DOI: 10.17650/2313-805X-2022-9-4-61-70

Autophagy activation in breast cancer cells *in vitro* after the treatment with PI3K/AKT/mTOR inhibitors

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Introduction. Current chemotherapy of breast cancer has a wide range of disadvantages, in particular, the development of therapy-related infections and hormonal imbalance. Combination of main cytostatic with glucocorticoids allows to broaden its therapeutic interval and to decrease the total toxicity of the treatment. However, long-term treatment with glucocorticoids leads to the development of severe side effects via activation of multiple molecular mechanisms. Thus, glucocorticoids activate prosurvival mTOR-dependent autophagy. Therefore, the evaluation of PI3K (phosphoinositide 3-kinases) / Akt (protein kinase B) / mTOR (mammalian target of rapamycin) inhibitors as adjuvants for breast cancer therapy is important for optimization of treatment protocol.
Aim. Analysis of the effects of PI3K/Akt/mTOR inhibitors, rapamycin, wortmannin and LY-294002 in combination with glucocorticoids in breast cancer cell lines of different subtypes.
Materials and methods. We demonstrated the inhibition of PI3K/Akt/mTOR signaling and the autophagy induction after the treatment of breast cancer cells with rapamycin, wortmannin and LY-294002 by Western blotting analysis of Beclin-1, phospho-Beclin-1 (Ser93 and Ser30).
Conclusion. PI3K/Akt/mTOR inhibitors in combination with Dexamethasone cooperatively inhibited mTOR signaling and activated autophagy in breast cancer cells *in vitro*.

Keywords: breast cancer, autophagy, glucocorticoid, mTOR, rapamycin, wortmannin, LY-294002, phosphoinositide 3-kinases, protein kinase B

For citation: Grigoreva D.D., Zhidkova E.M., Lylova E.S. et al. Autophagy activation in breast cancer cells *in vitro* after the treatment with PI3K/AKT/mTOR inhibitors. Uspekhi molekulyarnoy onkologii = Advances in Molecular Oncology 2022;9(4):61–70. DOI: 10.17650/2313-805X-2022-9-4-61-70

Активация аутофагии в клетках рака молочной железы *in vitro* после воздействия ингибиторами PI3K/AKT/mTOR

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Введение. Химиотерапия рака молочной железы имеет широкий спектр недостатков, в частности развитие сопутствующих инфекций и гормональных нарушений. Комбинация с синтетическими глюкокортикоидами позволяет расширить терапевтический интервал и снизить общую токсичность препаратов основной линии терапии. Однако длительное применение глюкокортикоидов способствует развитию ряда побочных эффектов, которые могут реализовываться за счет различных молекулярных механизмов. Так, глюкокортикоиды могут инициировать индукцию аутофагии, ведущую к выживанию опухолевых клеток. Запуск механизма аутофагии является mTOR-зависимым, в связи с чем актуальной является оценка возможности введения в качестве адъювантов в терапию рака молочной

железы ингибиторов сигнального пути PI3K (фосфоинозитид-3-киназа)/Akt (протеинкиназа B)/mTOR (мишень рапамицина млекопитающих).

Цель работы – анализ действия ингибиторов PI3K/Akt/mTOR рапамицина, вортманнина и LY-294002 в комбинации с глюкокортикоидами на запуск аутофагии в клеточных линиях рака молочной железы различного гистогенеза.

Материалы и методы. Методом Вестерн-блоттинга было показано, что рапамицин, вортманнин и LY-294002 ингибируют активность сигнального пути PI3K/Akt/mTOR и индуцируют аутофагию в клетках рака молочной железы, о чем судили по повышению уровня ключевого белка макроаутофагии, Beclin-1, и его фосфорилированных форм phospho-Beclin-1 по остаткам серина Ser93 и Ser30.

Заключение. В ходе работы было показано, что ингибиторы сигнального пути PI3K/Akt/mTOR в комбинации с дексаметазоном кооперативно подавляют сигнальный путь mTOR и активируют аутофагию в клетках РМЖ *in vitro*.

Ключевые слова: рак молочной железы, аутофагия, глюкокортикоид, мишень рапамицина млекопитающих, рапамицин, вортманнин, LY-294002, фосфоинозитид-3-киназа, протеинкиназа B

Для цитирования: Григорьева Д.Д., Жидкова Е.М., Лылова Е.С. и др. Активация аутофагии в клетках рака молочной железы *in vitro* после воздействия ингибиторами PI3K/AKT/mTOR. Успехи молекулярной онкологии 2022;9(4):61–70. DOI: 10.17650/2313-805X-2022-9-4-61-70. (На англ.).

INTRODUCTION

Incidence of breast cancer (BC) in 2020 is about 2.26 million new cases. It is the first common cancer accounting for approximately 12 % of all cancer worldwide [1]. Breast cancer subtypes are characterized by high heterogeneity in histogenesis, genetic abnormalities, clinical progression of disease and prognosis. Molecular classification of BC is based on the presence/absence of the expression of estrogen and progesterone receptors (ER, PR) as well as epidermal growth factor 2 (HER2). Hormone-dependent BC, characterized by the ER, PR and HER2 expression, is well curable [2]. The treatment usually includes ER antagonists and selective modulators tamoxifen, raloxifen and some others. Long-term therapy course requires the combination of the main anti-cancer drug with glucocorticoids (GC) [3]. ER-negative BC subdivides to triple negative BC (TNBC) and HER2-positive BC. There BC subtypes are associated with poor prognosis compared to luminal BC. HER2 amplification and hyperexpression in BC allows to apply targeted anti-HER2 therapy with the high efficacy [4, 5]. Triple negative BC accounts for 15 % of all BC cases and is characterized by higher aggressiveness and the percentage of relapses as well as poor prognosis. Triple negative BC treatment is the combination of surgery, radio- and chemotherapy with platina derivatives, paclitaxel and doxorubicin.

