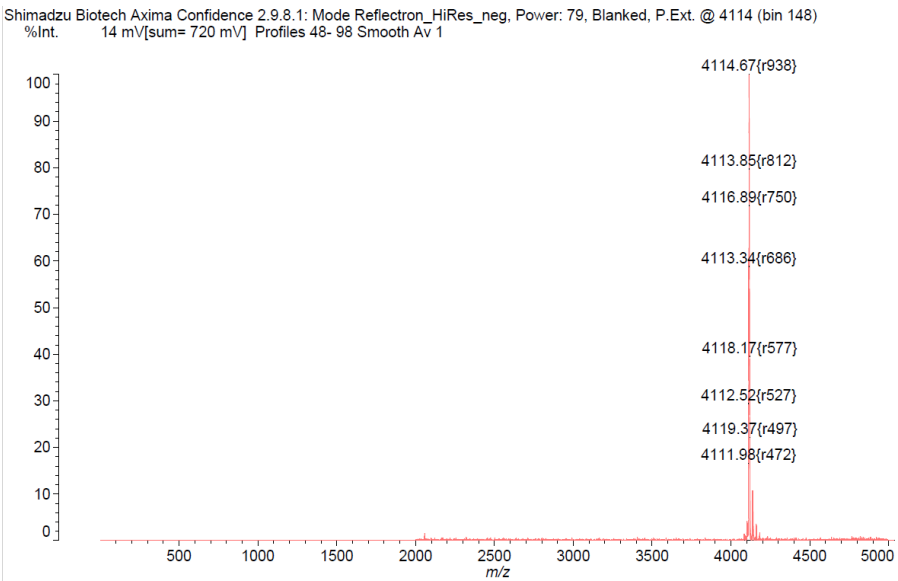

Figure 3 – MALDI-TOF MS mass spectra obtained from study of three series of test drug Quincenta®

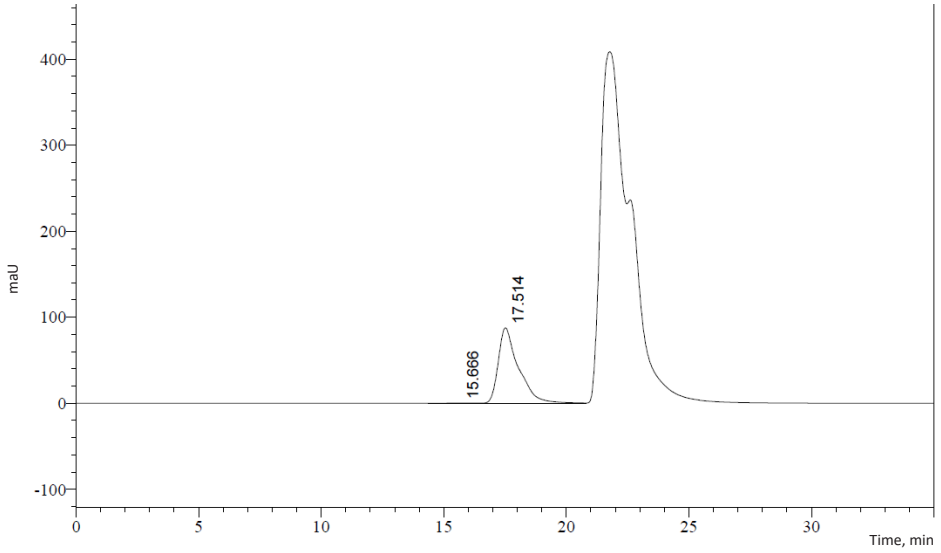

Figure 4 – Chromatogram obtained by determining content of high molecular weight proteins in reference drug

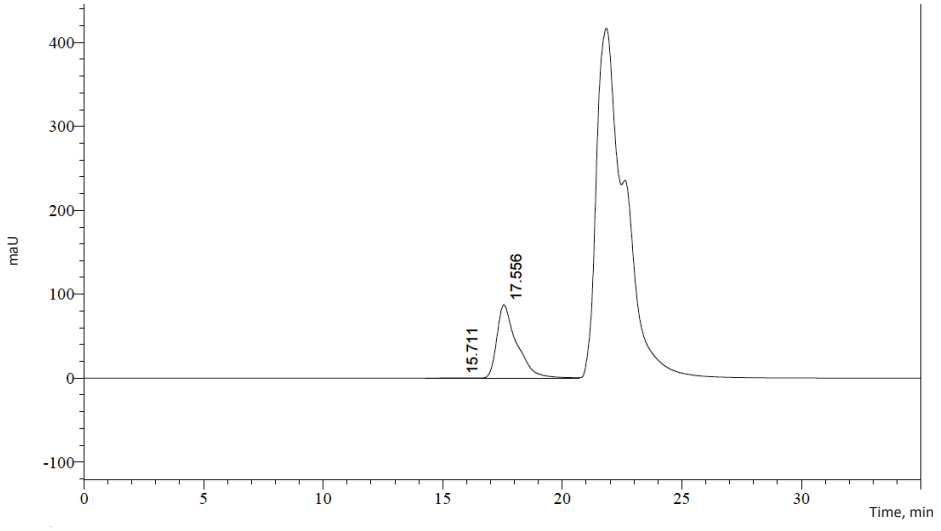

Figure 5 – Chromatogram obtained when determining the content of high molecular weight proteins in test drug

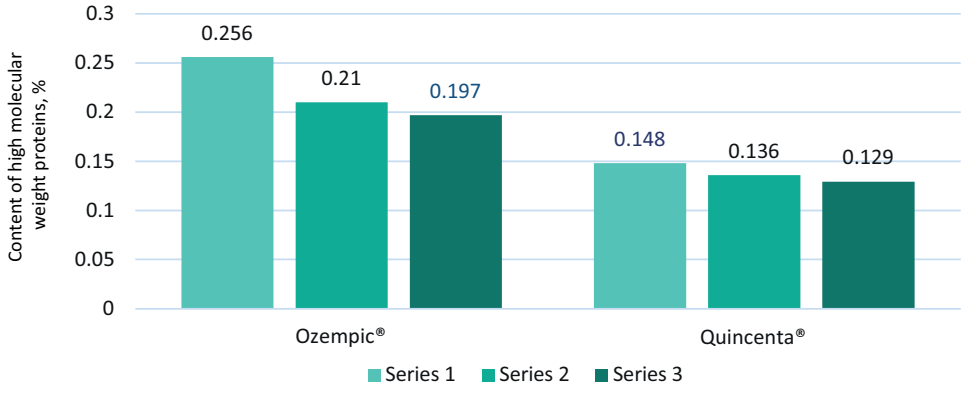

Figure 6 – Results of content analysis of high-molecular impurities in test and reference drugs

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Table 1 – Spectrophotometric analysis results of test / reference drugs in UV area

No	Object of analysis	Series	λ_{max}	λ_{min}
1	Reference drug	1	269.6	239.4
2		2	269.7	239.2
3		3	269.4	239.5
4	Test drug	1	269.6	239.7
5		2	269.3	239.2
6		3	269.1	239.6

Table 2 – Results of determining relative molar ratio of amino acids in samples of test and reference drugs

Amino acid name	Relative molar ratio of amino acids					
	Ozempic®			Quincenta®		
	Series No. 1	Series No. 2	Series No. 3	Series No. 1	Series No. 2	Series No. 3
Asp	1.1	0.9	1.2	0.9	1.0	1.1
Glu	5.0	5.3	5.4	5.2	5.0	4.9
His	1.1	0.9	1.2	1.0	1.1	0.9
Thr	1,9	2.0	2.3	2.2	1.8	2.1
Aeea	1,9	1.7	1,9	2.0	1.8	1.9
Tyr	1.0	0.9	1.1	1.1	0.8	1
Lys	1.0	1.1	1.0	1.2	0.9	1.1
Leu	2.1	1.8	2.1	1.9	2.2	2.2
Ser	2.7	2.5	2.9	3.1	2.8	2.6
Gly	4.1	4.4	3.9	4.0	4.2	3.8
Arg	2.0	1.7	2.2	1.8	2.1	1.9
Ala	3.0	2.7	2.9	2.8	2.9	3.3
Aib	1.0	0.8	1.0	0.9	1.1	1.1
Val	2.0	2.3	1.8	1.9	2.1	2
Ile	1.0	1.2	0.9	0.9	1.0	1.2
Phe	2.0	1.7	2.1	2.2	1.9	2
Trp	1.0	1.0	0.7	1.0	0.8	1.1

Table 3 – Results of quantitative determination of related impurities using reverse-phase high-performance liquid chromatography in the test and reference drugs

Index	Ozempic®			Quincenta®		
Series	1	2	3	1	2	3
Quantitative determination of semaglutide, mg/ml	1.35	1.34	1.33	1.37	1.39	1.35
Amount of impurities, %	2.683	2.312	2.751	0.944	1.124	1.036
Hydrophilic impurities, %	0.821	0.933	1.06	0.135	0.215	0.148
Hydrophobic impurities 1, %	1.737	1.308	1.62	0.716	0.834	0.825
Hydrophobic impurities 2, %	0.125	0.071	0.049	0.093	0.075	0.063

Table 4 – Comparative study results of biological activity of domestic and foreign drugs

Drug	Series No	EC_{50}	Activity % of RS
Ozempic®	1	1.379	93.62
	2	1.725	117.11
	3	1.232	83.64
Quincenta®	1	1.317	89.41
	2	1,731	117.52
	3	1.559	105.84
Semaglutide RS	–	1.473	–

Note: RS – reference standard.

Table 5 – Descriptive characteristics of volunteers' demographic and anthropometric data

Index	*n*	*p*	Quincenta®			Ozempic®		
			Mean value	CI –95.000%	CI 95.000%	Mean	CI –95.000%	CI 95.000%
Age, completed years	27	0.085940	35.6667	33.1337	38.1997	32.7404	30.4273	35.0541
Body weight, kg	27	0.119176	72.2222	68.3608	76.0837	68.6037	65.0962	72.1112
Height, cm	27	0.310119	173.7407	170.8119	176.6695	171.4815	168.5841	174.3788
BMI, kg^2/m	27	0.161126	23.7952	23.2588	24.3316	23.2274	22.6794	23.7754

Note: CI – confidence interval; BMI – body mass index.

Table 6 – Analysis of gender distribution

Gender	Groups comparison Pearson X^2 test	Quincenta®		Ozempic®	
		Frequency	Percentage	Frequency	Percentage
Male	0.5859	14	51.85185	12	44.44444
Female	0.5859	13	48.14815	15	55.55556

Table 7 – Value of calculated confidence intervals for calculated pharmacokinetic parameters

Parameter	Average ratio	90% CI		Valid range, %
		Low level	High level	
AUC_{0-t}	1.00	91.66	111.27	80–125
C_{max}	0.99	90.89	109.15	80–125

Another important advantage of semaglutide is a low risk of hypoglycemia, but the risk increases when it is combined with sulfonylureas and/or insulin [38].

But perhaps the most valuable feature came from the SUSTAIN 6 trial, which demonstrated that subcutaneous semaglutide, compared with placebo, was associated with a significant reduction in the incidence of CVD-related deaths, nonfatal myocardial infarctions, or nonfatal cerebrovascular accidents (p <0.001) (combined RR=0.74; 95% CI 0.58 to 0.95) and also with fewer cases of nephropathy [39]. A focused study (FLOW; NCT03819153) is currently investigating the effect of subcutaneous semaglutide on renal outcomes in people with T2DM and CKD.

A retrospective analysis also showed [40] that when taking semaglutide, there was a reduction in the relative and absolute risk of serious adverse cardiovascular events compared with comparators. Although the absolute risk reduction was small, there was a trend (p=0.06) for the greatest relative risk reduction in those with the lowest cardiovascular risk. This phenomenon may be explained by the fact that more advanced stages of diabetes may be more resistant to the beneficial effects of arGLP-1 on CVD outcomes.

Another pool of studies examined the effectiveness and safety of semaglutide in weight loss in overweight and obese patients without diabetes during the STEP clinical trial program. Thus, in the STEP 1,3,4, and 8 studies, semaglutide at a dose of 2.4 mg once a week led to an average weight loss of 14.9–17.4% from baseline by week 68, with 69–79% of participants achieved ≥10% weight loss, and 51–64% achieved ≥15% weight loss [41–44]. In the STEP 5 study, the mean weight loss with semaglutide 2.4 mg at week 104 was 15.2% of baseline compared with 2.6% with placebo [45]. An improvement in the main cardiometabolic risk factors was also found: a decrease in waist circumference, a decrease in blood pressure, normalization of lipid levels and C-reactive protein, as well as an improvement in physical functions and quality of life with a good safety profile of semaglutide [41–43, 45, 46].

Bandyopadhyay S. et al. [47] conducted a systematic review and meta-analysis to evaluate the effectiveness and safety of semaglutide therapy in patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis and found a significant decrease in the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), a significant decrease in liver lipid content, improvement in its elasticity, HbA1c and lipid profile indicators.

The identification of structurally related peptide impurities and their characterization is a critical challenge in the pharmaceutical development. These impurities may result from during the production degradation or storage and may affect the efficacy and safety of

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the finished product [48]. Semaglutide impurities can include peptides of imperfect structure, resulting from the insertion of an undesired amino acid or deletion (the absence of one or more amino acid residues), oxidation or racemization of amino acids [49].

There are methods for obtaining semaglutide by solid-phase synthesis that can reduce the formation of racemic impurities, simplify the purification of the target product, its purity and yield, and also reduce costs [50, 51].

The biological activity of peptide molecules directly correlates with their atomic size arrangement, while a configuration inversion at a particular peptide chiral center can cause a local spatial redistribution of critical functional groups [52]. That is why, when developing a method for producing semaglutide by chemical synthesis, a special attention is paid to controlling the racemization of amino acids.

During the analysis of literature data, it was revealed that in the foreign drug Ozempic® (Novo Nordisk A/S, Denmark), containing semaglutide of biotechnological origin, the formation of three isomers containing in its structure amino acids in the D-conformation D-His1, D-Ser8, D-Asp9, is possible. As it is known, the isomerization of even one amino acid in a certain peptide chain can have a significant impact on the overall conformation of the peptide molecule and affect its biological activity [53, 54]. Based on this fact, when developing technologies for obtaining the substance semaglutide, the main task for the specialists of Promomed Rus LLC was to minimize the amount of potential impurities.

At the study time of domestic and foreign drugs, it was found that Quincenta® (Promomed Rus LLC, Russia), which contains semaglutide of a synthetic origin, contains 2.5 times less impurities compared to the foreign drug.

Based on the results obtained in the course of comparative studies, it can be concluded that today it has been possible to achieve not only the production of a substance of the appropriate pharmacopoeial quality, but also to minimize the formation of by-products, thereby reducing the total amount of impurities in the finished medicinal product. It is important to note that peptide preparations obtained by microbiological synthesis consist of amino acids in the L-conformation, which corresponds to the life processes of natural microorganisms, while in the process of chemical synthesis D-isomers can be formed, which, as stated above, can critically change the drug quality and lead to unexpected pharmacological effects. Thus, control of spontaneous isomerization processes and obtaining a product of the required stereochemical purity are critical to the proper quality of the drug. In this regard, it is worth noting that at the moment, the Russian drug Quincenta® is the only domestic drug based on semaglutide, which uses its own technology for the production and purification of API, which ensures a high level of quality control of the resulting substance, the exclusion of undesirable impurities and isomerization and, consequently, contributes to achieving high efficiency and safety of the therapy.

Injection therapy for diabetes is the most effective treatment method. However, it is known that, despite its effectiveness, this method of therapy is characterized by a low level of adherence, and one of the ways to overcome this problem is the use of simple and convenient syringe pens [55]. That is why, for Russian drugs based on arGLP-1, in particular, the drug Quicenta®, a partner of the Promomed Group of Companies, the Medsintez Plant LLC, developed a special syringe pen that ensures a dosing accuracy, the administration ease of the drug, and also the consumption of the drug without unnecessary losses at the stage of selecting an individual dose during a long-term use. This multi-dose syringe pen has a unique advantage, as it allows a patient to fully use the active substance without leaving any residue from the cartridge.

According to the instructions for use of the drug, if after the injection the dose selector stops before the zero mark aligns with the pointer, it means that the patient, due to the characteristics of titration, did not receive the required dose of the drug. In this situation, the dose selector indicates the number of units that must be administered before the full dose of the drug is delivered from the new multi-dose pen. A similar design is not provided for in any current arGLP-1 dosage form registered on the domestic market. It is also worth noting that the above syringe pen is compatible with needles from any manufacturer, which is important from the point of view of a possible reduction in the availability of foreign-made needles. Thus, we can say that the design of the developed syringe pen for Russian drugs based on arGLP-1 from Promomed Rus LLC, in particular, the drug Quincenta®, not only helps to increase patient adherence to treatment, but also provides pharmacoeconomic advantages therapy, excluding an irrational disposal of unused medicine.

CONCLUSION

As a result of the studies, a sufficient amount of data was collected confirming the similarity of the physicochemical and biological properties of the drug with the chemically synthesized active substance semaglutide Quincenta® (a solution for a subcutaneous administration 1.34 mg/ml, Promomed Rus LLC, Russia) with the reference drug Ozempic® (a solution for a subcutaneous administration 1.34 mg/ml, Novo Nordisk A/S, Denmark). Based on this, it can be concluded that the quality, safety and effectiveness of the Russian drug with a synthetic analogue of the active substance are similar, and in some respects even exceed the reference drug.

