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Effects of Progesterone and Selective Ligands of Membrane Progesterone Receptors in HepG2 Cells of Human Hepatocellular Carcinoma

Tatiana A. Shchelkunova[1,a]*, Inna S. Levina[2], Ivan A. Morozov[3], Petr M. Rubtsov[3], Alexey I. Goncharov[1], Yury V. Kuznetsov[2], Igor V. Zavarzin[2], and Olga V. Smirnova[1]

[1] *Faculty of Biology, Lomonosov Moscow State University, 119234 Moscow, Russia*
[2] *Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russia*
[3] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia*
[a] *e-mail: Schelkunova-t@mail.ru*

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Abstract—Progesterone exerts multiple effects in different tissues through nuclear receptors (nPRs) and through membrane receptors (mPRs) of adiponectin and progestin receptor families. The effect of progesterone on the cells through different types of receptors can vary significantly. At the same time, it affects the processes of proliferation and apoptosis in normal and tumor tissues in a dual way, stimulating proliferation and carcinogenesis in some tissues, suppressing them and stimulating cell death in others. In this study, we have shown the presence of high level of mPRβ mRNA and protein in the HepG2 cells of human hepatocellular carcinoma. Expression of other membrane and classical nuclear receptors was not detected. It could imply that mPRβ has an important function in the HepG2 cells. The main goal of the work was to study functions of this protein and mechanisms of its action in human hepatocellular carcinoma cells. Previously, we have identified selective mPRs ligands, compounds LS-01 and LS-02, which do not interact with nuclear receptors. Their employment allows differentiating the effects of progestins mediated by different types of receptors. Effects of progesterone, LS-01, and LS-02 on proliferation and death of HepG2 cells were studied in this work, as well as activating phosphorylation of two kinases, p38 MAPK and JNK, under the action of three steroids. It was shown that all three progestins after 72 h of incubation with the cells suppressed their viability and stimulated appearance of phosphatidylserine on the outer surface of the membranes, which was detected by binding of annexin V, but they did not affect DNA fragmentation of the cell nuclei. Progesterone significantly reduced expression of the proliferation marker genes and stimulated expression of the p21 protein gene, but had a suppressive effect on the expression of some proapoptotic factor genes. All three steroids activated JNK in these cells, but had no effect on the p38 MAPK activity. The effects of progesterone and selective mPRs ligands in HepG2 cells were the same in terms of suppression of proliferation and stimulation of apoptotic changes in outer membranes, therefore, they were mediated through interaction with mPRβ. JNK is a member of the signaling cascade activated in these cells by the studied steroids.

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INTRODUCTION

The most studied function of progesterone is its effect on reproductive processes in a female organism. Its effects on tissue and organs of non-reproductive nature have been investigated only marginally. However, effects of progesterone and its synthetic analogues on the processes of proliferation, cell death, and carcinogenesis

Abbreviations: BxPC3, human pancreatic adenocarcinoma cell line; HepG2, human hepatocellular carcinoma cell line; nPR and mPR, nuclear and membrane progesterone receptors; PI, propidium iodide.

* To whom correspondence should be addressed.

have been demonstrated in a number of examined tissues [1]. Such effects of progestins often are multidirectional: stimulating or inhibitory depending on composition of progesterone receptors and phenotype of the particular cell [2-4]. Effect of progesterone is mediated through activation of its nuclear receptors (nPR) and five subtypes of membrane receptors of the adiponectin and progestin receptor families (mPRs). Among the latter the subtypes mPRα, mPRβ, and mPRγ are most abundant in the tissues. Participation of nuclear receptors in the processes of reproduction in female organisms, in the behavioral reactions, as well as in functioning of some tissues and organs of non-reproductive nature and tumor cells are being investigated from the beginning of 1960s [5]. Membrane receptors have been investigated for the last 20 years [6-8]. Many studies have been devoted to investigation of the differences in the ligand specificity of nPR and mPRs with the goal to identify selective agonists and antagonists [9-12], because very often both types of receptors are present simultaneously in the same cells. Function of mPRs in different tissues, as well as their role in carcinogenesis have not been fully elucidated yet. We examined expression of the genes of different types of progesterone receptors in more than 16 tumor cell lines, and identified a cell line of human pancreatic carcinoma, BxPC3, with maximum expression of mPRα and mPRγ, low expression of mPRβ, and absence of expression of nPR [13]. Binding of progesterone and its derivatives, synthesized in the Zelinsky Institute of Organic Chemistry, in the BxPC3 cells was investigated, and two compounds were selected that interacted with mPRs, but did not exhibit any affinity to nPR [14, 15]. Effects of these new selective ligands of progesterone receptors, LS-01 (19-hydroxipregn-4-en-20-one) and LS-02 (19-hydroxy-5β-pregn-3-en-20-one), were investigated in the BxPC3 tumor cells [16]. Cytotoxic effect of the investigated compounds was demonstrated with the effects of LS-02 being significantly different from the effects of progesterone and LS-01. In this study we have found another cell culture that did not have nPRs, but in which high expression of the *PAQR8* gene of the membrane β-receptor of progesterone was observed. This was the culture of the human hepatocellular carcinoma HepG2. The level of the mPRβ protein mRNA in the HepG2 cells was significantly higher than the level of this mRNA in all 16 human tumor cells lines investigated in our laboratory previously [13]. It could be suggested that this receptor have an important role in regulation of functions of these cells. Function of the β subtype of the membrane receptors and signaling pathways activated through them by progesterone have been investigated only marginally in comparison with the mPRα subtype. In the PC12 neuronal cells its functions is associated with the axon growth, and signalling pathway included phosphorylation of ERK [17]. Activation of this receptor together with mPRα in myometrium results in phosphorylation of myosin light chain and enhancement of contractile activity via a pathway involving activation of p38 MAPK, but not of ERK1/2 [18]. Expression of mPRβ in the endometrial cancer cells was shown to be associated with favorable prognosis of the disease progression [19]. Main goal of this study is elucidation of the function of mPRβ in the human hepatocellular carcinoma cells HepG2 and investigation of the signaling cascades activated upon its interaction with progestins. In order to confirm participation of mPRβ in the investigated effects, we used selective ligands of mPRs identified in our previous study. In this work we investigated effects of progesterone, LS-01, and LS-02 on proliferation and death of the HepG2 tumor cells, as well as activating phosphorylation of two kinases, which are presumed mediators of the effects of these steroids. Effects of the three steroids on viability of the cells was investigated using the XTT-test, on the process of apoptosis and necrosis by examining fragmentation of nuclear DNA, exposure of phosphatidylserine on the cell outer membrane, and permeability of the cell membrane to propidium iodide. Effects of progesterone and selective ligands of its receptors on expression of the genes of the proteins associated with proliferation and apoptosis were also investigated, as well as the possibility of participation of p38 MAPK and JNK in the signaling pathways activated by the receptor during its interaction with the hormone.