Therefore, therapy of hormone-resistant BC consists of cytostatic drugs associated with high systemic toxicity and severe adverse effects. Also modern BC treatment is characterized by the fast development of drug resistance.

Long-term treatment of BC includes GC. Their application allows to broaden the therapeutic range of main cytostatic drug, to diminish its side effects: nausea, vomits, inflammation [6–10]. Glucocorticoids also reveal antiproliferative effects on the cancer cells of various subtypes [6–10]. Synthetic GC are usually used in the therapy of solid tumors including BC because of immunosuppressive, anti-inflammatory and anti-vomiting effects as well as anti-proliferative action on cancer cells [6–10]. However, chronic treatment with GC lead to the different metabolic complications associated with the induction of the expression of a number GC-dependent genes: *REDD1* [11, 12], *FKBP51* [13], *KLF5* [14], *SGK1* [15], *MKP-1* [16, 17], *ROR1* [18], *YAP* [19] and others. Additionaly to direct regulation of gene expression by glucocorticoid receptor (GR) binding with GR-responsive elements in gene promotors and enhancers, GR could also regulate cell viability by the protein-protein interaction with key molecules of pro-proliferative and anti-apoptotic signaling pathways. Thus, GR suppresses the activity of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), AP-1, Wnt, mTORC (mammalian target of rapamycin) [20–22]. In our previous studies we demonstrated the efficacy of the combined application of GC and PI3K (phosphoinositide 3-kinases)/Akt (protein kinase B)/mTOR modulators with the ability to inhibit the expression of GC-dependent gene *REDD1*, to attenuate the viability of leukemia and lymphoma cells [7, 23, 24]. Moreover, we showed that side effects of GC are realized via multiple mechanisms including pro-survival autophagy activation [25].

Autophagy is the cell process of catabolism of cytoplasmic macromolecules and organelles. Autophagy is divided to macroautophagy associated with the autophagosome assemble and degradation of organelles and genetic material, microautophagy, which is realized via endosome and lysosome formation, and shaperon-related autophagy associated with the activation of heat shock proteins [26]. Macroautophagy induction promotes the shifts in the expression of oncogenes and tumor suppressor genes, the elimination of damaged organelles and the decrease of chromosomal instability [26, 27]. At the same time macroautophagy leads to cell death in tumors associated with the resistance to apoptosis induction [26–32]. Macroautophagy is activated in cells in conditions of hypoxia, stress and nutrient deficiency [26], and allows cells to resist the metabolic stress and the loss of sensitivity to treatment [28]. Autophagy activation is regulated by PI3K/Akt/mTOR signaling [29, 30] via the activation of Beclin-1, key component of PI3K III complex [31, 32]. It was demonstrated that Beclin-1 expression in BC cell line MCF-7 is lower

compared to the normal cells [33]. Along with this observation, Beclin-1 stimulation leads to the induction of autophagy, inhibition of proliferation *in vitro* and suppression of malignant transformation in xenograft model *in vivo* [33]. Mice with the loss of heterozygosity of *BECN1* gene demonstrated higher frequency of spontanic tumor development [14, 34, 35]. Low *BECN1* expression in HER2-positive BC is associated with HER2 amplification and poor prognosis [36].

Role of autophagy in the pathogenesis of BC is complicated due to difficulties in separation of microautophagy from macroautophagy in different tumors and in evaluation of the contribution of both processes in cell death and survival. Glucocorticoid-dependent autophagy via increase in REDD1 (regulated in development and DNA damage response 1) and FKBP51 expression was demonstrated in non-transformed cells of epidermis [12] and muscle [37]. It is known that GC-dependent kinase SGK1 (serum and glucocorticoid-inducible kinase 1) affects the activation of autophagy via PI3K/Akt/mTOR signaling [15]. Up-regulation of SGK1 is detected in many tumors including BC and is associated with metastasis and chemoresistance [15, 38, 39]. As GC-induced autophagy belongs to prosurvival autophagy type, we propose that GC induce microautophagy associated with the development of the drug resistance. Thus, GC activate the prosurvival autophagy in glioma and blood cancer cells [25, 40–42]. At the same time, the treatment of cancer cells with the combination of GC with PI3K inhibitors 3-methyladenine and chloroquine leads to the apoptosis induction [40].

Role of the autophagy in GC-induced resistance to chemotherapeutics remains unclear. Phosphoinositide 3-kinases inhibitors 3-methyladenine and hydroxychloroquine restore the sensitivity of lapatinib-resistance HER2-positive BC to lapatinib *in vitro* [43], PI3K inhibitor LY-294002 decrease the ER-related resistance of ovarian cancer to paclitaxel [44], and rapamycin (Rapa) restore the sensitivity of the cancer cells of various subtypes to chemotherapeutics [45–47]. Based of these data we assume that targeted regulation of autophagy by PI3K/Akt/mTOR inhibitors is promising for the optimization of GC-based combined BC therapy (fig. 1) [48, 49].

Therefore, targeted regulation of autophagy could be the option to restore the sensitivity of cancer cells to chemotherapeutics. The application of PI3K/Akt/mTOR inhibitors in combined anti-cancer therapy is promising for autophagy induction [48, 49].

The **aim of the study** – the present study is devoted to evaluation of autophagy activation by PI3K/Akt/mTOR modulators rapamycin (Rapa), wortmannin (WM) and LY-294002 (LY) individually and in combination with Dexamethasone (Dex) in BC cells.