Reducing the socioeconomic burden of diabetes and obesity is one of the most important tasks of the Russian healthcare system. Semaglutide may be the drug of choice for patients with diabetes and obesity, in particular, in combination with CVD, due to its high effectiveness in controlling glycemic levels and weight loss, restoring metabolic health parameters, and a proven protective effect in reducing the risks of cardiovascular events. The entry of Russian drugs – arGLP-1 into the market is a major step towards providing patients with the necessary therapy, especially in the face of a shortage of foreign predecessors. An open-label, randomized cross-over comparative study of bioequivalence, safety, tolerability and immunogenicity in healthy volunteers confirmed the equivalence of the test drug Quincenta® and the reference drug Ozempic®, and also demonstrated its high safety profile, tolerability and lack of immunogenicity. Based on the data obtained, the drug Quincenta® was registered in the Russian Federation.

The use of our own technology of chemical synthesis and purification of the resulting substance in the production of the drug determines the required stereochemical purity of the product, a reduction in the level of impurities, predictable properties and a low risk of adverse immune reactions. It is advisable to conduct further clinical studies to assess the effectiveness and safety of therapy for patients with diabetes mellitus and obesity or overweight, as well as to identify potential new possibilities for arGLP-1 therapy, including the Russian analogue of semaglutide.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

A.S. Ametov, L.A. Balykova – development of the clinical trial concept, analysis and description of the results, text correction; I.E. Shokhin – organization and conduct of physical and chemical research, interpretation of results; E.A. Rogozhina – organization and conduct of studies of physicochemical and biological properties, discussion of the design and results of the study; T.G. Bodrova – analysis and selection of literary sources, writing the text of the article, organizing and conducting physical and chemical studies, interpreting the results; M.E. Nevretdinova – analysis and selection of literary sources, interpretation of results, writing the text of the article; P.A. Bely – implementation of the research design, processing of research data; K.Ya. Zaslavskaya, V.S. Scherbakova – development of the design and concept of the study, writing the text of the article; D.V. Kurkin – analysis and description of the results; K.N. Koryanova – analysis and description of results, search and analysis of literary sources; E.S. Mishchenko – analysis and description of the results; E.Yu. Kesova, E.D. Kozlov, E.S. Samoshkina, D.N. Andreev, Yu.G. Kazaishvili, S.M. Noskov – development of the design and concept of a clinical trial. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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ОБЗОР

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Physiology and pharmacology of glucagon-like peptide-1 receptor

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Modern approaches to the treatment of type 2 diabetes mellitus (T2DM) are aimed not only at glycemic control, but also at reducing cardiovascular risks. The increasing prevalence of the disease and the need for effective treatment options highlight the importance of glucagon-like peptide-1 (GLP-1) receptor agonists in the pharmacotherapy structure.

The aim of the work was to review the literature regarding the physiology of GLP-1 and the therapeutic potential and development trends of its agonists.

Materials and methods. The search for the review materials was carried out using the abstract databases of PubMed, Google Scholar and e-Library. The search was carried out for publications from 2000 to 2023, using the following keywords: "GLP-1"; "GLP-1R agonists"; "GIP"; "exenatide"; "liraglutide"; "dulaglutide"; "semaglutide"; "lixisenatide"; "albiglutide"; "taspoglutide" taking into account various spellings.

Results. The interaction of almost all food components with enteroendocrine cells of the intestine leads to the secretion of incretins (primarily GLP-1) into the blood, triggering a complex of physiological reactions aimed primarily at the rapid utilization of incoming glucose (regulation of insulin and glucagon secretion), as well as the central regulation of dietary behavior (slowing gastric emptying and the formation of a feeling of satiety). A wide distribution of the GLP-1 receptor in various tissues and organs, its connection with intracellular signaling cascades aimed at launching energy-consuming remodeling (recovery) processes in endothelial cells, heart, neurons, beta cells, etc., is the basis for a wide range of pleiotropic effects of GLP-1 unrelated to its hypoglycemic effect. The discovery of synthetic GLP-1 receptor agonists with a long period of action has made it possible not only to therapeutically influence various parts of carbohydrate metabolism disorders, but also to increase the functional reserves of the target diabetes organs, reducing the risk of developing complications of the disease. Incretin-like drugs are well tolerated, with nausea being the most common side effect. The factors limiting a wider use of the drugs include their high cost and the preferred form of a subcutaneous solution. The current research is focused on the development of long-acting, oral, dual and triple agonists, fixed-dose combinations, and small molecule drugs.

Conclusion. GLP-1 receptor agonists are a class of effective and safe drugs for the treatment of diabetes and obesity, which is rapidly developing in the most advanced areas of pharmacy. A further development of this group and the solution of the identified problems will open up new opportunities for the treatment of diabetes and its complications.

Key words: GLP-1; glucagon-like receptor-1 agonists; diabetes mellitus; incretins

Abbreviations: ECD – extracellular N-terminal domain; FDA – U.S. Food and Drug Administration; FGF21 – fibroblast growth factor 21; Gcg – preproglucagon; GRP – gastrin releasing peptide; NTS – nucleus tractus solitarius; PI3K – phosphatidylinositol 3-kinase; PKA – protein kinase A; PKB – protein kinase B; PVN – paraventricular nucleus of the hypothalamus; PYY – peptide YY; SGLT1 – sodium-glucose linked transporter 1; TMD – transmembrane domain; ATP – adenosine triphosphate; AD – Alzheimer's Disease; PD – Parkinson's Disease; GABA – gamma-aminobutyric acid; GIP – glucose-dependent insulinotropic polypeptide; GM – genetically modified; GLP-1 – glucagon-like peptide-1; GLP-1R – glucagon-like peptide-1 receptor; BBB – blood-brain barrier; DPP-4 – dipeptidyl peptidase 4; GIT – gastrointestinal tract; T2DM – type 2 diabetes mellitus; FFA – free fatty acids; cAMP – cyclic adenosine monophosphate; CNS – central nervous system.

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REVIEW

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Физиология и фармакология рецептора глюкагоноподобного пептида-1

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Современные подходы к лечению сахарного диабета 2 типа (СД2) направлены не только на контроль гликемии, но и на снижение кардиоваскулярных рисков. Рост распространенности заболевания и необходимость в эффективных вариантах лечения подчеркивают важность агонистов рецепторов глюкагоноподобного пептида-1 (ГПП-1) в структуре фармакотерапии.

Цель. Анализ литературы, касающейся физиологии ГПП-1, а также терапевтического потенциала и тенденций развития его агонистов.

Материалы и методы. Поиск материала для написания обзора проводили с использованием реферативных баз PubMed, Google Scholar и e-Library. Поиск осуществляли по публикациям в период с 2000 по 2023 годы, с использованием следующих ключевых слов: «ГПП-1»; «агонисты ГПП-1Р»; «ГИП»; «эксенатид»; «лираглутид»; «дулаглутид»; «семаглутид»; «ликсисенатид»; «албиглутид»; «таспоглутид» с учетом различных вариантов их написания.

Результаты. Взаимодействие практически всех компонентов пищи с энтероэндокринными клетками кишечника приводит к секреции в кровь инкретинов (прежде всего ГПП-1), запускающих комплекс физиологических реакций, направленных, в первую очередь, на быструю утилизацию поступающей глюкозы (регуляция секреции инсулина и глюкагона), а также центральную регуляцию пищевого поведения (замедление опорожнения желудка и формирование чувства насыщения). Широкое распространение рецептора к ГПП-1 в различных тканях и органах, его связь с внутриклеточными сигнальными каскадами, направленными на запуск энергозатратных процессов ремоделирования (восстановления) в клетках эндотелия, сердца, нейронах, бета-клетках и др., является основой для широкого спектра плейотропных эффектов ГПП-1, не связанных с его гипогликемическим действием. Открытие синтетических агонистов рецепторов ГПП-1 с длительным периодом действия дало возможность не только терапевтически воздействовать на различные звенья нарушений углеводного обмена, но и увеличить функциональные резервы органов-мишеней СД, снижая риск развития осложнений заболевания. Инкретиноподобные препараты хорошо переносятся, самым распространенным побочным эффектом является тошнота. Факторы, ограничивающие более широкое использование препаратов, включают высокую стоимость и преимущественную форму – раствор для подкожного введения. Текущие исследования связаны с разработкой препаратов с пролонгированным действием, пероральной формы, двойных и тройных агонистов, фиксированных комбинаций, а также препаратов малых молекул.

Заключение. Агонисты рецепторов ГПП-1 представляют собой класс эффективных и безопасных лекарственных средств для терапии СД и ожирения, который стремительно развивается по самым передовым направлениям фармации. Дальнейшее развитие этой группы и решение обозначенных задач откроет новые возможности для лечения СД и его осложнений.

Ключевые слова: ГПП-1; агонисты глюкагоноподобного рецептора-1; сахарный диабет; инкретины

Список сокращений: ECD – внеклеточный N-концевой домен; FDA – Управление по санитарному надзору за качеством пищевых продуктов и медикаментов США; FGF21 – фактор роста фибробластов 21; Gcg – препроглюкагон; ГРП – гастрин-рилизинг пептид; NTS – ядро солитарного тракта; PI3K – фосфатидилинозитол-3-киназа; PKA – протеинкиназа A; PKB – протеинкиназа B; PVN – паравентрикулярное ядро гипоталамуса; PYY – пептид YY; SGLT1 – натрий-глюкозный котранспортер 1 типа; TMD – трансмембранный домен; АТФ – аденозинтрифосфат; БА – болезнь Альцгеймера; БП – болезнь Паркинсона; ГАМК – гамма-аминомасляная кислота; ГИП – глюкозозависимый инсулинотропный полипептид; ГМ – головной мозг; ГПП-1 – глюкагоноподобный пептид-1; ГПП-1Р – рецептор глюкагон-подобного пептида-1; ГЭБ – гематоэнцефалический барьер; ДПП-4 – дипептидилпептидаза 4; ЖКТ – желудочно-кишечный тракт; СД2 – сахарный диабет 2-го типа; СЖК – свободные жирные кислоты; цАМФ – циклический аденозинмонофосфат; ЦНС – центральная нервная система.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic and progressive metabolic disorder characterized by prolonged hyperglycemia, which increases the risk of developing macro- and microvascular complications. Between 80 and 90% of patients are overweight or obese at the time of T2DM diagnosis, so weight loss and a cardiovascular risk prevention are also key goals of T2DM treatment. At the same time, insulin and widely used drugs from the groups of thiazolidinedione and sulfonylurea derivatives contribute to the weight gain. Glucagon-like peptide-1 receptor (GLP-1R) agonists are incretin mimetics; they improve a glycemic control and are superior to other hypoglycemic drugs in many respects. GLP-1R agonists are recommended as one of the 2nd and 3rd line combination therapy options, primarily for patients with obesity and T2DM, and some (semaglutide and liraglutide) – to reduce the risk of cardiovascular events [1, 2].

Incretin mimetics (GLP-1R agonists and dipeptidyl peptidase-4 inhibitors – DPP-4) are well tolerated and do not cause hypoglycemia, but in 2013 the FDA (U.S. Food and Drug Administration) reported an increased risk of pancreatitis and precancerous cellular changes (metaplasia) of the ducts pancreas against the background of their use. At the same time, recent meta-analyses have shown that the incidence of acute pancreatitis increases with DPP-4 inhibitors, but not GLP-1R agonists. The inhibition of DPP-4 also affects the elimination of substrates other than glucagon-like peptide-1 (GLP-1): glucose-dependent insulinotropic polypeptide (GIP), some cytokines, growth factors and neuropeptides. DPP-4 (also called CD26) is also expressed on the surface of T-cells. Thus, DPP-4 inhibitors may have effects on the immune system and inflammation, which may also be influenced by genetic factors [3].

GLP-1R agonists are currently the drugs the effectiveness and safety of which have been confirmed by many clinical studies. Although they have a number of advantages over hypoglycemic drugs of other groups, a number of features limit their widespread use: nature, a method of production and, obviously, insufficient awareness of doctors and patients about modern antidiabetic drugs.

THE AIM of the work was to review the literature regarding the physiology of GLP-1 and the therapeutic potential and development trends of its agonists.

MATERIALS AND METHODS

The literature search (literature reviews, results of experimental and clinical studies) was carried out using the PubMed, Google Scholar and e-Library abstract databases. The search depth was 23 years – from 2000 to 2023. The list of keywords included (but was not limited to) various combinations and spellings: GLP-1 (GLP-1); GLP-1R agonists; GIP; exenatide; liraglutide; dulaglutide; semaglutide; lixisenatide; albiglutide; taspoglutide. The exclusion criteria included earlier publications and articles that did not directly address the topic of the work. 410 sources were analyzed and, after the systematization, the articles with similar information were removed. After screening, 120 sources were considered suitable for the inclusion in the review.

RESULTS AND DISCUSSION

Discovery of the preproglucagon family hormones

A timeline of major events related to the GLP-1 study is presented in Fig. 1.

At the beginning of the 20th century, when using insulin obtained from pancreatic extracts or in the form of crude preparations, the development of a hypoglycemic effect was preceded by an increase in glycemic levels (peak after 20 min), which was initially associated with a poor purification of the drug. In 1902, Ernest Bayliss and William Starling identified a substance produced by duodenal epitheliaL-cells upon contact with food components. This component stimulated the pancreas to secrete pancreatic juice when it entered the blood and was called secretin. In 1906, Benjamin Moore discovered that a repeated oral administration of porcine intestinal mucosal homogenate reduced glycosuria in diabetic patients, and also suggested that intestinal cells were able of secreting substances that stimulate an insulin secretion. In 1929, Edgard Zunz and Jean Labarre isolated a fraction from the intestinal extracts that reduced glycemic levels when administered to animals. It was called incretin, suggesting the ability to stimulate an insulin secretion. In 1923, Charles Kimball and John Murlin isolated a fraction of the pancreas that, when evaporated and reconstituted in water, had a potent hyperglycemic effect when administered to rabbits and dogs. The substance included in the fraction was called “glucose agonist” or “glucagon”. In 1965, Ellis Samols hypothesized that a glucagon-like intestinal material that stimulates the insulin secretion

might be related to the effect of incretin. The glucagon-like material isolated from the intestines consisted of proteins of several fractions and caused physiological effects opposite to those exerted by glucagon, which had been isolated from the pancreas. The concentration of the glucagon-like material in the blood increased in response to the entry of glucose into the intestine, but did not change when it was administered intravenously. Immunocytochemical studies showed that intestinaL-cells stained with antibodies to glucagon based on morphological and ultrastructural characteristics differed from pancreatic α-cells and were called "L-cells." Later, it was suggested that there is a large precursor molecule, proglucagon, which, during the translation, is split into several fractions differing in size and biological functions. Later, a 42-amino acid polypeptide that was capable of inhibiting gastric motility and hydrochloric acid secretion, was identified. It was named gastric inhibitory peptide or glucose-dependent insulinotropic polypeptide (GIP). It also increased the insulin secretion in a glucose-dependent manner, and its removal from intestinal extracts reduced the incretin effect by approximately 50% [4, 5].

An important discovery in the field of GLP-1R pharmacology was the isolation of exendin-4 (exenatide) by John Eng in 1992. Its clinical use became possible only in 2005 (AstraZeneca, UK). In 2006, Merck & Co (USA) received approval for the clinical use of sitagliptin, the first DPP-4 inhibitor, despite the fact that these enzymes were discovered back in 1966. Just 1 year later, in 2007, the same company registered the first combination drug from the group of incretin mimetics – a drug based on sitagliptin and metformin. In 2009, the first GLP-1R agonist with a high degree of homology to the human protein, liraglutide, appeared on the pharmaceutical market. In 2011, the first combination of an incretin mimetic and a non-hypoglycemic drug – a combination of sitagliptin and simvastatin – was registered. In 2014, incretin mimetics began to be registered for the first time for the indications not related to T2DM: liraglutide was registered as a drug for the treatment of obesity. In 2019, Novo Nordisk brought to the market the first oral GLP-1R agonist, semaglutide [4–6].

Expression of proglucagon family hormones

Preproglucagon (Gcg) is expressed in α-, β-cells of the pancreas, in enteroendocrine L-cells and in the brain [7, 8]. Specific enzymes – prohormone convertases, interacting with cleavage sites in the Gcg molecule, determine which peptide / hormone molecules are formed from it: glycentin (AK 1–69), the associated pancreatic polypeptide (GRPP; AK 1–30); glucagon (AK 33–61); oxyntomodulin (OXM; AK 33–69); the main fragment of proglucagon (MPGF; AK 72–158) and glucagon-like peptides 1 (GLP-1; AK 72–107/108) and 2 (GLP-2; AK 126–158) [9, 10]. These proglucagon fragments significantly influence a systemic metabolism, modulating food intake and satiety (GLP-1, glucagon, oxyntomodulin), maintaining fluid homeostasis (water intake and diuresis, GLP-1) [8, 11], thermogenesis (glucagon), metabolism lipids (GLP-1, glucagon, GLP-2), gastrointestinal motility (glucagon, GLP-1, GLP-2) and gastric emptying (glucagon, GLP-1, GLP-2). Glucagon and GLP-1, formed from the same precursor (preproglucagon, Gcg), are secreted by different cells and have opposite effects on blood glucose concentrations. This is achieved by cell-specific processes that determine a different expression and cleavage of proglucagon into fragments by enzymes from the group of convertases, i.e. prohormone convertase 1 (PC1 or PCSK1 or PC1/3) or 2 (PC2 or PCSK2). PC1 is expressed in brain and intestinal cells, participating in the formation of GLP-1, GLP-2, glycentin, oxyntomodulin and the intermediate peptide IP2. PC2 is expressed in the pancreas and determines the formation of glucagon, pancreatic polypeptide (GRPP), major proglucagon fragment (MPGF) and intermediate peptide (IP1) [4, 12].

The distribution (expression) of these enzymes in tissues is quite conservative under physiological conditions, but can change during hyperglycemia. In particular, the activity and/or expression of PC1 in α-cells (or isolated islets) is detected when cultured in a medium with a high concentration of glucose [13], which also leads to an increase in GLP-1 levels. An increased expression of PCSK1 in α cells leads to an increased production and secretion of GLP-1, an insulin and islet survival [14]. It has been noted that in the mice knockout for the glucagon receptor, the level of GLP-1 in α-cells is higher, therefore such animals are less sensitive to the toxic effects of streptozotocin [15]. Research findings suggest an important role for α cells in compensating for a high functional activity of β cells during the insulin resistance, pregnancy, and a cellular stress by modulating an intraislet GLP-1 production. Some studies note that to maintain adequate functioning of the islet apparatus, a communication

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of α- and β-cells is important, and glucagon, the main peptide, proglucagon-derived peptides (PGDP), being also a GLP-1R agonist, stimulates an insulin secretion, acting on β-cells [12]. In islets isolated from the mice with a β-cell-specific deletion of the glucagon receptor GcgR ($GcgR^{\beta cell-/-}$), a stimulation of the insulin secretion by glucagon is preserved but attenuated by treatment with the GLP-1R antagonist exendin (9–39), and in the islets isolated from GLP-1R knockout mice, a glucagon-dependent insulin secretion is reduced [4, 16].

GLP-1, GLP-2, glucagon, GIP, secretin and somatoliberin belong to the group of structurally related peptides capable of binding to structurally similar receptors of the GPCR class B family. The receptors of this family are named on the basis of its single and unique endogenous ligand (GLP-1, GLP-2, Gcg, GIP and somatoliberin), but under physiological conditions there is no significant cross-reactivity between peptide ligands and receptors of this family [17]. However, in the pancreas, glucagon is known to have a physiologically relevant cross-reactivity with GLP-1R with an EC_{50} of 36.4±0.22 nmol/L, but GLP-1 has no affinity for the glucagon receptor. It can be assumed that the interaction of glucagon with GLP-1R plays an important role for the insulin secretion [4, 18].

Proteins of the GLP-1 family are formed by processing from proglucagon. They differ in their ability to enhance the insulin secretion and are divided into GLP-1 (1–37) (or 1–36 amide), GLP-1 (7–36 amide) (“amidated” GLP-1) and GLP-1 (7–37) (“glycine-extended GLP-1”).

In humans, almost all circulating GLP-1 is one of the truncated forms, ~80% corresponds to GLP-1 (7–36 amide) and ~20% corresponds to the glycine-extended GLP-1 (7–37). The relative abundances of GLP-1 (7–36 amide), GLP-1 (7–37), and GLP-1 (1–37) vary among species. Longer and shorter forms of GLP-1 were found in the textracts of rat intestines and pancreas. GLP-1 (7–36 amide) and GLP-1 (7–37) equally effectively stimulate the secretion of insulin and C-peptide, exceeding the activity of GLP-1 (1–37). GLP-1 (amide 7–36) is rapidly metabolized to GLP-1 (amide 9–36), which is considered a weak partial agonist of the GLP-1 receptor, but relative to its parent structure, its plasma concentration may be five to ten times higher [4, 19, 20].

A cell-type selective Gcg expression is regulated by more than a dozen transcription factors that selectively bind to cis-acting elements in Gcg promoter and enhancer regions to stimulate or inhibit the Gcg expression. In addition to a number of homeodomain proteins, the Gcg expression is also stimulated by protein kinase A (PKA) in response to high levels of cAMP. Insulin stimulates the Gcg expression in the intestine and suppresses it in α cells. Some effectors of the Wnt pathway enhance the Gcg expression in the intestine but not in the pancreas [4, 21].

Glucagon receptor and GLP-1

Typically, all the effects of endogenous GLP-1 are realized through GLP-1R, a transmembrane receptor containing 463 amino acids associated with a G protein (GPCR). Like other class B GPCRs, GLP-1 receptors contain an extracellular N-terminal domain (ECD, an extracellular N-terminal domain or NTD, an N-terminal domain) of more than 100 amino acid residues, and a transmembrane domain (TMD), consisting of seven helices connected by extracellular and intracellular loops. The extracellular N-terminal domain of family B GPCRs contains a common fold stabilized by three disulfide bridges, which is essential for mediating the binding affinity of the receptor to the ligant peptide. GLP-1 binds to both the extracellular N-terminal domain (ECD) and the extracellular half of the TMD. The structure-activity relationship studies of the GLP-1R activation have revealed an extensive interaction of the GLP-1 C terminus with the peptide-binding groove of the N-terminal extracellular domain (ECD) of the receptor. Binding of GLP-1 to the ECD brings the N-terminus of the GLP-1 peptide closer to the TMD, and their interaction causes a conformational change in the helical bundle, allowing an interaction of the intracellular half of the TMD with the G protein. The key insulinotropic effect of GLP-1 through the G-protein coupled receptor B is through the formation of cyclic adenosine monophosphate (cAMP), which, in combination with elevated Ca^{2+} levels, promotes exocytosis of insulin-containing vesicles [20, 22, 23]. In addition to pancreatic islet cells, GLP-1R is also expressed in the brain, kidney, stomach, liver, skeletal muscles, and adipose tissue. In the pancreas, the maximum level of GLP-1R expression is observed in β-cells, less in acinar cells and minimum in ductaL-cells. This receptor was also found in the walls of the kidneys and lungs arteries, the sinus node of cardiomyocytes (limited to the sinoatrial node), and the duodenum (in the Brunner’s gland). A low expression of GLP-1R was noted in parietal and smooth muscle cells of the stomach and in the intestinal plexus [4, 24, 25].

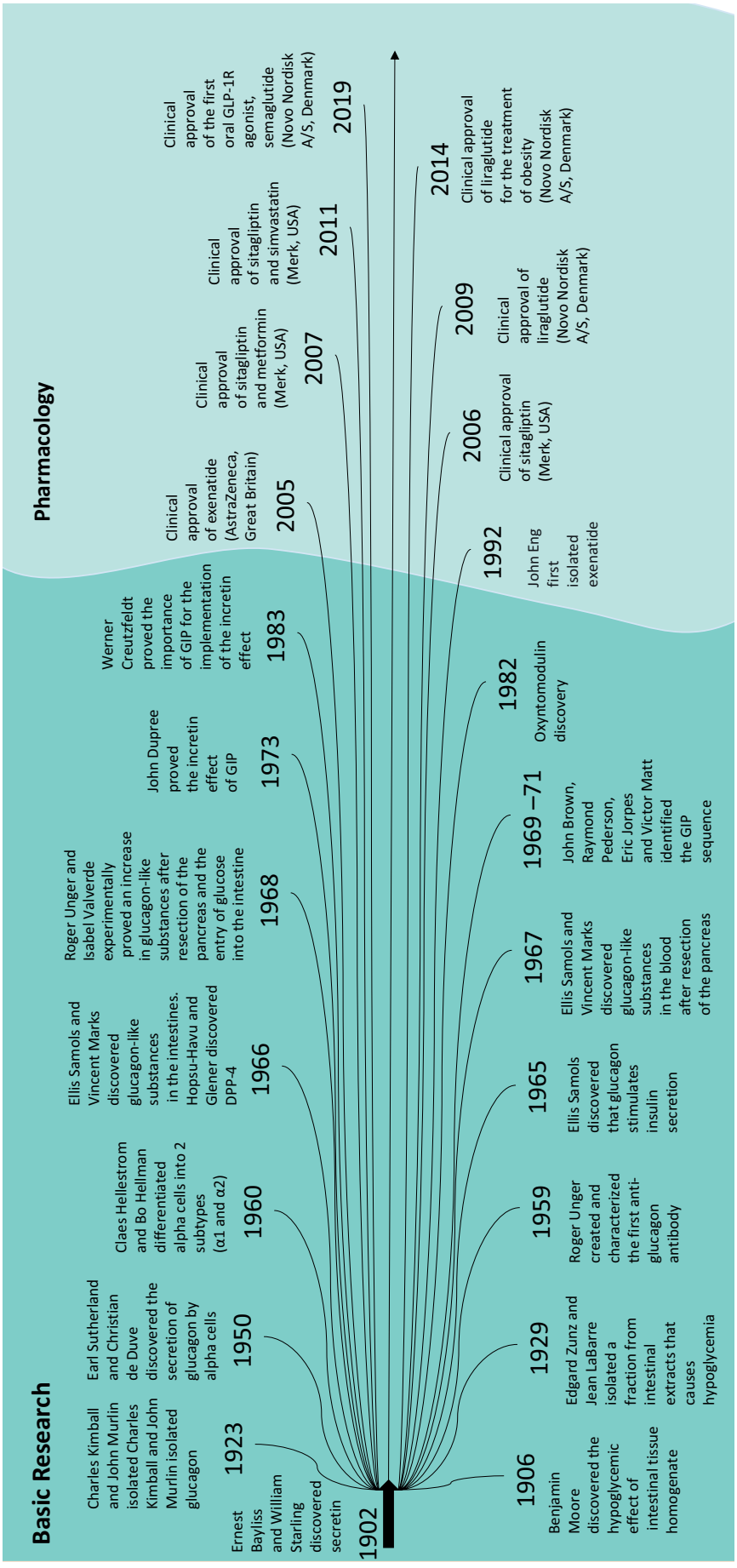

Figure 1 – Key dates of discoveries related to the development of GLP-1R agonists

Note: GIP – glucose-dependent insulinotropic polypeptide; GLP-1R – glucagon-like peptide-1 receptor; DPP-4 – dipeptidyl peptidase 4.

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	Sales volume of agonists GLP-1 (packages, A)			Sales volume of agonists GLP-1 (rubles, B)			Sales volume of agonists GLP-1 (rubles, C)		
Exenatide (Bayeta)	7110 (26.26%)	2786 (1,97%)	827 (0.44%)	31137 (13.57%)	16499 (1.08%)	4887 (0.24%)	4235.0 (10.15%)	6594.2 (11.16%)	8314.5 (9.48%)
Lixisenatide (Lixumia)	507 (1.87%)	86 (0.06%)	17 (0.001%)	1885 (0.82%)	299 (0.02%)	81 (0.001%)	4135.6 (9.91%)	4089.3 (6.92%)	4975.1 (5.67%)
Insulin glargine and lixisenatide (Soliqua SoloStar)	–	3543 (2.50%)	7925 (4.18%)	–	17628 (1.15%)	27586 (1.36%)	–	5006.8 (8.47%)	3679.1 (4.20%)
Liraglutide (Victoza)	19404 (71.68%)	72630 (51.31%)	74408 (39.26%)	194952 (84.99%)	630084 (41.05%)	638013 (31.57%)	10025.2 (24.03%)	8827.2 (14.93%)	9609.2 (10.96%)
Liraglutide (Saxenda)	50 (0.18%)	20097 (14.20%)	29156 (15.38%)	1411 (0.62%)	449220 (29.27%)	648294 (32.07%)	23316.2 (55.90%)	23996.5 (40.60%)	22030.9 (25.12%)
Insulin degludec and liraglutide (Sulfotai)	–	–	23 (0.001%)	–	-	421 (0.02%)	–	–	19362.4 (11.30%)
Dulaglutide (Trulicity)	–	42399 (29.96%)	64258 (33.91%)	–	421130 (27.44%)	575917 (28.49%)	–	10594 (17.92%)	9910.70 (11.30%)
Semaglutide (Ozempic)	–	–	12898 (6.81%)	–	-	126004 (6.23%)	–	–	9804.78 (11.18%)

Figure 2 – Market of GLP-1R agonists in the Russian Federation

Note: the data was officially purchased from the DSM Group, based on them, calculations were made and diagrams were presented; data are presented in Russian rubles.
As of August 1, 2022, 1 US dollar (USD) corresponded to 61.3 Russian rubles (RUB).

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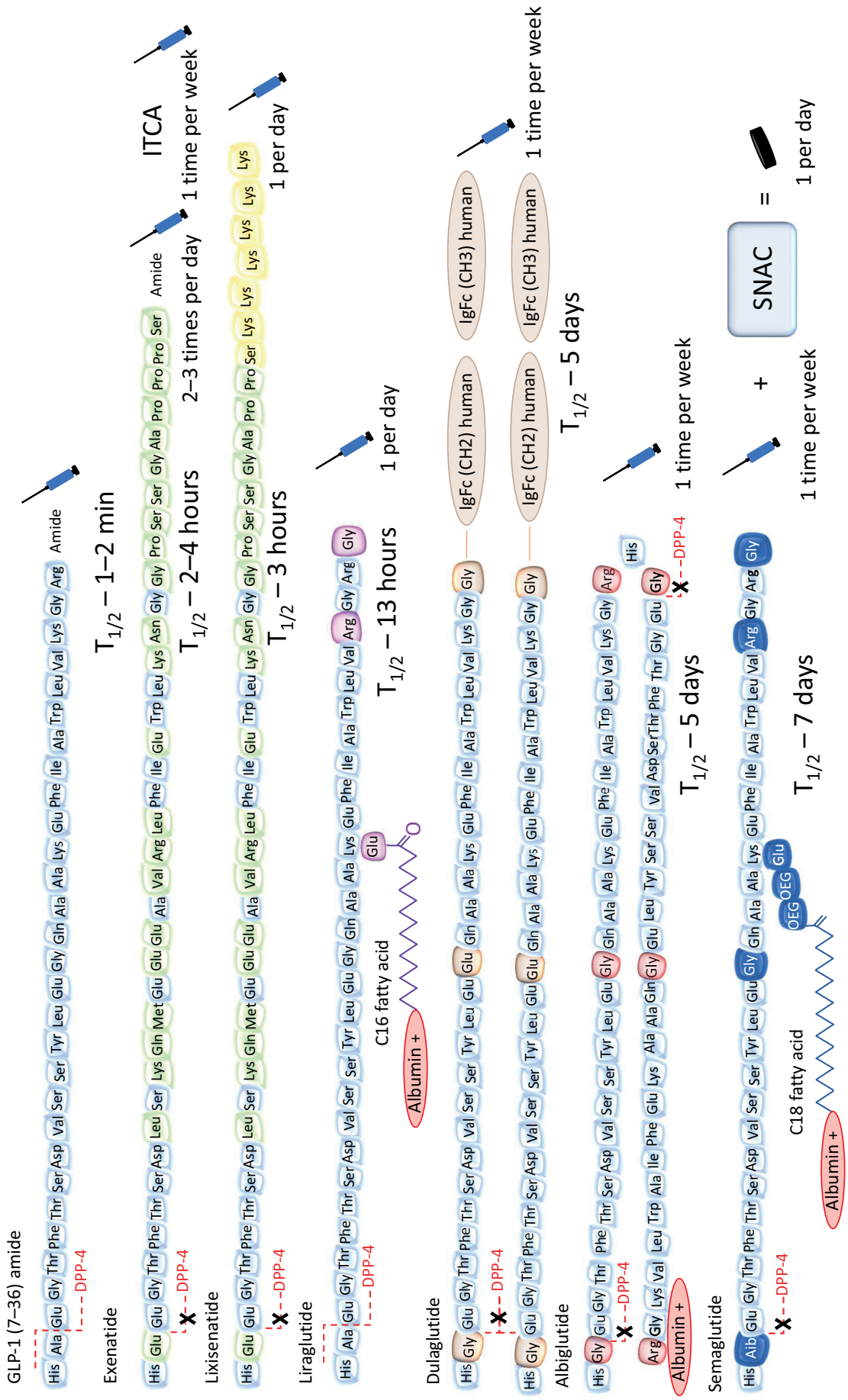