MATERIALS AND METHODS

Cell cultures. Breast cancer cells were cultured in DMEM (MCF-7 and MDA-MB-231 cell lines) or RPMI-1640 (HCC-1954 cell line) with 10 % fetal embryonic serum, penicillin (50 ME/ml) and streptomycin (50 ME/ml) ("Paneco", Russia) at 37 °C and 5 % CO_2.

Cell treatment. Cells were pretreated with solvent, Rapa, WM, LY (10 nM, "LC Labs", USA) for 4 h and then were treated with Dex (10 mM, "KRKA", Czech Republic) for 24 h as described [39].

Western blotting. Western blot analysis was performed as following: after the incubation cells were washed with PBS (phosphate buffered saline), then were lysed in RIPA (radioimmunoprecipitation assay) buffer with protease and phosphatase inhibitors ("Sigma-Aldrich", USA). Protein concentration was evaluated as described in [50]. Proteins were resolved in 10 % PAGE (polyacrylamide gel electrophoresis) in Tris-glycin buffer with 1 % SDS (sodium dodecyl sulfate) and transferred on PVDF (polyvinylidene fluoride) membrane (pore diameter 0,22 um). Membranes were blocked with 5 % non-fat milk in TBS

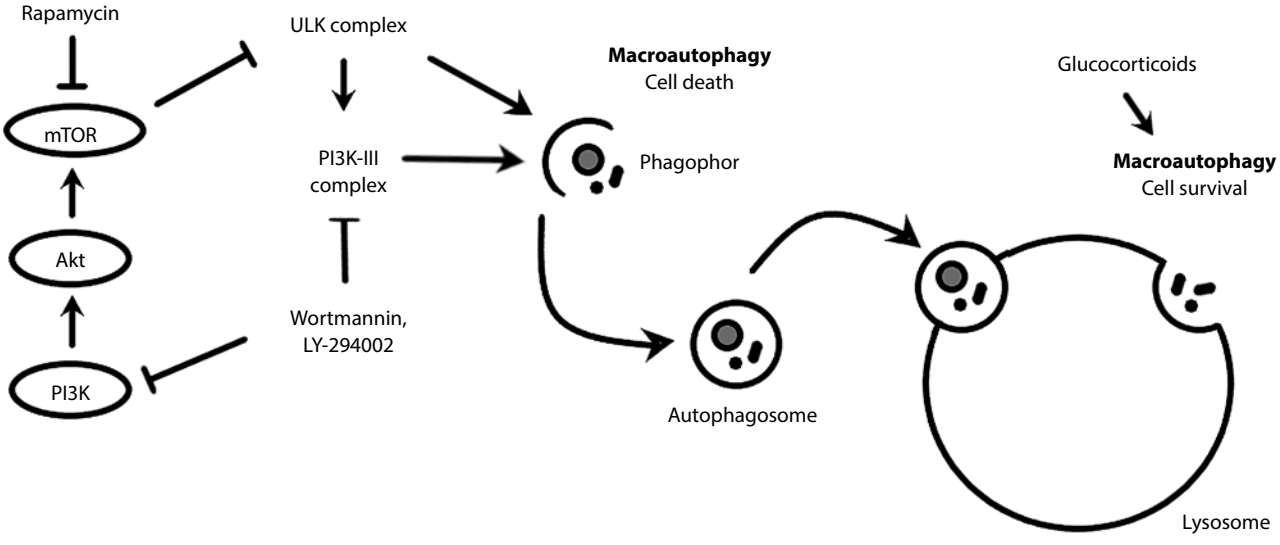


Fig. 1. *Regulation of autophagy (adapted from [25, 26, 29]). PI3K – phosphoinositide 3-kinases; mTOR – mammalian target of rapamycin complex; Akt – protein kinase B; ULK – uncoordinated 51-like kinase*