Figure 3 – Structure and features of drugs based on GLP-1 analogues

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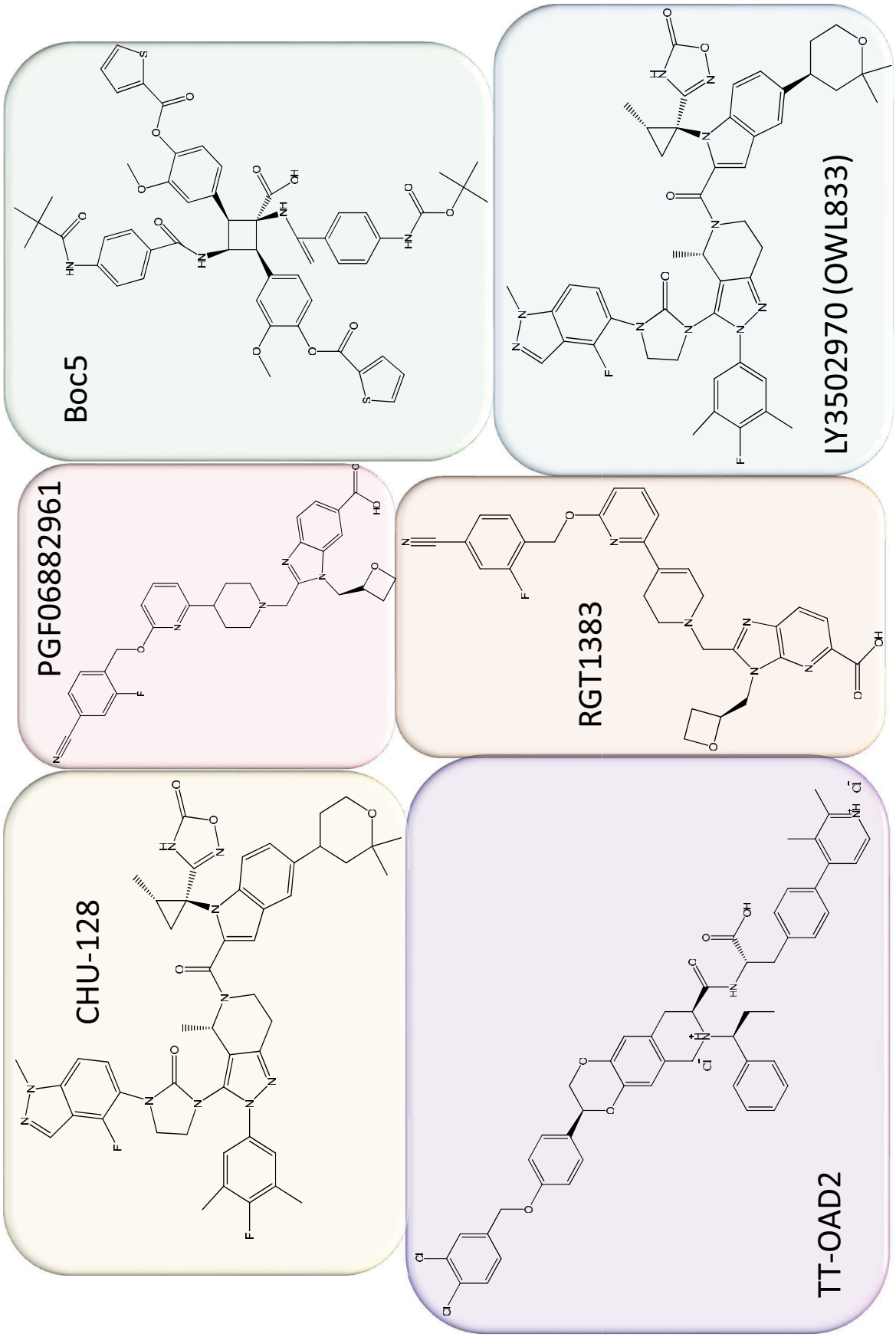

Figure 4 – Structures of non-peptide GLP-1R agonists

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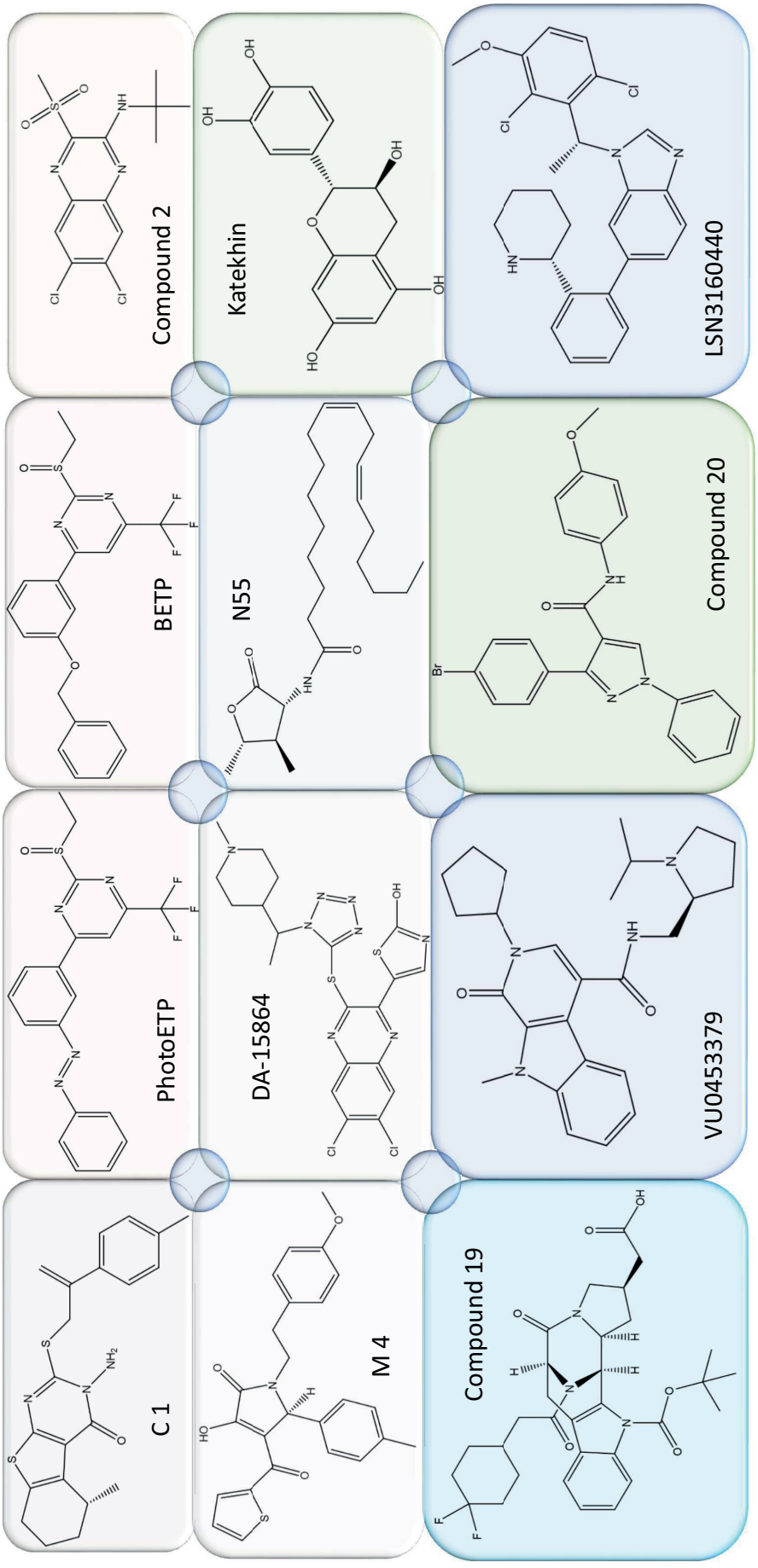

Figure 5 – Allosteric modulators GLP-1R

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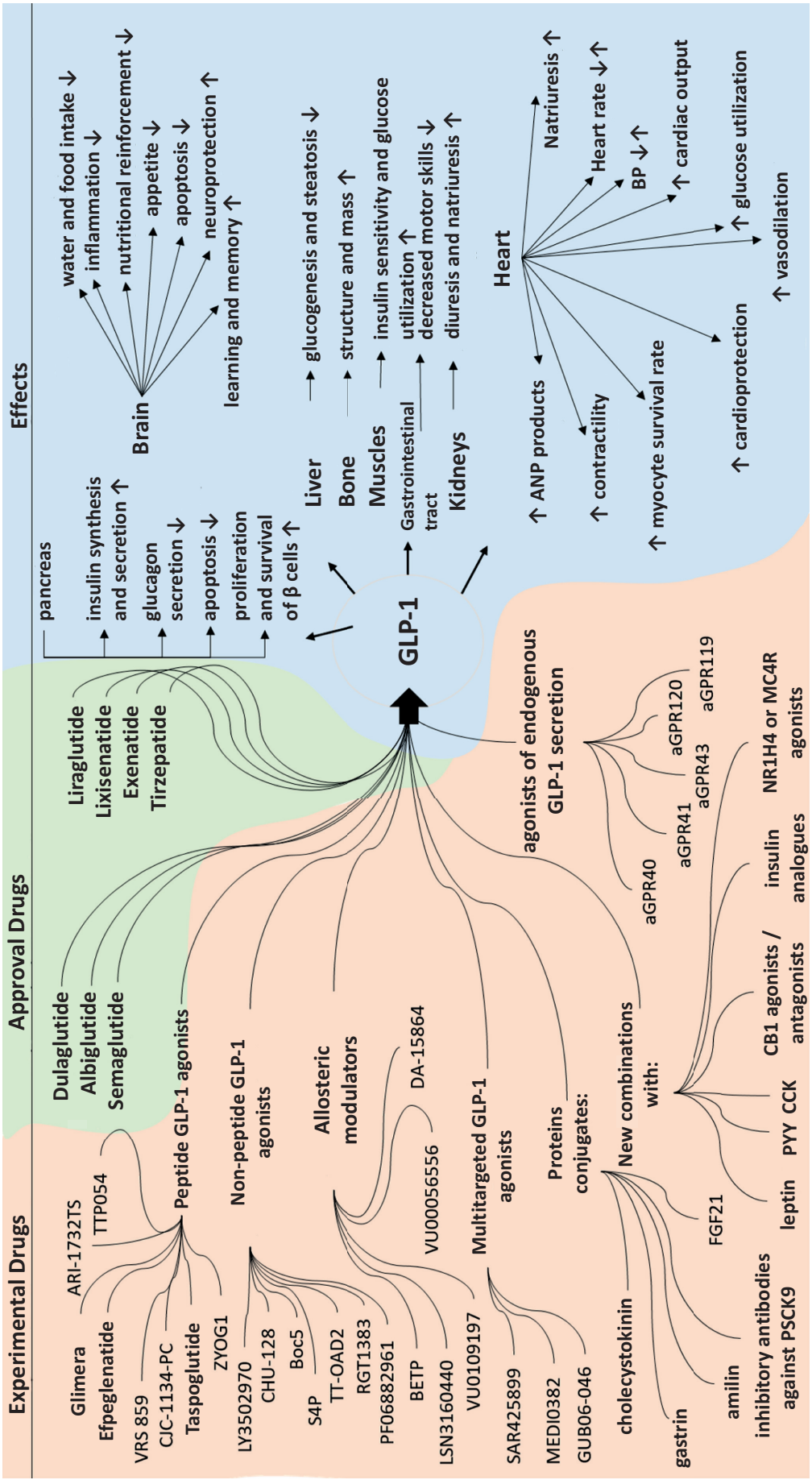

Figure 6 – Approaches to the creation of GLP-1R agonists and their effects
Note: BP – blood pressure; GLP-1 – glucagon-like peptide-1.

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Table 1 – Localization and function of receptors GPR40, GPR41, GPR43, GPR119, GPR120 [35, 36]

Receptor	Localization	Physiological effect upon activation	Agonist
GPR40 (FFA1, FFAR1)	L- and K-cells of the intestine, beta cells of the pancreas	↑ secretion of incretins and glucose-stimulated insulin secretion, improvement of regenerative abilities and ↓ apoptosis of beta cells	medium- and long-chain fatty acids, saturated and unsaturated (C10–C22)
GPR41 (FFA3, FFAR3)	L-cells, monocytes, neutrophils, adipocytes, alpha- and beta-cells	↑ secretion of incretins and leptin, physiological effects have not been fully studied	short chain fatty acids (C3=C4=C5>C2=C1)
GPR43 (FFA2, FFAR2)	L- and K-cells, adipocytes, leukocytes, Alpha- and beta-cells	↑ secretion of incretins and leptin, ↓ intestinal motility, ↑ secretion of GLP-1, ↓ activation of leukocytes, ↓ lipolytic activity in adipose tissue and ↓ plasma levels of free fatty acids; physiological effects have not been fully studied	short chain fatty acids (C2=C3>C4>C5=C1)
GPR119	L- and K-cells, beta-cells	↑ incretin secretion and glucose-stimulated insulin secretion, improved regeneration and ↓ apoptosis of beta-cells	fatty acid amide derivatives and phospholipids
GPR120 (FFA4, FFAR4)	L-cells, adipocytes, macrophages	↑ secretion of incretins, ↑ glucose uptake by adipocytes and acquisition of an anti-inflammatory phenotype by tissue macrophages	medium- and long-chain saturated (C14-C18) and unsaturated (C16–C22) fatty acids

The studies performed on transgenic animals with a controlled expression of green fluorescent protein (GFP) have established the presence of its activity in pancreatic β- and δ-cells, vascular smooth muscles, atrium, gastric antrum, intestinal neurons, vagal ganglia and dorsal roots. [25, 26].

The density of GLP-1R expression in different organs can vary greatly between humans and animals. For example, thyroid C cells in rodents express GLP-1R at very high densities (more than 10 000 times higher than in humans), and a chronic administration of GLP-1R agonists to rodents caused a marked proliferation of thyroid C cells and hypersecretion of calcitonin. It appears that in rodents a gastrointestinal stimulus (gastrin, GLP-1) is required for a normal calcitonin secretion, whereas in humans, this function has disappeared. Numerous clinical studies have reported no increase in the calcitonin secretion or the incidence of C-cell carcinoma during therapy with GLP-1R agonists [19]. However, the labeling of some drugs, such as tirzepatide (a GIP and GLP-1 receptor agonist), contains warnings about the risk of C-cell thyroid tumors in certain categories of patients.

The activation of GLP-1R through Gs stimulates the synthesis of cAMP, through the Gq/11 pathway it increases intracellular Ca^{2+} and through the recruitment of β-arrestin it promotes the activation of the ERK signaling pathway [27, 28]. A ligand-dependent functional selectivity of GLP-1R leading to the launch of signal transduction pathways specific for various ligands (relative to the endogenous hormone), causing diverse cellular responses, has been proven. This was revealed by analyzing a cAMP production, Ca^{2+} accumulation, β-arrestin recruitment and $ERK_{1/2}$ phosphorylation. Exendin-4 (exenatide) and oxyntomodulin, unlike GLP-1, are biased for β-arrestin signaling, which also promotes the cell proliferation and survival through mechanisms involving the $ERK_{1/2}$ activation [27, 28]. This property of GLP-1R is important because different ligands, by modulating specific signaling pathways, can have different pharmacological effects.

GLP-1 secretion

L-cells are distributed with an increasing gradient along the length of the gastrointestinal tract, occurring at low frequency in the duodenum (proximal-distal neuronal and/or humoral signals providing the early phase of GLP-1 secretion during meals), increasing in the jejunum, reaching a maximum in the ileum and colon gut. GLP-1 immunoreactive cells are also located in the gastric mucosa, although in small numbers [7]. The apical surface of the L-cell faces the intestinal lumen and, upon a contact with chyme (with lipids and carbohydrates), secretes GLP-1 [25, 29]. Distal L-cells may play an important role in slowing the passage of chyme as it reaches the ileum and colon through the “ileal brake” mechanism (a negative feedback mechanism for slowing an intestinal transit in response to the increased levels of undigested nutrients in the ileum). This phenomenon is also reproduced by a proximal ileal transposition or the use of α-glucosidase inhibitors, where the exposure of more L-cells to undigested nutrients leads to a greater GLP-1 secretion [7, 30].

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Incretins are broken down quite quickly after the secretion. Within the intestine, 75% of active GLP-1 is destroyed, half of the passing GLP-1 is broken down in the liver, and only 10–15% of active GLP-1 reaches the pancreas [31]. Fasting plasma levels of GLP-1 are in the range of 5–10 pmol/L and increase after meals to 15–50 pmol/L (half-life for GLP-1 is 2 min). The level of GIP in this case varies from 20–30 to 300 pmol/L after a meal, returning, like GLP-1, to the initial level within 3 h (half-life for GIP is 5–7 min) [32].

In the portal vein, total GLP-1 concentrations peak approximately 15 min after an intragastric infusion of a liquid meal and return to the baseline levels within 90 to 120 min. There is a controversy as to whether a glucose-induced GLP-1 secretion is impaired in patients with T2DM, but a clinical trial showed that GLP-1 response to oral glucose was reduced in patients with prediabetes or T2DM, but a meta-analysis of 22 clinical trials did not confirm this [33].

Proteins, fats, and carbohydrates influence the GLP-1 secretion through different mechanisms. Accordingly, more GLP-1 is released after the ingestion of a mixed meal [26].

Effect of carbohydrates on GLP-1 secretion

The cellular mechanisms of a glucose stimulation of the GLP-1 secretion by L-cells partially coincide with the mechanisms underlying the insulin secretion by pancreatic islets. Glucose and fructose dose-dependently increase the secretion of GLP-1 by enteroendocrine cells through a universal mechanism – the closure of ATP-sensitive $K-_{ATP}$ channels, which is accompanied by a membrane depolarization, the opening of voltage-dependent Ca^{2+} channels (VDC), an increase in Ca^{2+} influx and the triggering of vesicular exocytosis with the secretion of GLP-1 into the bloodstream. Sulfonylureas, by inhibiting the $K-_{ATP}$ activity, increase the insulin secretion, but there is no clear evidence that they affect the GLP-1 secretion. In addition, glucose (even in low concentrations) stimulates the electrical activity of L-cells, promoting the secretion of GLP-1 through the induction of small inward currents dependent on the sodium-glucose cotransporter (SGLT1). Sucralose and other sweeteners did not affect the GLP-1 secretion in primary L-cell cultures or in humans [26, 30, 34].

Effect of lipids on GLP-1 secretion

L-cells, like other enteroendocrine cells, react through specific receptors with a decreasing efficiency (as listed) to the following free fatty acids: α-linolenic (C18:3), docosahexane (C22:6), palmitoleic (C16:1), oleic (C18:1), stearic (C18:0) and octanoic (C8:0) acids. In humans, saturated fatty acids are less effective than unsaturated fatty ones. The induction of the GLP-1 secretion by free fatty acids (FFAs) is strongly dependent on the cytosolic Ca2+ concentration. FFAs increase the GLP-1 secretion by stimulating the influx of extracellular Ca2+ through Ca2+ channels on the cell surface [26, 35, 36].

In response to FFA, the secretion of GLP-1 is mediated by receptors for long-chain FFA – GPR40 (FFAR1) and GPR120 (FFAR4), short-chain FFA – GPR41 (FFAR3) and GPR43 (FFAR2), as well as for the derivatives of fatty acid amides and phospholipids GPR119 [37, 38]. In the animals knockout for these receptors, the secretory response of GLP-1 to FFA is significantly reduced, and the use of agonists to different receptors (with different secondary messengers) leads to the development of a synergistic effect on the GLP-1 secretion [26, 27, 30].

Effect of amino acids on GLP-1 secretion

The secretion of GLP-1 can be stimulated by proteins, tri- and dipeptides and amino acids. This was observed in the experiments on the cultures of primary L-cells of the mice colon, in GLUTag cells, the isolated perfused ileum, as well as *in vivo* in mice, rats and humans [26, 39]. Glutamine, asparagine, phenylalanine and glycine have a stimulating effect on the GLP-1 secretion, with glutamine and glycine being the most active. In the studies on NCI-H716 cells, the stimulating effect of leucine, isoleucine, valine, skim milk, casein and whey on the GLP-1 secretion was proven. The release of GLP-1 is stimulated by L-arginine (also an insulin secretagogue), which has been demonstrated in the experiments in the isolated rat intestines and when administered orally. These effects were absent in the GLP-1R knockout mice [26, 30].

In response to proteins or individual amino acids, the stimulation of the GLP-1 secretion is based on the activation of Ca^{2+} calmodulin-dependent kinase II [26], i.e. the peptide-mediated GLP-1 secretion is a Ca^{2+}-sensitive process and involves L cell signaling through the Ca^{2+}-sensing receptor (CaSR) and peptide transporter 1 (PEPT1). The stimulation of GLP-1 secretion from purified murine L-cell cultures by glycine-sarcosine (Gly-Sar) is blocked in the absence of extracellular Ca^{2+} and is inhibited by the treatment of L-type Ca^{2+} channels with nifedipine. The oligopeptide stimulation of GLP-1

release is reduced in cultured L-cells treated with a CaSR antagonist and increased in the peptide transporter 1 (PEPT1)-deficient mice [4, 26].

Effect of endocrine stimuli on GLP-1 secretion

The specific distribution of L-cells in the intestine suggests the existence of a proximal-distal coordination loop in which neuronal and/or endocrine factors arising in the upper intestine, influence the secretion of GLP-1 by L-cells in the distal region [7, 29]. However, the putative shunt (if it exists) is likely important during the early postprandial phase, when L-cells of the distal intestine are not yet in direct contact with nutrients in the intestinal lumen. L-cells are found in close proximity to both enteric neurons and intestinal microvessels [7], suggesting a possible role for a neuroendocrine regulation of the GLP-1 secretion. The presence of the neuroendocrine regulation of the GLP-1 secretion is confirmed by the results of studies in rodents, in which a direct contact of L-cells in this part of the intestine with nutrients in its lumen is excluded. The introduction of glucose or fat into the duodenum of such rodents very quickly causes the secretion of GLP-1 by L-cells at the level comparable to that observed when they were introduced into the ileum [7, 40].

Neurotransmitters from vagal and intestinal neurons (including acetylcholine and gastrin-releasing peptide) increase the GLP-1 secretion. Acetylcholine receptors, including the muscarinic receptors M1, M2, and M3, are expressed in rat L-cells and human NCI-H716-cells. A nonspecific muscarinic receptor antagonist (atropine) or a selective M1 antagonist (pirenzipine) suppressed the lipid-induced GLP-1 secretion in rats, which was not observed for M2 or M3-selective antagonists [7]. In NCI-H716 cells, the GLP-1 secretion is stimulated by bethanechol (an M2 agonist), which is blocked by the pretreatment with pirenzipine or gallamine (an M2 antagonist) [41]. Together, these data indicate the involvement of muscarinic receptors M1 and M2 in GLP-1 secretion by L cells [4,7].

In the isolated perfused porcine ileum, the GLP-1 secretion is inhibited by the administration of norepinephrine. This effect is blocked by the co-infusion of a non-selective α-adrenergic receptor antagonist (phentolamine) [41]. The GLP-1 secretion is stimulated by a β-adrenergic agonist (isoprenaline) and this effect is blocked by the co-infusion of a β-adrenergic antagonist (propranolol) [41]. These data suggest that the intestinal GLP-1 secretion is stimulated by cholinergic and β-adrenergic receptors signaling but they are inhibited by an α-adrenergic receptor activation.

Gastrin-releasing peptide (GRP) is produced and released by GRP-ergic neurons of the intestinal nervous system and also stimulates the secretion of GLP-1, which was blocked by the administration of a GRP antagonist (BW10). The ability of GRP to increase the GLP-1 secretion has been demonstrated in rat L-cell cultures and in rat ileal preparations. Using the isolated perfused canine pancreas, it was found that GRP also directly stimulates the insulin secretion and delays gastric emptying. The GLP-deficient mice had a decreased glucose tolerance, the first-phase insulin secretion, and GLP-1 in response to oral glucose [42].

Thus, the secretion of GLP-1 is carried out by the L-cells located in the proximal small intestine after the hummus has just left the pylorus. Postprandial glucose concentrations may exceed the absorptive capacity of the proximal intestine, resulting in glucose reaching distant L-cells more quickly. Neuroendocrine reflexes may be involved in the regulation of the GLP-1 secretion. The local increase in GIP stimulates the vagal afferent transmission with a subsequent activation of its efferent and intestinal neurons, which release acetylcholine and/or GLP, which stimulate the secretion of GLP-1 from the distal region [7].

Other factors influencing the GLP-1 secretion include the activation of the olfactory receptor OR51E1 by nonanoic acid, which stimulates the secretion of GLP-1 and PYY in L-cells [43]. Ghrelin stimulates the GLP-1 secretion in L-cell cultures. The peripheral administration of ghrelin enhances the glucose-stimulated GLP-1 secretion, which is not observed in the GLP-1R knockout mice and is blocked by the administration of a ghrelin receptor antagonist (GHRP6) [40].

Effects of GLP-1

Insulinotropic effects of GLP-1

Binding of GLP-1 to its receptor in β-cells activates adenylate cyclase, increasing the concentration of cAMP and activating PKA (phosphorylates the SUR1 subunit of K_{ATP}-channels, shifting the balance towards their closure and depolarization of the cell membrane, which leads to the opening of voltage-gated Ca^{2+} channels (VDC) and exocytosis insulin granules), as well as enhancing signaling through metabolic proteins directly activated by cAMP (Epac) [27, 34, 44]. Up to 50% of GLP-1-induced insulin secreted depends on signaling through Epac, members (Epac1 and Epac2; expressed in β-cells) which contain an

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evolutionarily conserved cAMP-binding domain, allowing them to regulate various biological functions in a cAMP-dependent manner. Epac proteins stimulate the release of Ca^{2+} from the endoplasmic reticulum, increasing the insulin secretion by increasing the pool of intracellular Ca^{2+} [27, 44]. During hyperglycemia, the Ca^{2+} influx into β-cells through VDC channels is significantly increased; Epac2 opens RYR Ca^{2+} channels in the endoplasmic reticulum, further increasing an intracellular Ca^{2+} concentration and, consequently, insulin exocytosis. A calcium-induced calcium release (CICR) determines the dependence of the insulinotropic effect of GLP-1 on the glucose concentration. Therefore, in isolated perfused rat pancreas, at the glucose concentrations <2.8 mmol/L, GLP-1 cannot stimulate an insulin release, which changes when it increases (>6.6 mmol/L) [21, 34, 45]. GLP-1-stimulated insulin exocytosis is partially inhibited by a PKA inhibitor (H89) and is completely blocked by the combination of H89 with anti-Epac2 antiserum (cAMP-GEFII) [4, 45].

In addition to the distinct effect of GLP-1 on the insulin secretion, GLP-1R, by activating PKA, stimulates insulin synthesis in β-cells, which probably occurs due to an increase in the expression of Pdx1 (insulin promoter factor 1) [46].

Effect of GLP-1 on β-cell proliferation and apoptosis

As the age increases, the rate of β-cell replication decreases. In the experimental studies, it was noted that GLP-1R agonists increase the β-cell mass by stimulating their proliferation and inhibiting apoptosis [6, 47, 48]. GLP-1R agonists, by activating the transcription factor CREB, stimulate the expression of the insulin receptor substrate 2 (Irs2, a substrate of insulin-like growth factor 1 and insulin receptor tyrosine kinases), which promotes the growth and survival of β-cells. In mice deficient in Irs2 [47], the β-cell destruction and increased apoptosis were observed. They were accompanied by severe hyperglycemia, which was not eliminated even with a long-term administration of exendin-4, which, by enhancing the CREB phosphorylation, improves the Irs2 function. Streptozotocin-induced β-cell apoptosis was reduced by the administration of GLP-1R agonists, which reduced the oxidative stress and increased the β-cell survival by stimulating anti-apoptotic signaling mechanisms – a PI3-kinase-dependent phosphorylation of protein kinase B (PKB), leading to the inactivation of the proapoptotic protein BAD and the suppression of FoxO1 [44, 49, 50]. Thus, after the activation of GLP-1R in the β-cell, multiple signaling pathways are triggered. They can preserve the mass of β-cells under pathological conditions (the activation of PKA, PKB, CREB, the expression of Pdx1 and Irs2, the inactivation of BAD, etc.). However, according to many authors, the inhibition of apoptosis has a greater therapeutic potential, since the β-cell proliferation decreases with the age and is less pronounced in humans compared to laboratory animals [4, 44, 48, 51].

Effect of GLP-1 on glucagon secretion

The GLP-1 receptor is present in only 10% of pancreatic α-cells [25] and most authors are inclined to believe that GLP-1 inhibits the glucagon secretion not through GLP-1R, but through endocrine mechanisms. GLP-1 stimulates the secretion of somatostatin, which, through paracrine mechanisms, reduces the release of glucagon. This effect was blocked by a somatostatin 2 receptor antagonist (CYN154806) [21, 25]. The effect of GLP-1 on the glucagon secretion can be mimicked by forskolin-induced changes in cAMP. Low concentrations of forskolin (1–10 nmol/L) suppress the glucagon secretion by up to 60%, while high concentrations (0.1–10 μmol/L), on the contrary, stimulate it. The PKA inhibitor (8-Br-Rp-cAMPS) attenuates the glucagon secretion-inhibitory effect of GLP-1. The blockade of N-type Ca2+ channels by ω-conotoxin, but not L-type Ca^{2+} channels by nifedipine, reduces the stimulation of the glucagon secretion by glucose and blunts the effects of GLP-1. Thus, GLP-1 may inhibit the glucagon secretion from α-cells through the PKA-dependent modulation of the N-type Ca^{2+} channel activity in addition to the paracrine action of somatostatin [52]. In addition to the above, GLP-1 indirectly suppresses the glucagon secretion due to its insulinotropic effect (increases the secretion of insulin, amylin, zinc and GABA). Insulin inhibits the glucagon release by activating phosphatidylinositol 3-kinase (PI3K). In α-cells, insulin further enhances the translocation of GABA-A receptors, and when released from β-cells, it enhances the inhibition of the glucagon secretion by glucose. Insulin co-crystallizes with Zn^{2+} in the secretory granules of β-cells and is co-secreted with it during hyperglycemia, and in this case, Zn^{2+} plays an important role in the suppression of the glucagon secretion by insulin [4, 51].

Thus, GLP-1 inhibits the glucagon secretion through several mechanisms involving somatostatin, insulin, Zn^{2+}, GABA and amylin. The significant involvement

of the GLP-1 receptor on α-cells in this process is not supported by all authors.

Cardiovascular effects of GLP-1

The expression of GLP-1R has been noted in blood vessels, as well as in the atria, ventricles, endocardium, endothelium, and smooth muscle cells of coronary vessels [25, 32]. Many studies have revealed the cardioprotective effects of GLP-1R agonists, which are associated with improvements in the endothelial function and myocardial metabolism, as well as the cardiomyocyte survival. In a myocardial ischemia-reperfusion injury, the cytoprotective effect of GLP-1 is associated with the activation of a number of RISK-pathway kinases (Reperfusion Injury Salvage Kinase / RISK / Pathway) – protein kinase A, phosphoinositol 3-kinase (PI3K), protein kinase B and $ERK_{1/2}$, which contributed to a decrease in the permeability of the mitochondrial membrane, protecting cardiomyocytes from apoptosis during the reperfusion injury [53]. Some researchers associate a cardioprotective effect with a GLP-1R-mediated activation of the transcription factor Nrf2 (GLP-1R / PKA(PKB) / CREB / Nrf2), which regulates the expression of antioxidant enzyme genes (glutathione-S-transferase, UDP-glucuronyltransferase, heme oxygenase-1 and etc.) [49]. There is a hypothesis about the vasoactive and cardioprotective role of the GLP-1 metabolite, which is not supported by all researchers.

In healthy volunteers, a single administration of GLP-1, its metabolite or exenatide does not affect the blood flow in the mesenteric or renal arteries, but causes vasodilation of the abdominal cavity and internal organs, which underlies the hypotensive effect of GLP-1 [54]. A meta-analysis of 60 clinical studies found that GLP-1R agonists reduced a diastolic blood pressure by 1.84 to 4.60 mmHg. Art. and increase the heart rate by 2–3.35 beats/min [55].

The mechanisms underlying the effect of GLP-1 on the blood pressure, are not fully understood, but may include vasodilation caused by the nitric oxide secretion, the ability to stimulate the urine and sodium excretion through the kidneys [11, 19].

Effect of GLP-1 on food intake and body weight

GLP-1R is expressed at high densities in the frontal cortex, hypothalamus, thalamus, hippocampus, amygdala, cerebellum, and substantia nigra. These regions are key in the central regulation of the energy homeostasis and autonomic functions [56, 57].

In the hypothalamus, the receptor is mainly distributed in the following nuclei: arcuate (ARC, arcuate nucleus of the hypothalamus, associated with the regulation of appetite), lateral (LHA, lateral hypothalamic area – a hunger center), paraventricular (PVN, paraventricular nucleus of the hypothalamus) – secretes oxytocin, which suppresses appetite entering the ventromedial nucleus (VMH, ventromedial nucleus of the hypothalamus – a saturation center), as well as somatostatin – slows down a gastric motility, dorsomedial (DMH, dorsomedial hypothalamic nucleus – regulation of the blood pressure and the heart rate) and suprachiasmatic (SCN, suprachiasmatic nucleus – circadian rhythms) [8, 57, 58].

The receptor is found in the dorsal complex of the vagus nerve, in the nucleus of the solitary tract (NTS, nucleus of the solitary tract) – one of the nuclei of the medulla oblongata, the processes of its neurons are part of the facial, glossopharyngeal and vagus nerves. The NTS is the entry point for sensory nerves from the internal organs, serves as a switch for vagal reflexes and, due to its connections with the hypothalamus, is a link in the formation of appetite. The peripheral administration of leptin or gastric distension activates GLP-1-producing neurons in the NTS [21, 58].

GLP-1R is expressed at a lower density in the periventricular zones: the subfornical organ and *area postrema* (AP, “posteriormost field,” “chemoreceptor zone” of the brain stem, responsible for the gag reflex). As a neuropeptide, GLP-1 can regulate many autonomic and neuroendocrine functions. It has been shown experimentally and clinically that GLP-1, by inhibiting the activity of the vagus nerve, reduces a gastric motility and the secretion of gastric glands and pancreatic juice [7, 57].

The central regulation of food intake by GLP-1 agonists is not limited to GLP-1R signaling in the hypothalamus and hindbrain. GLP-1 also influences the nutrition by affecting areas of the brain involved in reward, motivation, and addiction, such as the ventral tegmental area (VTA), the nucleus accumbens (NAcc, or Nac, *nucleus accumbens,* a group of neurons in the ventral striatum (belongs to the basal ganglia of the brain), involved in the processes associated with reward, pleasure, addiction, aggression, fear and the placebo effect), lateral septum (LS, lateral septum – a brain area connecting CA3 with the ventral tegmental area for communication reward signals with the context in which they arise) [4, 57, 58].

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There are different opinions about the ability of endogenous GLP-1 to cross the blood-brain barrier (BBB). The studies using radiolabeled GLP-1 have demonstrated its rapid uptake by brain through the endothelium that expresses GLP-1R on its surface. Other authors suggest the passage of GLP-1 into the brain tissue in certain areas with an incomplete BBB, i. e. in the area of the periventricular organs of the brain stem, also known for its high density of GLP-1 receptors. A similar tropism was observed with synthetic GLP-1R agonists (liraglutide and semaglutide). Interestingly, at these sites, the agonist accumulation (required for imaging) is lower in GLP-1R knockout animals. Thus, these experiments also reveal the site of the first activation of the agonist receptor. The large molecule GLP-1R agonists (dulaglutide and albiglutide) are expected to have a low ability to cross the BBB, resulting in less central side effects and less influence on appetite. However, the capillary fenestrae in the pancreatic islets appear to be wide enough to provide an access to these large molecules [19, 59].