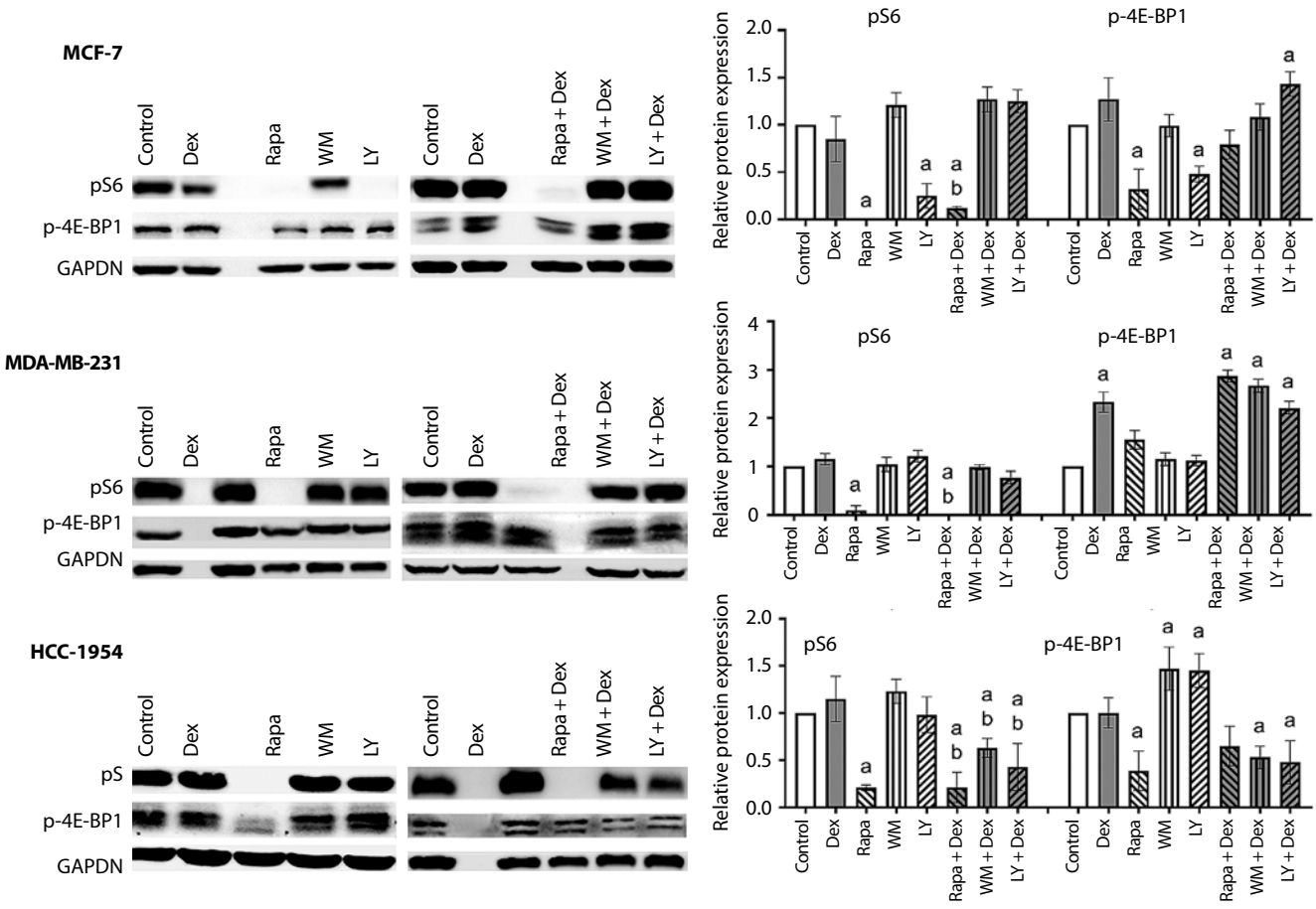


Fig. 2. *The effects of PI3K (phosphoinositide 3-kinases)/Akt (protein kinase B)/mTOR (mammalian target of rapamycin complex) inhibitors on the level of p-4E-BP1 and pS6 in breast cancer cells individually and in the combination with Dexamethasone (Dex). The Beclin-1 level was evaluated by Western blotting with the specific antibodies. Densitometry results were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The treatment effects were compared by one-way ANOVA: a – statistically significant difference from the control; b – statistically significant difference from the samples treated with Dex (p <0.05). Rapa – rapamycin; WM – wortmannin; LY – LY-294002; pS6 – phospho-S6 ribosomal protein*

(TBS) and incubated with primary antibody overnight at 4 °C. The following antibodies ("Cell Signaling Technology", USA) were used: p-Beclin-1 (Ser30), #54101, p-Beclin-1 (Ser93), #14717, Beclin-1, #4122, pS6 (phospho-S6 ribosomal protein), #5364, p-4E-BP1, #2855. Then membranes were incubated with anti-rabbit/anti-mouse IgG secondary antibodies ("Abcam", UK). To verify equal protein loading and adequate transfer, the membranes were probed with anti-glyceraldehyde-3-phosphatehehydrohenase (GAPDH, ab181602, "Abcam", UK). Protein bands were visualized by Clarity™ Western ECL Substrate ("Bio-Rad", USA) on ImageQuant™ LAS 4000 ("General Electric", USA). Quantitative analysis were performed by ImageJ software.

Antiproliferative activity. Cell were cultured in 24-well plates (25 000 cell/well) and treated as described above. Antiproliferative effects were evaluated by trypan blue staining using cell counter ("Bio-Rad", USA).

Induction of apoptosis. Cells were cultured in 24-well plates (50 000 cells/well) and treated as described above. For PI (propidium iodide) staining cells were resuspended in 70 % ethanol, fixed for 2 h at –20 °C, placed in PBS containing 5 μL PI, 0,1 % sodium citrate and 0,3 % Triton-X100 and incubated for 30 min at room temperature. Analysis by FACScan flow cytometer (Becton Dickinson) was carried out to discriminate between live and apoptotic cells.

Statistical analysis. Mean and standard deviation values were calculated using Microsoft Excel software. The treatment effects in each experiment were compared by one-way ANOVA or *t*-test.

RESULTS

Effects of combined application of Dexamethasone and PI3K/Akt/mTOR modulators on mTOR activity. Effects of Rapa, WM, LY and Dex on mTOR inhibition on BC cells were evaluated by the phosphorylation level of key down-stream targets of mTOR: 4E-BP1 (eukaryotic initiation factor 4E (eIF4E) binding protein-1 (Thr37/46), p-4E-BP1) and S6 (phospho-S6 Ribosomal Protein (Ser240/244), pS6) using Western blotting. It has to be mentioned that MCF-7 and HCC-1954 expressed the mutant *PIK3CA* leading to hyperactivation of PI3K/Akt mTOR signaling.

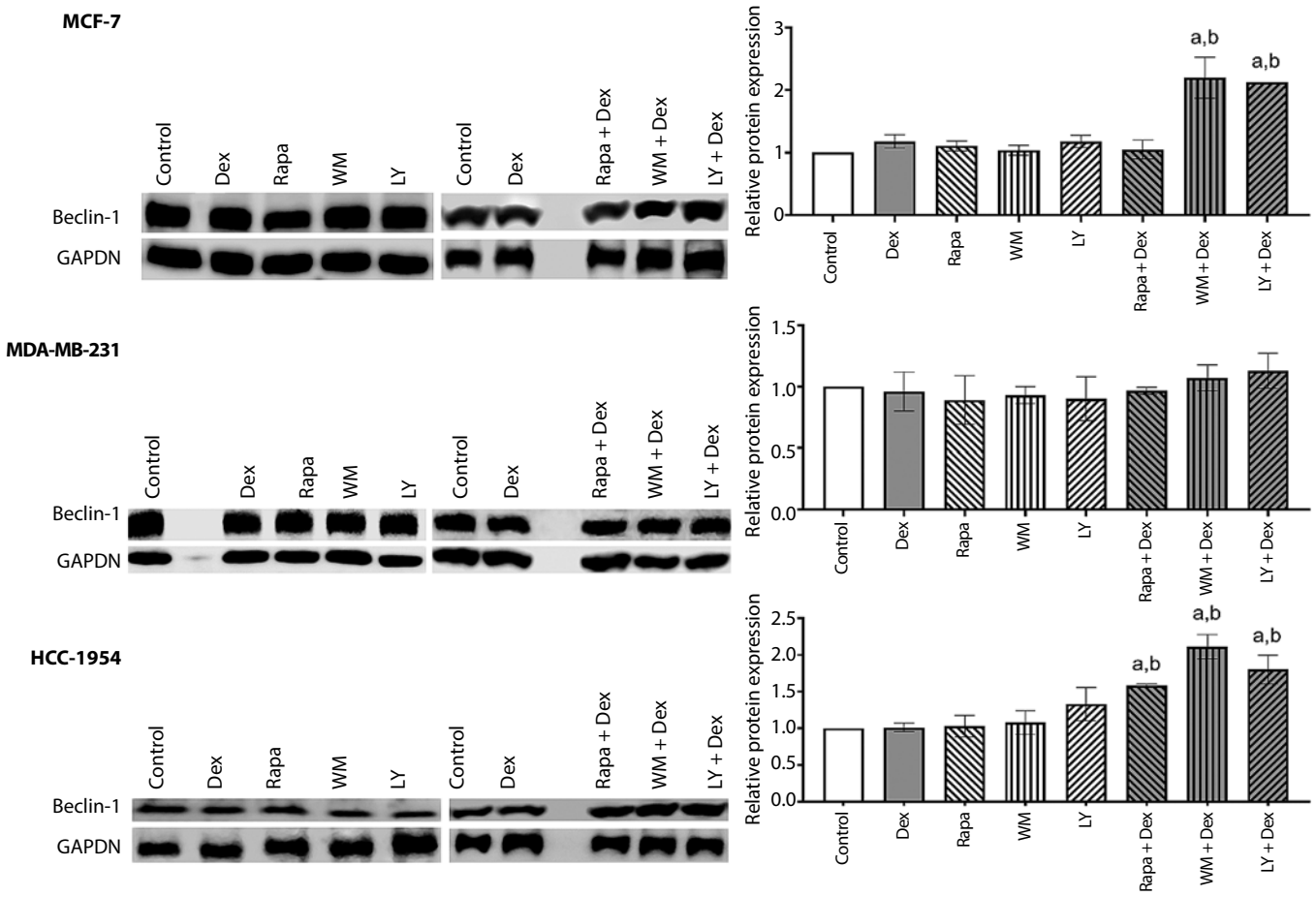


Fig. 3. *The effects of PI3K (phosphoinositide 3-kinases)/Akt (protein kinase B)/mTOR (phosphoinositide 3-kinases) inhibitors on the expression of Beclin-1 protein in breast cancer cells individually and in the combination with Dexamethasone (Dex). The Beclin-1 level was evaluated by Western blotting with the specific antibodies. Densitometry results were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The treatment effects were compared by one-way ANOVA: a – statistically significant difference from the control; b – statistically significant difference from the samples treated with Dex (p <0.05). Rapa – rapamycin; WM – wortmannin; LY – LY-294002*

Dexamethasone increased the level of p-4E-BP1 (phospho eukaryotic initiation factor 4E (eIF4E) binding protein-1) in TNBC cells MDA-MB-231. PI3K/Akt/mTOR inhibitors did not affect GC-induced phosphorylation of 4E-BP1 (fig. 2). Rapamycin suppressed the phosphorylation of ribosomal protein S6 in all three BC cell lines individually and in combination with Dex, and inhibited the phosphorylation of 4E-BP1 in MCF-7 and HCC-1954 cells. Wortmannin and LY in combination with Dex decreased the level of p-4E-BP1 and pS6 in HCC-1954 cells (fig. 2). The level of mTOR activity suppression varied between different BC subtypes.

Effect of Dexamethasone and PI3k/Akt/mTOR on the activation of autophagy. Beclin-1 is the main regulator of autophagy in cells [31, 51], and its cleavage in stress conditions induced the shift in cell metabolism to apoptosis activation [31].

Incubation of BC cells with all studied molecules individually and in combination did not lead to Beclin-1 cleavage as well as they did not affect Beclin-1 expression in MDA-MB-231 cells (fig. 3). At the same time combination of WM and LY with Dex induced the increase in Beclin-1 protein level in MCF-7 and HCC-1954 cells. Rapamycin stimulated the expression of Beclin-1 in combination with Dex in HCC-1954 cell line.

PI3K/Akt/mTOR inhibitors combined with Dex induced the phosphorylation of Beclin-1 by Ser93 residue in cells with *PIK3CA* hyperexpression. Thus, we demonstrated the 1.9 ± 0.5-fold increase in p-Beclin-1 level after incubation of MCF-7 cells with Rapa + Dex, 3.0 ± 0.7-fold increase with WM+Dex, and 2.5 ± 0.2-fold increase with LY + Dex. Weaker effects were showed in HCC-1954 cells: the average increase in p-Beclin-1 (Ser93) level was 1.5-fold (fig. 4).

Dexamethasone and PI3K/Akt/mTOR inhibitors induced the phosphorylation of Beclin-1 by Ser30 in HCC-1954 cells after individual treatment. We demonstrated the 1.5-fold increase in p-Beclin-1 (Ser30) level in MCF-7 and MDA-MB-231 cell after the treatment with Rapa. The similar effect was described in these cell lines after the treatment with WM+Dex (fig. 5).