Mice with global GLP-1R deficiency had a normal body weight and showed no apparent metabolic abnormalities when maintained on a standard *ad libitum* diet, except mild hyperglycemia during fasting and glucose loading. With an experimental nonspecific blockade of GLP-1R, a specific deletion or GLP-1R inactivation in the central nervous system (including only in the nucleus of the solitary tract – NTS), as well as vagotomy, GLP-1 lost its hypophagic effect [7, 58, 60]. In contrast, a direct intraparenchymal administration of subthreshold doses of GLP-1R agonists into the NTS, the ventral tegmental area (VTA), a paraventricular nucleus (PVN), a lateral nucleus (LN), a ventromedial nucleus (VMN), dorsomedial (DMN), nucleus accumbens (NAcc), ventral hippocampus and lateral septum reduces the food intake, which is blocked by the GLP-1R antagonist exendin (9-39) [58, 61]. The anorexigenic effect of liraglutide is blunted by the genetic removal of GLP-1R from glutamatergic neurons, but is completely preserved when it is removed from GABAergic neurons [62].

The administration of GLP-1R agonists causes the neuronal activation (measured by the cFos activation) in the paraventricular nucleus (PVN), amygdala, area postrema (AP), nucleus tractus solitarius (NTS), and arcuate nucleus (ARC) [7, 51]. The GLP-1R-induced neuronal activation is accompanied by an increased phosphorylation of PKA and MAPK with a corresponding decrease in the AMPK activity (which increases during fasting, inhibits glycolysis, and stimulates food intake). At the same time, the PKA / MAPK activity inhibition by the administration of Rp-cAMP or UO126 weakens the effects of GLP-1R agonists [56, 63].

The activation of GLP-1R inhibits a variety of reward-related behaviors in rodents, such as the amphetamine-associated operant behavior, alcohol consumption and seeking, and place preference (Amp-CPP) test. This is not observed in mice with a CNS-specific deletion of GLP-1R [57, 64]. The activation of the nucleus accumbens (NAcc) by GLP-1R reduces food intake by reducing food palatability. Fed rats given a choice between a regular or high-fat diet and given exendin-4 preferred the regular diet [60]. The injection of exendin (9–39) into the NAcc increases the amount of the energy-dense food eaten and increases the frequency of licking during the first meal, which is an indicator of strong palatability [51, 57, 58, 65]. Thus, GLP-1R agonism reduces homeostatic and hedonic feeding, and modulates food intake not only through the hypothalamus and hindbrain, but also through signaling in the mesolimbic system, in which the GLP-1R activation influences the behavior related to reward and taste perception. .

A direct electrical stimulation of the nucleus tractus solitarius (NTS) induces glutamatergic excitatory postsynaptic currents (EPSCs) in Gcg^+ positive neurons. Leptin induces a rapid depolarization of Gcg+ neurons in the NTS, as detected by voltage and current measurements in the whole-cell horizontal or coronal sections of the brainstem, impairing its ability to directly stimulate the central GLP-1 secretion. Hindbrain Gcg^+ neurons do not have GLP-1R and cannot be directly activated by GLP-1. The administration of PYY, melanotan II, or ghrelin did not stimulate these neurons in isolated NTS brain slices, but they responded to the stimulation by leptin, cholecystokinin, and epinephrine [8, 66]. The leptin receptor expression was detected in GLP-1-secreting NTS neurons. This may mean that peripheral endocrine stimuli (e.g., leptin), through this mechanism, may trigger the central activation of GLP-1-secreting NTS neurons.

A cholecystokinin-induced activation of NTS Gcg^+ neurons is blocked by the glutamate receptor antagonist (DNQX) or the inhibition of the α1-adrenergic signaling [66], these neurons perceive various peripheral signals and, in response to them, regulate the energy balance. A permanent blockade of GLP-1R in the central nervous system or its viral knockdown is accompanied by an increase in body weight in rats, while a chemogenetic stimulation of Gcg^+ neurons, on the contrary, caused

a decrease in food consumption [67]. An acute chemogenetic inhibition of these neurons did not increase food intake, but did increase it after stress-induced hypophagia [68]. Thus, glutamatergic GLP-1-producing neurons in the NTS are activated by a number of peripheral signals and regulate many aspects of feeding behavior.

Effect of GLP-1 on energy homeostasis

Non-shivering thermogenesis requires a functional brown adipose tissue, so it is reasonable to believe that GLP-1R contributes to the control of the energy expenditure by regulating the activity of brown adipose tissue through the receptors in the CNS, since it is expressed in brain nuclei involved in the control of brown adipose tissue metabolism. The administration of GLP-1 into the dorsomedial nucleus of the hypothalamus (DMH) increased thermogenesis in brown adipose tissue, and the disruption of local GLP-1R expression on rat DMH neurons led to an increase in body weight, which was accompanied by a decrease in the heat production by brown adipose tissue [58, 69].

Thus, GLP-1Rs of brain are involved in the control of the energy expenditure, primarily by regulating food intake and also regulating the energy expenditure. But the studies of these GLP-1 effects are limited due to their low penetration ability through the BBB and their distinct effect on the appetite and gastrointestinal motility, as well as the physiology of GLP-1R in rodents.

Effect of GLP-1 on gastric emptying

The activation of GLP-1a leads to a decrease in gastric motility and the hydrochloric acid secretion, both when administered peripherally and centrally. The observed effect was not reproduced in GLP-1R knockout mice, or after vagotomy in humans, and blocking the GLP-1 receptor, on the contrary, led to the accelerated gastric emptying [63]. However, with a long-term administration, GLP-1R agonists have a reduced effect on gastric motility due to developing tachyphylaxis [70].

Effect of GLP-1 on hypothalamic-pituitary-adrenal axis

Nucleus tractus solitarius (NTS) neurons are involved in regulating the hypothalamic-pituitary-adrenal axis in response to stress, and GLP-1R signaling in the brain plays an integral role in the acute CNS response to stress. Preproglucagon-positive NTS neurons have dense projections to the PVN area of the hypothalamus, where they innervate corticoliberin-releasing neurons [4, 51, 57, 58]. GLP-1R is also expressed in PVN neurons, which are colocalized with corticoliberin [71]. The administration of GLP-1 into the CNS of rats and mice activates the hypothalamic-pituitary-adrenal axis and increases the corticosterone secretion. In rodents and humans, peripherally administered GLP-1R agonists transiently stimulate the hypothalamic-pituitary-adrenal axis and increase concentrations of corticosterone, aldosterone, and ACTH [59]. Mice with a PVN-selective deletion of GLP-1R have an impaired stress-induced activation of the hypothalamic-pituitary-adrenal axis, which prevents stress-induced weight loss, reduces cardiovascular responses and anxiety [71]. The combined administration of dexamethasone and exendin-4 leads to a greater anorexigenic effect and weight loss than the GLP-1 agonist alone. By the administration of anti-dopamine-β-hydroxylase-saporin (DSAP), the ablation of catecholamines in hindbrain neurons blunts the ability of exendin-4 to increase the corticosterone secretion but potentiates the hypophagic effect of exendin-4.

Effects of GLP-1 on learning, memory and neuroprotective effects

The GLP-1 receptor is expressed in the hippocampus, an area of the brain involved in spatial learning and memory. The central GLP-1R agonism improves some aspects of learning and memory in the Morris water maze and increases latency in the passive avoidance test. The improvement in learning and memory by the GLP-1 administration is blocked by the pre-administration of exendin (9–39) and is absent in GLP-1 receptor-deficient mice [72].

Central GLP-1R signaling is neuroprotective. GLP-1 and exendin-4 enhance the differentiation and neurite outgrowth in rat pheochromocytoma (PC12) and human neuroblastoma SK-N-SH cells. The effect of GLP-1R agonists is blocked when PC12 cells are co-incubated with exendin (9–39). GLP-1 and exendin-4 protect cultured hippocampal neurons from glutamate-induced apoptosis [56, 73]. The duration and severity of seizures induced by the systemic administration of the neurotoxin kainic acid was greater in GLP-1R knockout mice than in wild-type mice and shorter in the mice that had undergone the targeted restoration of the GLP-1R expression in the hippocampus using an adeno-associated virus (AAV).

The neuroprotective effects of the central GLP-

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1R agonism are mediated by the ability to increase the formation of cAMP and enhance the activation of PI3-kinase and ERK. The GLP-1R agonism increases cAMP levels in cultured hippocampal neurons and PC12 cells. The pharmacological inhibition of either PI3-kinase or ERK blocks the stimulatory effect of GLP-1 and exendin-4 on neurite outgrowth in PC12 cells. In PC12 cells, the stimulation of the axonal growth by GLP-1 is partially suppressed by the PKA inhibitor (H89), suggesting the cAMP-mediated activation of PI3K and ERK after the GLP-1R agonism and is not entirely dependent on PKA signaling [56].

The impaired insulin sensitivity may be a causative factor in the neurodegeneration in Huntington's disease patients [74]. The overexpression of mutant huntingtin protein disrupts insulin signaling and stimulates neuronal apoptosis in human neuronal SK-N-MC cells, and liraglutide improves the insulin sensitivity and increases the viability of such cells by mechanisms including: reducing neuronal glucotoxicity, oxidative stress and mutant protein aggregation through the stimulation of AMPK-mediated autophagy [73, 74].

In a neurodegeneration rat model, GLP-1 and exendin-4 reduced an ibotenic acid-induced depletion of cholinergic neurons of the basal forebrain, which was reflected in the greater preservation of the choline acetyltransferase immunoreactivity of these cells. The administration of GLP-1R agonists into the hippocampus prevents learning and memory impairment caused by the administration of amyloid β [75]. GLP-1R agonists, when administered to mice, reduce the progression of Alzheimer's disease (AD) [76, 77]. In asthma, a glucose transport across the BBB is reduced, and a 6-month administration of liraglutide in asthma patients largely neutralized this process [78]. In another study, a 12-week treatment with liraglutide in the patients at risk of AD did not affect cognitive processes, as no differences had been noted between cohorts [79]. Clinical studies of the neuroprotective effects of GLP-1R agonists in AD are ongoing [80].

GLP-1 analogues have shown some effectiveness in the treatment of Parkinson's disease (PD), which is characterized by the degeneration of dopaminergic neurons, which is experimentally achieved by administering the dopaminergic neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). In the mice treated with this toxin, a 7-day infusion of exendin-4 into the lateral ventricle significantly reduced the damage to the dopaminergic system and the development of motor disorders [81]. Similar neuroprotective effects of GLP-1 agonists have been noted in various models of PD [82]. In primary cultures of neurons treated with the dopaminergic toxin 6-hydroxydopamine (6-OHDA), exendin-4 increased their survival and levels of tyrosine hydroxylase, a key enzyme in dopamine synthesis [81]. Several clinical studies have confirmed the ability of GLP-1R agonists to improve clinical symptoms of PD, as well as motor and cognitive functions [83].

The administration of liraglutide or semaglutide in a model of ischemia-reperfusion injury of the brain led to a dose-dependent reduction in the infarct size by up to 90% (and, accordingly, a neurological deficit after 24 hours) after a 90-minute (a moderate severity of ischemia) occlusion of the middle cerebral artery. However, a preliminary administration of the GLP-1R antagonist exendin (9–39) reduced the protective effect of the drugs, and the neuroprotective effect of GLP-1 agonists was significantly reduced when choosing a more severe type of ischemia – for 120 or 180 min [84].

Effect of GLP-1 on water intake and kidney function

In humans, GLP-1R has been identified in proximal tubule cells and preglomerular vascular smooth muscle cells. GLP-1 has a dose-dependent effect on the renal function by reducing water intake and stimulating a urine and sodium excretion (stimulation of proximal tubular natriuresis) for a short time after its intracerebroventricular or intraperitoneal administration. Moreover, the effect was centrally mediated and did not depend on food intake, and was blocked by exendin (9–39). GLP-1 probably induces natriuresis and diuresis by inhibiting sodium-hydrogen exchanger 3 (NHE3), located on the brush border of renal proximal tubular cells. This effect may partly explain the hypotensive effect of GLP-1R agonists. Through the modulation of cAMP/PKA signaling, GLP-1 influences the inflammation, including the kidney and blood vessels, likely protecting the kidney from the oxidative damage [11, 85].

In clinical trials, GLP-1R agonists primarily reduced the incidence of albuminuria in the absence of a clear evidence of an effect on severe renal outcomes [11, 19, 85].

Effect of GLP-1 on bone tissue

In addition to glucose homeostasis, incretins are involved in the regulation of the energy-consuming

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process of bone remodeling: they inhibit the resorption and stimulate the bone tissue formation. The existence of an enteroendocrine-bone axis is indicated by the accumulated data on the influence of factors such as long-term parenteral nutrition, bariatric surgery, and a hereditary dysfunction of the GIP on the incidence of osteoporosis [30, 86]. In the animals' knockout for receptors for two incretins, $GIPR^{-/-}$ and $GLP\text{-}1R^{-/-}$, a decrease in the bone strength and a slowdown in collagen synthesis were observed [87].

The GLP-1 receptor is expressed in some human osteoblast cell lines (MG-63 and TE-85), which, however, has not been observed for the Saos-2 line. At the same time, GLP-1 increased the viability of MG-63 and TE-85 cells. GLP-1 receptor knockout mice suffer from osteopenia and have increased skeletal fragility. Liraglutide slowed bone loss in the rats with glucocorticoid-induced osteoporosis and in ovariectomized diabetic rats. It should be noted that, unlike in humans, in rodents, the activation of GLP-1R in thyroid C cells promotes the release of calcitonin, which inhibits the bone resorption. This somewhat reduces the translational potential of the studies results of GLP-1R agonists obtained at the preclinical stage [86]. GLP-2, which is co-secreted with GLP-1, also plays an essential role in functioning of the enteroendocrine-bone axis. The GLP-2R receptor (GLP-2R) is widely expressed in human osteoclasts and can regulate their activity. In clinical studies, GLP-2 was shown to reduce markers of the bone resorption in healthy and postmenopausal women [10, 86].

Most of the data in the literature are on the GIP effect on bone tissue remodeling. It dose-dependently reduced the differentiation and the bone resorptive activity of murine and human osteoclasts and also inhibited the parathyroid hormone-induced increase in the bone resorption. GIP also reduced apoptosis in the human bone marrow mesenchymal stem cells and Saos-2 osteoblastic cells. The administration of GIP to healthy people led to a decrease in the level of type I collagen C-telopeptide (CTX I), a marker of the bone tissue resorption, and an increase in the level of type 1 procollagen N-terminal propeptide (P1NP), a marker of the bone matrix formation. Thus, GIP may have an anabolic effect on the bone in addition to the inhibiting bone resorption [86].

GIP and GLP-2 are key regulators of postprandial bone remodeling in humans in the enteroendocrine-bone axis. Their combined administration to healthy people resulted in an additive reduction in the bone resorption, exceeding the effect of each hormone separately. Coagonists of the GIP and GLP-2 receptors being developed are considered a promising approach for the treatment of osteoporosis [9], but currently published results are contradictory. Thus, the GIP/GLP-1/GLP-2 receptors may become promising pharmacological targets for the prevention of fractures in the patients with osteoporosis and possibly also other bone diseases, such as patients with diabetes [88].

Pharmacology of GLP-1

In the structure of the pharmaceutical market of hypoglycemic agents in the Russian Federation, GLP-1R agonists in 2016 accounted for 0.09% (27 071) in packages and 2.3% (229.3 million) in rubles, and in 2019 and 2020, these figures were significantly higher than 0.3% (141 541), 4.41% (189 512) and 8.55% (1.5 billion), 10.56% (2 billion), respectively (Fig. 2 A, B). Moreover, the cost of such drugs is the highest on the market (Fig. 2 B). It should be noted that in 2016 there were only 4 drugs on the market – exenatide (Bayeta®, Astra Zeneca, UK), lixisenatide (Lixumia®, Sanofi, France), liraglutide (Victoza® and Saxenda®, Novo Nordisk A/S, Denmark), and in 2020, dulaglutide (Trulicity®, Eli Lilly and Company, Switzerland), semaglutide (Ozempic®, Novo Nordisk A/S, Denmark) and combinations with insulin analogues (degludec or glargine) were added. Considering the data presented (Fig. 2), the market for hypoglycemic drugs containing GLP-1R agonists can be considered rapidly developing. Currently, the modification of degradation-resistant GLP-1R agonists is mainly aimed at improving pharmacokinetic parameters.

GLP-1R agonist drugs

Exenatide is a synthetic peptide of 39 amino acids (Fig. 3) (first discovered as exendin-4 in the saliva of the Arizona common snake *(Heloderma suspectum),* the first 30 of which are 53% homologous to the mammalian GLP-1. Exenatide has glycine at the second amino acid position. on the N- end, which protects the peptide from the DPP-4-mediated degradation and inactivation. They also differ in a number of amino acids in the central and C-terminal domains, which include Leu10, Lys12, Gln13, Met14, Glu16, Glu17, Tyr19, Arg20, Leu21, Glu24, Lys27, Asn28 and Gly30. The C terminus of exendin-4 is 9 amino acids larger than GLP-1, which maintains the secondary structure through a tryptophan cage formation and increases the selectivity for GLP-1R [89–

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91]. The exendin-4 of half-life in rats is 18–41 minutes after intravenous, 125–174 min after intraperitoneal and 90–216 minutes after the subcutaneous administration, its bioavailability is higher than that of GLP-1 (7–36 amide) and GLP-1, plasma clearance is 4–8 ml/min. The mode of application is twice a day. Amylin Pharmaceuticals has developed a once-weekly formulation of exenatide under the brand name Bydureon® (AstraZeneca, marketed since 2012). In this formulation, exenatide is formulated in a sustained-release microsphere containing a 50:50 poly(D,L-lactide-glycolide) polymer (37.2 mg per dose) along with sucrose (0.8 mg per dose) [6, 90, 92].