Cytotoxic effects of Dexamethasone and PI3K/Akt/mTOR inhibitors in breast cancer cells. Dex did not reveal significant cytotoxic effects in BC cells *in vitro* [7] but

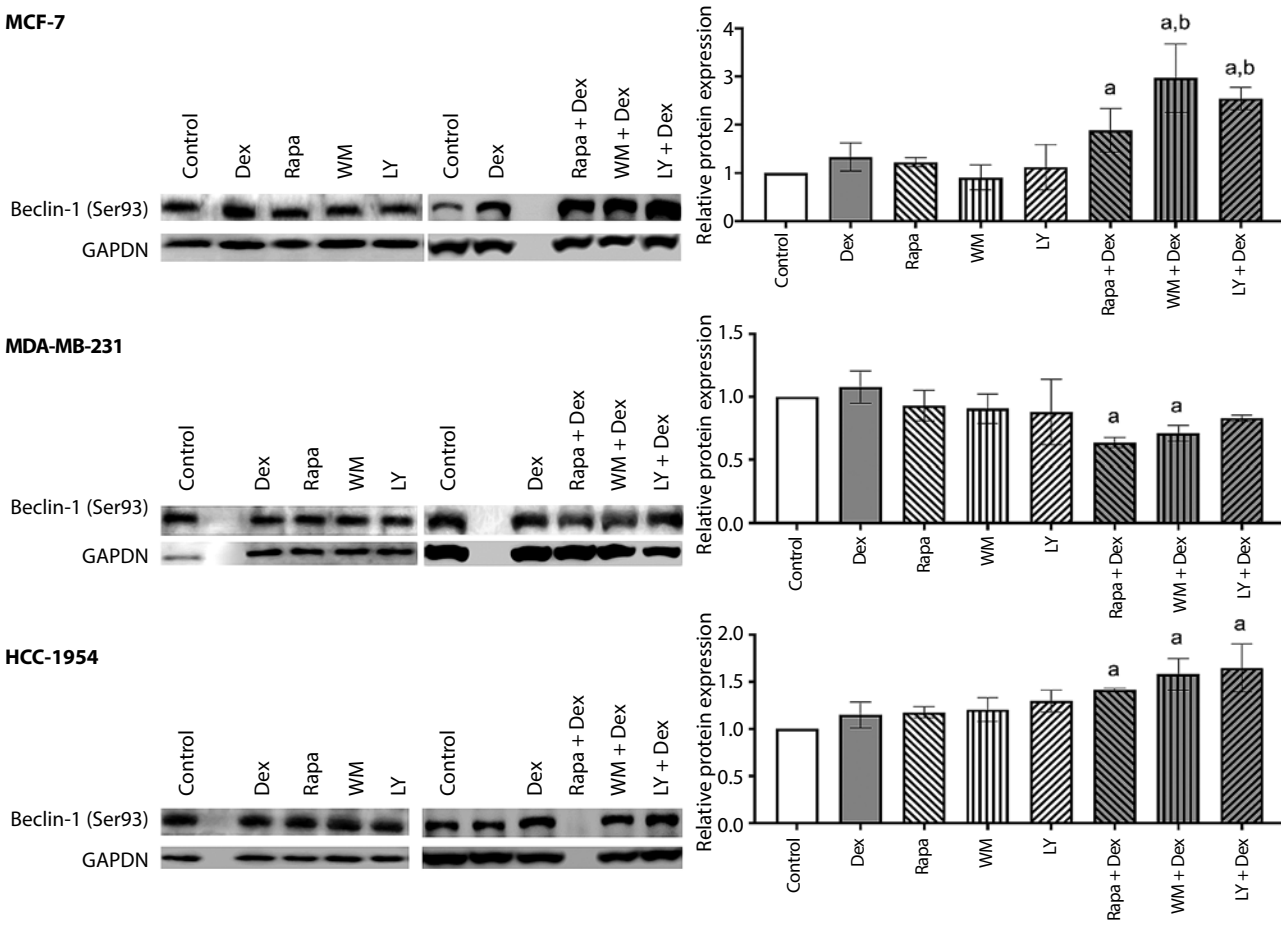


Fig. 4. *The effects of PI3K (phosphoinositide 3-kinases)/Akt (protein kinase B)/mTOR (phosphoinositide 3-kinases) inhibitors on the Phospho-Beclin-1 (Ser93) level in breast cancer cells individually and in the combination with Dexamethasone (Dex). The phosho-Beclin-1 level was evaluated by Western blotting with the specific antibodies. Densitometry results were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The treatment effects were compared by one-way ANOVA: a – statistically significant difference from the control; b – statistically significant difference from the samples treated with Dex (p <0.05). Rapa – rapamycin; WM – wortmannin; LY – LY-294002*

induces the growth arrest in G1 phase. Rapamycin inhibited the proliferation of HCC-1954 and MDA-MB-231 cells by 50 and 30 %, respectively (fig. 6, *a*) as well as induced growth arrest in G1 phase in all studied cells (fig. 6, *b*). Combination of PI3K/Akt/mTOR inhibitors with Dex decreased the proliferative activity by 20–30 % in MCF-7 cell line.

DISCUSSION

It is well-known that partial mTOR inhibition leads to the development of chemoresistance in cancer cells [52, 53]. The suppression of S6 and 4E-BP1 is associated with antiproliferative effects of Rapa [54–56]. *In vitro* Rapa inhibited the phosphorylation of S6 и 4E-BP1 individually and in combination with Dex in BC cells. LY decrease the level of phosphorylated mTOR targets in MCF-7 cells. At the same time, LY and WM inhibited mTOR activity when used in combination with GC. The data obtained demonstrated partial mTOR inhibition and showed higher sensitivity of S6 to partial inhibitors of PI3K/Akt/mTOR signaling [54]. We demonstrated for the first time the potency of combined application of PI3K/Akt/mTOR modulators and GC as these compounds did not reveal antagonistic mode of action.