At the time of its introduction, exenatide was a promising treatment for T2DM, the significant drawback of which was the requirement for 2 daily injections. Therefore, Intarcia Therapeutics developed the ITCA 650 device – an osmotic mini-pump the size of a match (4x44 mm), implanted subcutaneously into the abdominal wall and delivering microdoses of exenatide into the body for up to 6 months. Four clinical trials showed significant reductions in HbA1c and body weight, but the FDA denied its approval in 2017 due to manufacturing issues [6, 90], and in 2021, the FDA again denied the approval for this approach due to the increased risk development of vascular diabetes complications on the use of ITCA 650.

VRS 859 (exenatide-XTEN, Versartis Inc., USA) is a combined protein, with a uniform and stable absorption, containing exenatide and a hydrophilic end of 864 amino acids (XTEN technology, Amunix Inc), which increased the half-life from 2.4 to 139 h in humans and should theoretically lead to the long-lasting glycemic control [90]. The studies of this compound in mice have shown that after the intraperitoneal administration of the drug at a dose of 120 nmol/kg, the glucose tolerance persists for up to 48 hours (after the administration of exenatide, a similar improvement lasts up to 1 hour). It is suggested that a single subcutaneous dose of 100 mg of VRS-859 can provide plasma drug levels sufficient to provide a glycemic control for 1 month [92].

Efpeglenatide (HM11260C, Langlenatide, LAPS-Exendin, LAPS-Exd4). In this drug, exendin-4 is coupled to the non-glycosylated Fc fragment of human immunoglobulin through a non-peptide linker (unlike dulaglutide, which has only one peptide variant fused to the Fc carrier) to reduce the immunogenic potential. Efpeglenatide has a half-life of >150 h and is in phases II and III of clinical trials (a weekly or monthly dosage) [90]. In obese nondiabetic patients, in a 20-week study, efpeglenatide (4, 6, 8 mg once every 7 or 14 days) significantly reduced body weight (6.2–7.8 kg *vs* placebo –0.8 kg), no serious adverse events were observed [6, 51].

Albenatide (CJC-1134-PC, ConjuChem, USA) is exendin-4 linked to the C-terminus of recombinant human albumin, forming a special conjugate complex (Preformed Conjugate-Drug Affinity Complex, PC-DAC) through a linker with a maleimide terminal, which is used for a chemical conjugation with a single cysteine residue in albumin. In humans, the half-life of CJC-1134-PC is approximately 8 days. The results of clinical trials have not yet been published [6, 31, 92].

Thus, despite the fact that exenatide was the first registered drug from the group of GLP-1R agonists and its relatively small similarity (in terms of the amino acid sequence) with the human hormone, it continues to be studied and modified, new dosage forms / combinations are being developed; they prolong and simplify its application and increase its efficiency.

Lixisenatide is an analogue of exenatide in which the proline at position 38 is omitted and six consecutive lysine residues with pharmacokinetic characteristics comparable to exenatide, are added to the C-terminus. Used once a day; increasing the administration frequency does not increase the effectiveness of a hyperglycemia control [6, 92]. In Russia, a fixed combination solution for a subcutaneous administration of lixisenatide (33 and 50 μg/ml)+insulin glargine (100 U/ml) is registered.

Liraglutide is designed on the native GLP-1 sequence (7–37) with a (conservative) substitution of lysine at position 28 for arginine. At the second position from the N-terminus, alanine is retained, but the lysine at position 20 is linked through a gamma-glutamic acid spacer to palmitic acid (C16:0), which, by binding to albumin, makes the drug less sensitive to DPP-4 proteolysis. Liraglutide is 97% homologous to GLP-1. The molecule modification contributed to the increased bioavailability, and its half-life increased to ~ 12 h. Used once daily at doses of 0.6–1.8 mg/day, and at a dose of 3 mg/day, liraglutide has been approved for the treatment of obesity since 2014 [92 ,93].

Semaglutide is an analogue of liraglutide, 94% homologous to GLP-1. The DPP-4-sensitive alanine at the second N-terminal position in liraglutide is replaced by aminoisobuturic acid (Aib), and the palmitic (C16:0) fatty monoacid in liraglutide is replaced by dicarboxylic

stearic acid (C18:0). These chemical modifications extend the half-life of semaglutide to 160 hours. It is used once a week. High-dose semaglutide is undergoing clinical trials for efficacy in obesity without T2DM. Based on semaglutide, the first oral GLP-1R agonist was created – drug Rebelsas® (Novo Nordisk A/S, Denmark), the effects of which are comparable to GLP-1R agonists administered subcutaneously. To facilitate the absorption in the gastrointestinal tract (to increase lipophilicity) and protect the peptide drug from the enzymatic destruction, the absorption enhancer N-(8-[2-hydroxybenzoyl]amino) sodium caprylate was used [89, 93].

Dulaglutide consists of two identical disulfide-fused GLP-1 molecules that are linked by a polypeptide chain (glycine and serine spacer) to a heavy chain fragment (Fc) of modified human immunoglobulin G4 (IgG4) to reduce an immunogenic potential. The GLP-1 fragments of dulaglutide are 90% homologous to the native one (some fragments of native GLP-1 are replaced by parts of exendin-4). Glycine at the second N-terminal position protects the molecule from DPP-4 inactivation, while glutamic acid at position 16 stabilizes the secondary structure and improves its potency. The glycine substitution at position 30, along with the native glycine at the C terminus of GLP-1 (7–37), serves as a leading sequence for the spacer that anchors the Fc fragments of IgG4. Such modifications improve the bioavailability, slow down the renal clearance and reduce the immunogenic potential. It is to be used once a week [6, 92].

Albiglutide is a head-to-tail tandem of two GLP-1 molecules, in which the C-terminus of the first molecule is fused to the N-terminus of the second. Each of the two GLP-1 molecules is replaced by glycine in the DPP-4-sensitive fragments. The C-terminus of the second GLP-1 is covalently fused to human albumin, which slows down renal clearance, increasing the half-life to ~120 h in humans [92]. It is to be appied once a week.

Taspoglutide (R1583/BIM51077; Hoffmann-La Roche, Switzerland) is a long-acting analog of human GLP-1 containing aminoisobutyric acid, 10% (Aib 8–35) of human GLP-1 (7–36 amides) with 93%. homology to the native polypeptide. In a phase III study, it effectively reduced HbA1c and body weight when administered weekly subcutaneously at doses of 10 and 20 mg. However, undesirable reactions such as nausea, vomiting and allergic manifestations occurred more often than when taking exenatide at a dose of 10 mcg twice a day. The development of taspoglutide was discontinued in 2010 [89].

Glymera (Glymera, PB1023, PhaseBio Pharmaceuticals, USA) is a recombinant analogue of GLP-1, a polypeptide consisting of 636 amino acids, genetically fused with a physiologically inert polymeric elastin-like peptide of E. coli, and is administered subcutaneously once a week (in phase III clinical trials). The efficacy of weekly doses (50, 70 and 100 mg) was compared with once-daily liraglutide and placebo in a phase IIb study (600 patients with T2DM, 20 weeks). The effectiveness of the glymera administration was inferior to liraglutide [90].

Common adverse reactions to GLP-1 analogs are nausea, vomiting, and diarrhea. These effects are dose-dependent, and in some cases are perceived as potentially beneficial by reducing meal frequency and quantity, thereby promoting weight loss. Compared with short-acting GLP-1 analogues (such as exenatide), long-acting ones are less likely to cause nausea and vomiting, but more often cause diarrhea [51].

In the literature, there is also limited information about the developed peptide GLP-1R agonists ZYOG1 (Zydus Cadila, India) and ARI-1732TS (Arisaph Pharmaceuticals, USA), the studies of which are at phase 1 and the preclinical stage, respectively [90].

According to the information available in the State Register of Medicines, the following GLP-1R agonists and their combinations – INN (trade name – name of the holder or owner of the registration certificate of the medicinal product) are registered in Russia:

– exenatide (Bayeta®, Astra Zeneca, UK);

– lixisenatide + insulin glargine (Soliqua SoloStar®, Sanofi Winthrop, France);

– liraglutide (Victoza®/Saxenda® – Novo Nordisk A/S, Denmark; Quinliro® – Biokhimik JSC, Russia; Enligria® – Promomed Rus LLC, Russia);

– liraglutide+insulin degludec (Sultophy® – Novo Nordisk A/S, Denmark);

– dulaglutide (Trulicity® – Swix Healthcare LLC, Russia);

– semaglutide solution for the subcutaneous administration; (Ozempic® – Novo Nordisk A/S, Denmark; Quincenta® – Promomed Rus LLC, Russia; Semavik® – GEROPHARM LLC, Russia),

– semaglutide tablets (Rebelsas® – Novo Nordisk A/S, Denmark).

As part of the import substitution of foreign drugs with Russian analogues, GLP-1R agonists are of great

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interest to domestic pharmaceutical companies. A particular attention is drawn to the most studied drug, liraglutide, as well as semaglutide, a GLP-1R agonist with a long period of action [94, 95]. At the same time, as for liraglutide, taking into account a relatively small size of the peptide and its lack of tertiary structure, it is considered expedient to produce API through chemical synthesis, which is assessed as a highly productive, scalable and commercially viable process that can produce a high-purity product. The results of a comparative study of liraglutide obtained in this way (Enligria®, a solution for a subcutaneous administration 6 mg/ml, Promomed RUS LLC, Russia) showed similar to the original drug (Saxenda®, a solution for a subcutaneous administration 6 mg/ml, Novo Nordisk A/S, Denmark) physicochemical and biological properties [94].

Non-peptide GLP-1R agonists

As stated above, the activation of GLP-1R by endogenous GLP-1 requires an extensive action on the receptor complex, including the interaction of the C terminus of GLP-1 with the peptide-binding groove of the N-terminal extracellular domain (ECD) of the receptor, followed by the approach and interaction of the N terminus of the peptide GLP-1 with a transmembrane domain (TMD). This allows the interaction of the intracellular half of the TMD with the G protein, a signal transmission, and ultimately leads to exocytosis of insulin-containing vesicles. Mimicking the initial multiple extensive interactions with the ECD and TMD of the GLP-1 receptor seemed unrealistic for non-peptide small molecules, for which, in addition, different features of interactions with the receptor complex had been proposed [20, 22, 23]. However, several non-peptide agonists are currently in clinical trials: PF 06882961 (Pfizer, USA), TTP-273 (vTv Therapeutics/Huadong Medicine, China) and OWL-833 (Chugai/Eli Lilly, USA), which indicates significant progress in overcoming this problem [96].

The non-peptide agonists LY3502790, PF-06882961 and CHU-128 (Fig. 4) are characterized by a specific interaction with the GLP-1 receptor: the activation of the G-protein signaling activity only in the GLP-1 receptor with Trp33 (ECD). This was an unexpected finding because primate-specific Trp33 (ECD) served as a critical point for binding small molecules, but not native GLP-1. Non-peptide agonists induce changes in the GLP-1R conformation through van der Waals interactions and hydrogen bonds of Trp33 (ECD) with extracellular loops ECL 1 and ECL2 instead of a direct interaction of the peptide with ECL2 [96]. TTP-273 has unique kinetic and signaling properties and has a distinct binding mode compared to GLP-1 [97].

TTP273 (Transtech Pharmaceuticals (TTP), later renamed as vTv Therapeutics, USA) is being developed as an oral GLP-1R agonist with a half-life of about 6 hours. Its 2-week administration caused a pronounced dose-dependent decrease in glycemic levels, the blood pressure (systolic by 8 mm Hg, placebo – 2 mm Hg, diastolic up to 5 mm Hg, placebo – 1 mm Hg), triglyceride levels (by 2.8 mmol/L compared with placebo 1,7 mmol/L) and body weight: by 2 kg compared to placebo – 0.6 kg. In a 12-week multicenter study, T2DM patients receiving metformin additionally received TTP273 (150 mg once or twice daily) or placebo. While taking TTP273, the following placebo-adjusted values were observed: HbA1c –0.86 and –0.71%, respectively (placebo – HbA1c +0.15%). A weight reduction was observed by an average of 0.9 and 0.6 kg when taking TTP273 once and twice daily, respectively. Study 2, LOGRA (aLlosteric Oral GLP-1R Agonist), assessed the safety and efficacy of TTP273 in T2DM patients at a stable dose of metformin, but the results have not yet been published. TT-OAD2 is a weaker analogue of TTP273 from the same developer with slow kinetics, but a revealed structure. In HEK293 cells (with a high density of GLP-1R), the compound affects cAMP without recruiting β-arrestin-1 [20, 90, 97, 98].

Compound RGT1383 is a full GLP-1R agonist, comparable to GLP-1, increases cAMP with an EC50 value of about 0.2 nmol/L and a partial agonist of the β-arrestin recruitment at the level of ~ 30%. RGT1383 binds to the orthosteric binding pocket through an inward movement of the extracellular loop of ECL3 and the extracellular end of TM7. In addition, the Trp33 extracellular N-terminal domain (ECD) plays a critical role in binding RGT1383 to the human GLP-1 receptor [20, 97].

Danuglipron (PF-06882961, Pfizer Inc, USA) is a member of a series of pyrimidine derivatives that, in *in vitro* studies, exhibits a GLP-1R agonism higher than that of some closely related peptides (exendin-4, liraglutide and endogenous oxyntomodulin). PF-06882961 increases cAMP production (EC_{50}=13 nM) and partially increases Ca^{2+} levels, pERK1/2 recruitment and β-arrestin. The binding sites for PF-06882961 and LY3502970 or CHU-128 largely overlap, although each occupies a different position. This significant overlap

explains the species specificity of compounds PF-06882961 and LY3502970 [99]. Orforgliprone LY3502970 (OWL833, Chuai Pharmaceutical, Japan and Eli Lilly, USA) is a non-peptide partial agonist of GLP-1R for the oral use. In efficacy studies, the oral administration of this compound reduced glucose levels in humanized GLP-1R transgenic mice, as well as insulinotropic and hypophagic effects in non-human primates, at levels comparable to exenatide [20, 22]. The analysis of seven randomized controlled trials of orforgliprone and danuglipron showed significant reductions in body weight and HbA1c levels with a low risk of hypoglycemia, but a high incidence (more than 50%) of gastrointestinal adverse events (nausea and vomiting) may significantly limit the prospects of such drugs [100].

Some of the first non-peptide GLP-1R agonists described are substituted cyclobutane compounds, exemplified by the compounds Boc5 (a full agonist) and S4P (a partial agonist). These compounds do not activate cells without GLP-1 receptors or cells expressing glucagon receptors (GcgR) or GLP-2 receptors, and their agonism is blocked by exendin (9–39). Despite a high degree of mimicking the effects of peptide GLP-1R agonists, the Boc5 compound and its more active derivative have not received any further development as oral drugs [20].

Allosteric modulators of GLP-1R are being developed as drugs that, by binding to various allosteric sites of the receptor, can enhance the action of endogenous peptide agonists of the GLP-1 receptor (Fig. 5). The first compounds exhibiting a similar pharmacological activity were developed by Novo Nordisk (based on quinoxalines). The compound synthesized by this company is a complete and highly selective GLP-1R agonist (exendin (9–39), the effect is absent in the animals knockout for the target receptor), increases the binding affinity of GLP-1 to the receptor, but not its activity. The designated compound is less active than GLP-1, exenatide or liraglutide in stimulating the insulin secretion by BRIN-BD11 cells. Quinoxaline derivatives require the optimization to improve their chemical stability and pharmacokinetics. The 2-thio-quinoxaline analog compound DA-15864 increases a glucose-stimulated insulin secretion and acts synergistically with GLP-1, significantly increasing peak plasma insulin levels. It has also been reported that the combined use of quinoxaline-based allosteric modulators with exendin-4 has a pronounced neuroprotective effect, which was mediated by the stimulation of GLP-1R through the cAMP-PKA-CREB signaling pathway [20].

The pharmaceutical company Domain Therapeutics has developed a series of quercetin-like flavonoids (flavones, isoflavones and catechins), which are allosteric modulators of GLP-1R [20], but have not been developed as drugs.

Eli Lilly (USA) has released a number of agonists and positive allosteric modulators of GLP-1R based on pyrimidine, which are optimized to increase the affinity for GLP-1R and the effectiveness of inactive GLP-1 (9–36) – the main metabolite of GLP-1(7–36). The BETP activity is not blocked by exendin-4 (9–39); in *in vivo* studies, the compound stimulates an insulin secretion in rats and the oxyntomodulin-stimulated insulin secretion, indicating its ability to initiate biased signaling through oxyntomodulin-mediated GLP-1R. Competitive binding studies showed that LSN3160440 cooperatively modulates the binding affinity and efficiency of GLP-1 (9–36) to activate the GLP-1 receptor. The compound LSN3160440 in *in vitro* and *in vivo* studies enhanced the activity and effectiveness of GLP-1 (9–36) in activating its receptor. Co-addition of LSN3160440 and GLP-1(9–36) to isolated mouse β-cells or the administration to Wistar rats significantly increased a glucose-dependent insulin secretion (at the levels comparable to GLP-1). This compound is the only reported allosteric receptor modulator that simultaneously interacts with both the orthosteric ligand and the receptor [20, 101].

Malik F. et al. developed an innovative high-throughput screening system that identified compounds VU00056556 and VU0109197 with a common hexahydroquinolone carboxylate core that interacted with GLP-1R more tightly than native GLP-1. Once the lead compound was identified, its structure was optimized, resulting in VU0453379, which exhibits a biased GLP-1R agonism (a highly selective agonist) and weakly affects a β-arrestin recruitment, with an acceptable metabolic and pharmacokinetic profile. This compound is the first to cross the BBB, which may be important for the development of central GLP-1R agonists [20].