Also we observed higher cytotoxic effects of Dex, Rapa, WM and LY when applied in combination rather than individually in BC cells *in vitro* (fig. 6). Taken together with the absence of Beclin-1 cleavage, these results demonstrated the autophagy contribution to growth arrest in G1 phase (fig. 3).

Phosphorylation of Beclin-1 by Ser30 and Ser93 residues is associated with the activation of autophagy [57]. Our results showed the autophagy induction in BC cells by PI3K/Akt/mTOR inhibitors as well as cooperative effects of Dex and PI3K/Akt/mTOR inhibitors on autophagy activation in BC cells with PI3K excessive activation (MCF-7 and HCC-1954).

Dex ability to activate autophagy but not BC cell death *in vitro* demonstrated the induction of prosurvival microautophagy leading to the development of chemoresistance of cancer cells. The combination of GC with PI3K/Akt/mTOR inhibitors allows to activate GC-dependent macroautophagy related to PI3K/Akt/mTOR suppression.

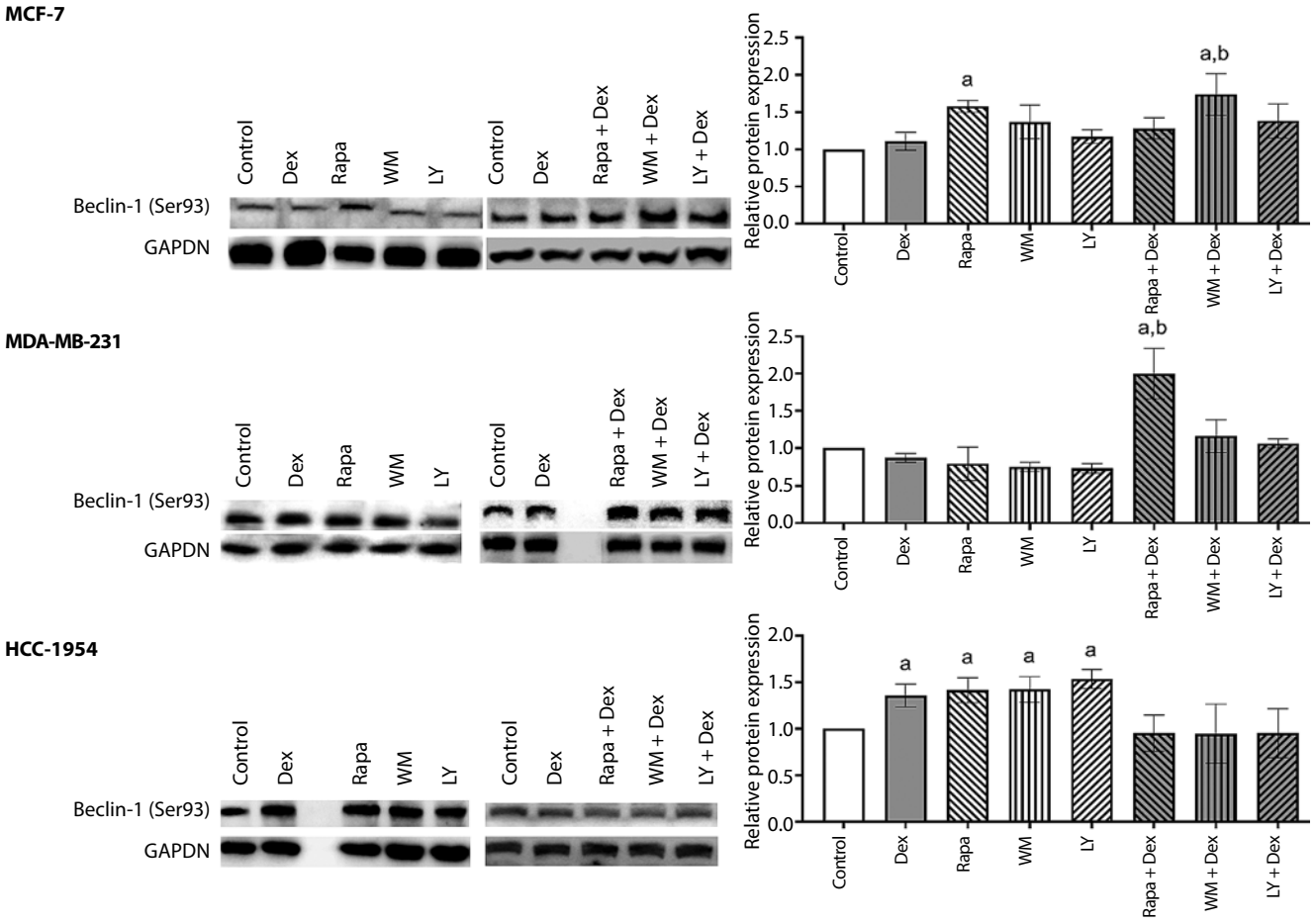
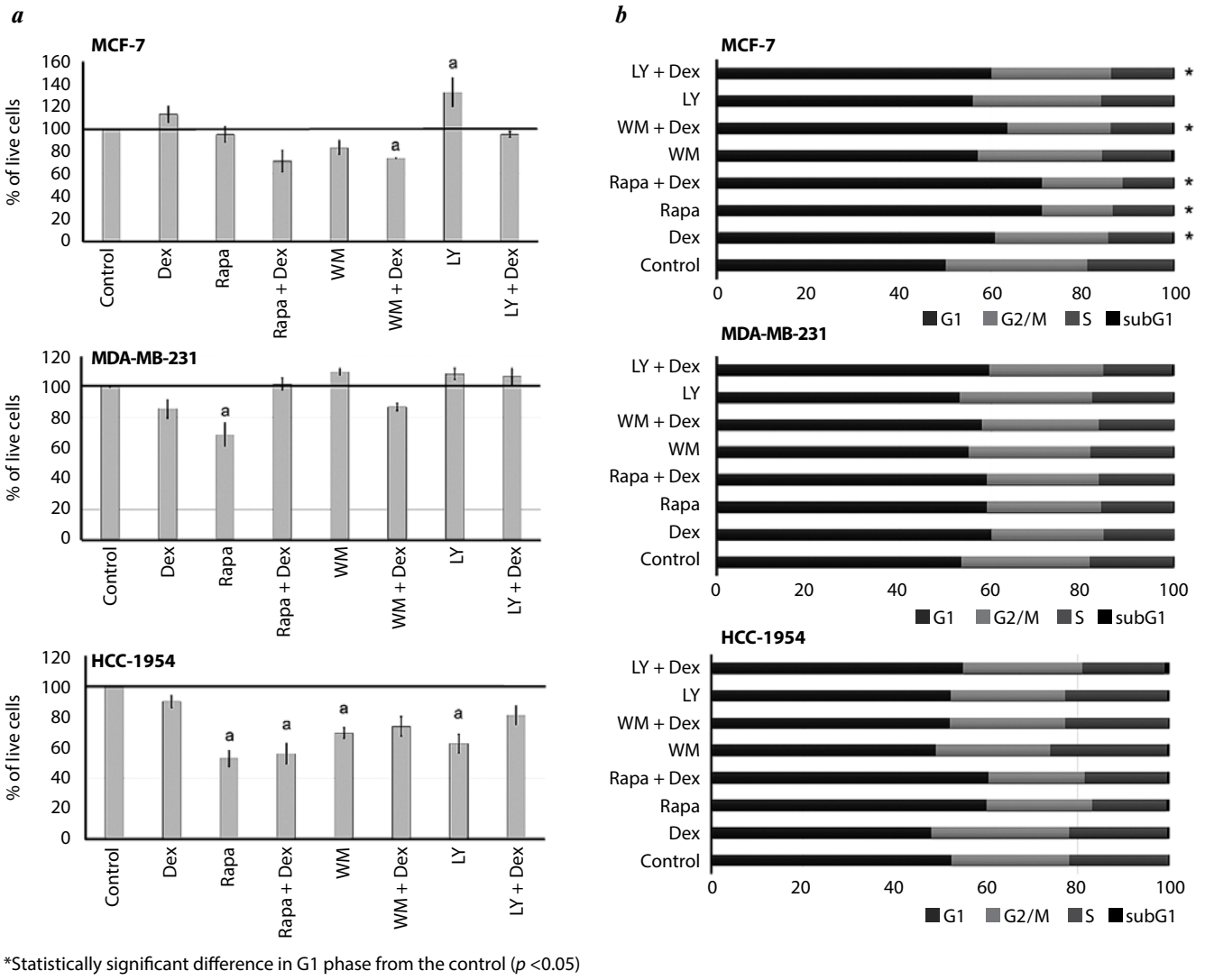