Compounds HIT-465 and HIT-736 (their structure has not been disclosed) have a high bioavailability and a long half-life and are biased modulators of GLP-1R, but the allosteric binding site is different from that of the compound developed by Novo Nordisk A/S [20].

Ethanolic extracts from fenugreek seeds (*Trigonella foenum-graecum* L.) enhance GLP-1 signaling, and their fractionation and purification led to the isolation of compound N55 (N-linoleoyl-2-amino-γ-butyrolactone).

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In *in vitro* studies, this compound promotes a GLP-1-dependent cAMP accumulation and dose-dependent endocytosis of GLP-1 receptors. Compound N55 has a unique mechanism of action – instead of binding to the allosteric site of GLP-1R, like other known modulators, N55 directly binds to GLP-1(7–36)NH_2. Binding of N55 to GLP-1 may induce conformational changes in GLP-1 (7–36)NH_2, thereby inhibiting its degradation and exposure to trypsin. Therefore, N55 represents a new class of allosteric modulators of GLP-1R, and similar effects on GLP-1 may have a potential to control the activity of this receptor [20, 102].

A group of scientists from Sanofi-Aventis Deutschland GmbH developed compound N14 based on 3,4,5,6-tetrahydro-1H-1,5-epiminoazocino[4,5-b] indole, which is the most potent non-covalent allosteric modulator of GLP-1R, it stimulates an insulin secretion and has acceptable pharmacokinetic and pharmacodynamic characteristics [20].

All known non-peptide GLP-1R agonists bind to it predominantly in the helical bundle of the receptor, with a binding pocket that overlaps with that of GLP-1 in a manner that is either similar or completely different. The multiple active conformations of GLP-1R result in varying efficacy and biased agonism of the substances. Allosteric binding sites for GLP-1R are located at several locations throughout its structures – on the GLP-1 peptide itself and in intra- and extracellular areas – and allosteric modulators influence the affinity and effectiveness of orthosteric ligands.

Combination therapy based on GLP-1

Currently, combination forms with insulin degludec (Sultophy®, Novo Nordisk A/S, Denmark) or glargine (Soliqua SoloStar®, Sanofi, France) are used. The creation of such combinations is logical and understandable from the point of view of increasing the effectiveness of hypoglycemic therapy (due to the synergy in the action of insulin and GLP-1R agonists) and marketing (expanding the product portfolio by combining two of the company's drugs into one). Combining GLP-1R analogues with basal insulin reduces HbA1c faster and more significantly compared to monotherapy [103].

Various approaches to combining GLP-1R agonists include their combination with amylin, glucagon [104], leptin, salmon calcitonin, PYY, cholecystokinin, melanocortin-4 receptor (MC4R) agonists [105], various insulin analogues [103], adrenomedullin [107] and β3-adrenergic receptor agonists [108], cannabinoid receptor 1 (CB1) agonists/antagonists [109], and bile acid receptor agonists (farnesoid-x (FXR), NR1H4) [110].

Multitarget molecules based on GLP-1

The creation of multitarget molecules that will interact with multiple receptors is potentially more attractive than monotherapy or combinations of individual drugs for several reasons. First, it is easier to obtain a marketing authorization for a molecule than for a combination. Second, each substance in the combination has unique pharmacokinetic profiles that are equalized when the molecules are fused, limiting an interindividual variability in metabolism and pharmacokinetic interactions of individual structures. At the same time, the activity of one molecule is constant, and in combination it can be titrated by changing the ratio of the components in the mixture, which is especially important if one of them has a narrow therapeutic window.

The possibility of using glucagon as part of a multifunctional molecule with hypoglycemic effects was not initially considered, but its catabolic properties (lowering lipid levels) are attractive, especially if its hyperglycemic effects can be reduced. One possible candidate that interferes with or compensates for the counterinsular action of glucagon is GLP-1, while at the same time providing the full range of effects inherent to this hormone. Thus, the co-administration of GLP-1 and glucagon led to a decrease in food intake and an increase in the energy expenditure. A long-term administration of selective monoagonists in combination to obese primates caused a greater reduction in body weight compared to the use of these drugs alone [111].

The structural similarity of GLP-1 to glucagon allows their pharmacological effects to be integrated by combining them into a single molecule. Preclinical studies of GLP-1/glucagon coagonists confirmed the feasibility of this approach, which led to the creation of a large number of similar molecules (based on glucagon, oxyntomodulin, etc.), which are currently undergoing clinical trials. The most notable compounds are SAR425899 (Sanofi-Aventis Deutschland GmbH) and MEDI0382 (AstraZeneca), which have shown promising results in their effectiveness in reducing hyperglycemia and body weight. Compound SAR425899 was also found to cause dose-dependent severe gastrointestinal side effects, which may limit its use in relation to compound MEDI0382. The research on these two compounds is ongoing [92, 104, 112].

Novo Nordisk A/S has developed several long-acting dual agonists (NN1177, NN1151, NN1359) that differ in their affinity for the GLP-1 and glucagon receptors. These compounds have been successfully tested in preclinical trials, but several systemic problems have been identified due to the species specificity and a number of receptors involved, making it difficult to optimally select the GLP-1 / glucagon activity ratio of these compounds. The pharmacodynamic effects of coagonists vary among species and are dependent on the compound exposure and study duration (a tolerance development), making the identification of an optimally balanced clinical candidate difficult [104, 113].

The compound GUB06-046 is a coagonist to the secretin receptor (SCTR) and GLP-1; its use significantly reduces body weight, increases glucose utilization and increases β-cell mass [114].

The creation of monomolecular GLP-1 and GIP agonists is advisable from the point of view of enhancing insulinotropic effects. GIP is also involved in the regulation of a bone tissue remodeling and has a therapeutic potential for osteoporosis. The question of the therapeutic value of stimulation or receptors inhibition for GIP remains open, since in T2DM patients, the sensitivity of tissues to GIP decreases, which can be restored against the background of normalization of glycemia [51, 104, 115].

Tirzepatide (LY3298176) is an experimental GIP analogue and is a linear polypeptide of 39 amino acids. The dibasic fatty acid portion (eicosandioic acid) is linked via glutamic acid to two (2-(2-aminoethoxy) ethoxy)acetic acid units to the side chain of a lysine residue. This arrangement provides a much longer half-life, increasing the time between the doses due to its high affinity for albumin. The drug is administered weekly subcutaneously. Phase 3 trials were completed worldwide in 2021. Tirzepatide has a greater affinity for GIP receptors than for GLP-1 receptors, resulting in a greater reduction in hyperglycemia compared to the selective GLP-1R agonists. Tirzepatide mimics the actions of natural GIP, in relation to the GLP-1 receptor it stimulates an increase in cAMP, but not β-arrestin, and it also increases the levels of adiponectin, an adipokine involved in the regulation of glucose and lipid metabolism, with a maximum increase of 26% (10 mg dose) from the baseline at 26 weeks [93].

In May 2022, the FDA approved of Mounjaro™ injection (tirzepatide, Eli Lilly and Company, USA) for once-weekly dosing (the six doses are: 2.5, 5, 7.5, 10, 12.5, and 15 mg) at as an adjunct to diet and exercise to improve a glycemic control in T2DM adults. It is the first and the only FDA-approved GIP and GLP-1 receptor agonist which has been demonstrated to be effective and safe through the SURPASS program. The effects of the drug were compared with a semaglutide injection 1 mg, insulin glargine and insulin degludec. The efficacy of the drug was evaluated at doses of 5 mg, 10 mg and 15 mg, used alone or in combination with metformin, SGLT2 inhibitors, sulfonylureas and insulin glargine. In a SURPASS study (SURPASS-4, NCT03730662), compared with the baseline HbA1c (8.5%), the drug reduced it by an average of 2.1% (5 mg), 2.3% (10 mg), and 2.4% (15 mg) compared to 1.4% for insulin glargine. It also reduced patients' weight from the baseline of 90.3 kg by an average of 6.4 (5 mg), 9 (10 mg) and 10.4 kg (15 mg) compared with an increase of 1.8 kg for insulin glargine [112, 116].

Based on the encouraging efficacy of the GLP-1/glucagon and GLP-1/GIP coagonists, it was hypothesized that a single molecule with a triple agonism at all three of these receptors could provide a greater efficacy than the corresponding coagonists. In such a molecule, the glucagon fragment is responsible for the modulation of lipid metabolism [104, 106], the GLP-1 and GIP fragments compensate for the hyperglycemic effect of glucagon, have an insulinotropic effect and jointly promote weight loss. The monomeric peptide exhibits a triple agonism at the GLP-1, GIP, and Gcg receptors, which reduces body weight more significantly than the corresponding coagonists and monoagonists in preclinical studies [105]. Clinical studies of such drugs are ongoing.

Retatrutide (LY3437943) is an agonist of GLP-1, GIP and glucagon receptors. In phase II clinical trials, the patients with a body mass index higher than 30, experienced a 17.5% reduction in body weight after 24 weeks of the treatment and a 24.2% reduction after 48 weeks, indicating a significant potential of triple agonists in the treatment of excess body weight [117].

An alternative to producing chimeric or hybridized peptides with scrambled sequences is fusion molecules or conjugates. As noted above, the sequences similarity of the proglucagon peptide family and the structures of their receptors make it possible to construct chimeric peptides with an agonism at multiple receptors with the sizes comparable to native peptides. In particular, the results of the compounds studies obtained by fusion of the GLP-1 molecule with gastrin, amylin, cholecystokinin, FGF21 and inhibitory antibodies against PCSK9 have

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already been published. The GLP-1 / gastrin molecule (also active against the related intestinal hormone xenin) has the potential to restore β-cells [118]. The GLP-1/amylin molecule and GLP-1 / cholecystokinin significantly reduce food intake and blood glucose levels [104]. The fusion of GLP-1 with FGF21 or GLP-1 with anti-PCSK9 led to a more pronounced (than after the use of GLP-1R agonists) normalization of dyslipidemia and body weight [119]. In a phase I clinical trial, the treatment of overweight and obese patients with a GLP-1 / anti-PCSK9 drug reduced LDL cholesterol but did not improve glucose metabolism [120].

Despite the promising results of preclinical studies of various GLP-1 conjugates, it is necessary to establish the cellular mechanisms of their effects, pharmacokinetic parameters and compatibility / reliability of the data translation into clinical practice.

Approaches to the development of GLP-1R agonists and their effects are shown in Fig. 6.

Stimulators of endogenous GLP-1 secretion

In addition to the above-mentioned pharmacological approaches to influencing the GLP-1 receptor, leading pharmaceutical companies are trying to realize the possibility of increasing the secretion of incretins by enteroendocrine cells of the intestine by stimulating a special group of receptors localized on them. Under normal conditions, their physiological activators are FFAs supplied with food or formed as a result of fermentation of dietary fiber under the influence of intestinal microbiota. These receptors (GPR40, GPR41, GPR43, GPR119 and GPR120) were discovered during the implementation of the Human Genome Project, and subsequently their significant role in the regulation of incretin biosynthesis and carbohydrate metabolism was established. The activation of GPR40 receptors, in addition to incretin-mediated effects, has hepato- and neuroprotective effects, GPR41 and GPR43 affect the leptin metabolism, an adipocyte differentiation, the nervous and immune systems [35, 36]. The metabolic and pleiotropic effects of GPR119 [35, 37] and GPR120 agonists are being actively studied [26, 27, 30].

CONCLUSION

Thus, the peptide, discovered due its ability to stimulate the insulin secretion, has evolved into a class of drugs with a pronounced effectiveness against the progression of diabetes and overweight / obesity. The generally accepted description of its role is as follows: GLP-1 is released from the intestine into the bloodstream after eating to increase the insulin secretion and suppress the glucagon secretion to effectively utilize the intestinal glucose and reduce glycemia to normal values (incretin effect), and GLP-1 also acts on afferent neurons of the vagus nerve and/or directly to the brain to suppress appetite and create satiety. In addition, a wide distribution of the GLP-1 receptor in various tissues and organs, its connection with intracellular signaling cascades aimed at launching energy-consuming anabolic processes, provides cardio-, endothelial- and neuroprotective effects of GLP-1, unrelated to its hypoglycemic effect. The use of more potent synthetic GLP-1R agonists has revealed the significant therapeutic potential of these pleiotropic properties of GLP-1, with a confirmation in clinical studies. GLP-1R agonists are a class of medications that provide not only areliable glycemic control and weight loss for patients, but are also accompanied by a reduction in the risk of developing cardiovascular complications of diabetes. GLP-1 agonists stimulate insulin biosynthesis and the β-cell proliferation, and also inhibit their apoptosis. Incretin-like drugs are well tolerated, and the most common side effect of this class is nausea, which is due to the central effect of GLP-1 on the gastric tone.

In addition to the described examples, the development of new incretin mimetics continues: GLP-1R agonists (VRS 859, efpeglenatide, CJC-1134-PC, taspoglutide, glymera, TTP054, ZYOG1, ARI-1732TS), including the non-peptide nature (LY3502970, CHU-128, Boc5, S4P, TT-OAD2, RGT1383, PF06882961), allosteric receptor modulators (DA-15864, BETP, LSN3160440, VU00056556, VU0109197), new combinations (with leptin, salmon calcitonin, PYY, cholecystokinin, insulin analogues, adre nomedullin, agonists β3-adrenergic receptor, cannabinoid receptor 1 (CB1) agonists / antagonists, MC4R agonists and NR1H4 agonists), molecules based on GLP-1R agonists with a multi-target mechanism of action (SAR425899, MEDI0382, GUB06-046, Tirzepatide), including in the form conjugates with other proteins (gastrin, amylin, cholecystokinin, FGF21 and inhibitory antibodies against PCSK9).

GLP-1R agonists are consistently increasing their presence on the Russian pharmaceutical market. Thus, from 2016 to 2020, their share increased from 0.09 to 0.41% or from 2.3 to 10.56% of sold hypoglycemic agents of all groups in physical (packaging) and value (rubles) terms, respectively. The dominant position is occupied

by the pharmaceutical company of Denmark, in whose product portfolio for 2020 there were 5 positions, which allows this company to occupy more than 50% of the market in volume terms and almost 70% in value terms.

Thus, GLP-1R agonists represent a class of not only effective and safe drugs for the treatment of T2DM and obesity, but also rapidly developing in the most advanced areas of pharmacy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Denis V. Kurkin – idea and planning of the structure of the work, design of graphic material, editing and approval of the final version of the manuscript; Dmitry A. Bakulin, Yuliya V. Gorbunova, Valeria B. Saparova, Ksenia N. Koryanova, Anastasia N. Chumachenko, Olga V. Ivanova, Elizaveta V. Pavlova – collecting materials and writing a draft manuscript; Evgeniy I. Morkovin, Andrey V. Strygin, Yuriy A. Kolosov – collecting materials and editing the final version of the manuscript; Marina A. Dzhavakhyan, Andrew V. Zaborovsky, Igor E. Makarenko, Roman V. Drai, Vladimir I. Petrov – consultations on highly specialized issues, approval of the final version of the manuscript.

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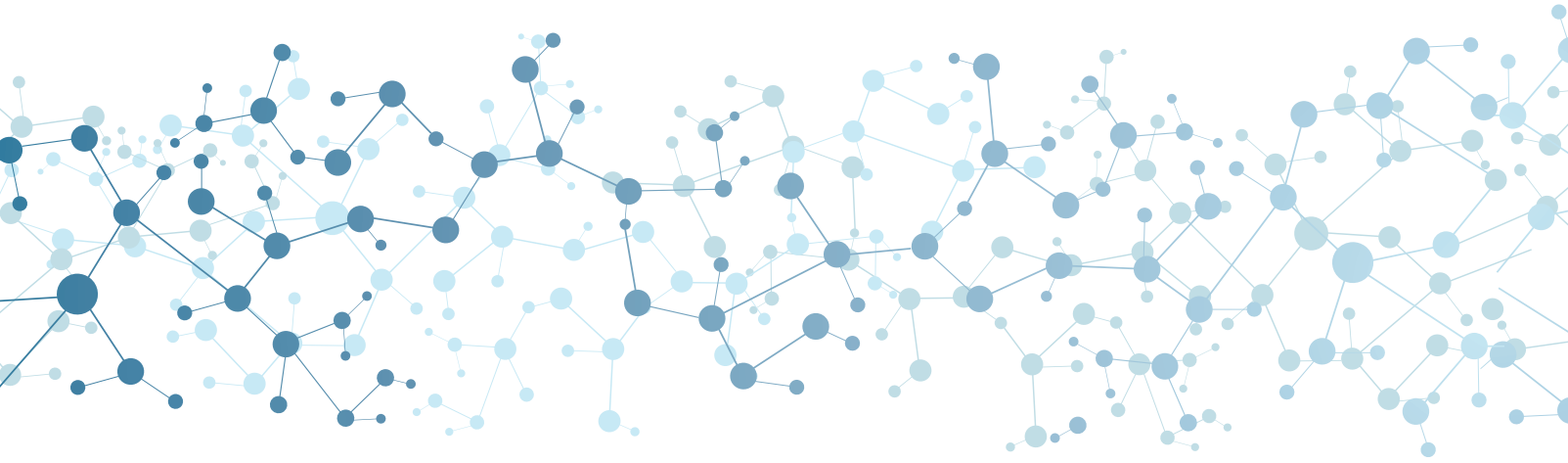


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