Fig. 5. *The effects of PI3K (phosphoinositide 3-kinases)/Akt (protein kinase B)/mTOR (phosphoinositide 3-kinases) inhibitors on the Phospho-Beclin-1 (Ser30) level in breast cancer cells individually and in the combination with Dexamethasone (Dex). The phosho-Beclin-1 level was evaluated by Western blotting with the specific antibodies. Densitometry results were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The treatment effects were compared by one-way ANOVA: a – statistically significant difference from the control; b – statistically significant difference from the samples treated with Dex (p <0.05). Rapa – rapamycin; WM – wortmannin; LY – LY-294002*

CONCLUSION

We demonstrated that the combination of PI3K/Akt/mTOR with Dex cooperatively suppressed mTOR signaling and activated autophagy in BC cells *in vitro*. Overall, our data provide the rationale for novel GC and PI3K/Akt/mTOR-based therapy for BC and further investigation of this approach.



*Statistically significant difference in G1 phase from the control ($p < 0.05$)

Fig. 6. *Antiproliferative effect of PI3K (phosphoinositide 3-kinases)/Akt (protein kinase B)/mTOR (phosphoinositide 3-kinases) inhibitors individually and in combination with Dexamethasone (Dex) in breast cancer cells: a – cells were cultured with solvent, Dex, PI3K/Akt/mTOR inhibitors and their combinations. Cells were counted after the 24 h of treatment. Number of the viable cells is presented as percentage to solvent-treated control. a – statistically significant difference from the control ($p < 0.05$); b – cell cycle phases were detected by flow cytometry with PI staining. The treatment effects were compared by t-test. Rapa – rapamycin; WM – wortmannin; LY – LY-294002*

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Conflict of interest. The authors declare no conflict of interest.
Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

Funding. This work is supported by the Russian Science Foundation (grant No 17-75-20124).
Финансирование. Исследование выполнено при поддержке Российского научного фонда (грант № 17-75-20124).

Compliance with principles of bioethics. The study protocol was approved by the biomedical ethics committee of N.N. Blokhin National Medical Russian Research Center of Oncology, Ministry of Health of Russia.
Соблюдение правил биоэтики. Протокол исследования одобрен локальным комитетом Научно-исследовательский институт онкологии ФГБУ «Национальный медицинский исследовательский центр онкологии им. Н.Н. Блохина» Минздрава России.

Article submitted: 22.05.2022. **Accepted for publication:** 17.11.2022.
Статья поступила: 22.05.2022. **Принята к публикации:** 17.11.2022.