MATERIALS AND METHODS

Materials. The following reagents were used in the study: DMEM medium with phenol red, Versene solution, 100× L-glutamine, 0.05% trypsin-EDTA solution in Hanks' balanced salt solution (PanEko, Russia); fetal bovine serum (FBS) and fetal bovine serum treated with dextran coated charcoal (DFBS) (HyClone, USA); 100× mixture of antibiotics (penicillin, streptomycin) and antimycotics, DMEM medium without phenol red (Gibco, USA); progesterone (Sigma-Aldrich, USA); reagents for immunoblotting and electrophoresis (Thermo Scientific, USA; GE Healthcare, United Kingdom); goat antibodies against rabbit IgG (H + L), and goat antibodies against mouse IgG (H + L) conjugated with horseradish peroxidase (HRP) (Wuhan Elabscience Biotechnology, China); 19-hydroxypregn-4-en-20-one (LS-01) and 19-hydroxy-5β-pregn-3-en-20-one (LS-02) were synthesized in the Zelinsky Institute of Organic Chemistry (Russia) [14, 15].

Cell cultivation and treatment with hormones. Cells of human hepatocellular carcinoma, HepG2 (ATCC, USA) were cultivated in a DMEM with phenol red containing 10% FBS, 0.365 g/liter glutamine, and solution of antibiotic and antimycotic mixture under standard

condition at 37°C in an incubator in the presence of 5% CO_2. After the third passage, cells were seeded into a DMEM medium without phenol red that contained 10% DFBS and solution of antibiotics. After three passages in the medium without steroids cells were inoculated into microplates or Petri dishes, cultivated to 50-70% confluency, and treated with hormones or diluent (ethanol, no more than 0.2% *v/v*) for 24-72 h. Next culture fluid was removed, and cells were used for analysis. In different assays steroid concentrations vary from 10 nM to 20 μM. High concentration allow seeing effect more clearly, as progesterone affinity to mPRs is 5-30-fold lower than its affinity to nPR [13, 14]. It is likely that mPRs activation occurs during pregnancy and close to the spots of the synthesis of progesterone, and where local concentration of this hormones is 10-100-fold higher than in the circulating blood [20]. Under experimental conditions similar concentrations were observed in the blood serum from animals treated with steroids, but survival and weight gain in such animals were maintained at the level observed for the control animals [21].

XTT-test. HepG2 cells were seeded into a 96-well plate (Corning, USA) at concentration of (5-6) × 10^3 cells per well. Next day the medium was replaced with the medium containing progesterone or one of the selective ligands at different concentration. On completion of incubation, XTT-test was conducted using a Cell Proliferation Kit (Biological Industries, USA) according to the manufacturer's instructions. Absorption in the wells was measured 1 and 2 h after the start of incubation at 450 and 620 nm with a Multiscan EX plate reader (Thermo Electron Corporation, USA).

TUNEL-analysis. HepG2 cells were inoculated into 6-well plates (Corning) at concentration (5-7) × 10^5 cells per well. Next day medium in the wells was replaced with the medium containing progesterone, LS-01, and LS-02 at different concentrations. After incubation cells were detached from the well surface with trypsin solution, washed, and fixed in a 1% (*w/v*) solution of paraformaldehyde for 15 min on ice, washed with a phosphate buffered saline (PBS), and left overnight in 70% ethanol at –20°C. Next day the TUNEL assay was performed with the help of an APO-BrdU™ TUNEL Assay Kit with Alexa Fluor® 488 Anti-BrdU (Invitrogen, USA, A23210) in accordance with the manufacturer's instruction. Cells were washed, incubated overnight at room temperature in a solution containing BrdUTP, terminal deoxynucleotidyl transferase and reaction buffer. After incubation cells were washed with a washing buffer and treated for one hour in the dark with Alexa Fluor™ 488 dye-labeled anti-BrdU antibodies diluted 20-fold. Next a buffer with propidium iodide and RNAse A were added to the tubes, and 30-min later a number of cells interacted with the labeled antibodies was determined with a LSR Fortessa (BD Biosciences, USA) flow cytometer.

Staining with Annexin V conjugate. Cells were seeded into a 24-well plate at concentration 7 × 10^4 cells per well. Next day the cells were treated with hormones. After incubation cells were detached from the well surface with trypsin solution, washed with cold PBS, and apoptotic and necrotic cells were determined with the help of an Annexin V-FITC conjugate kit (Invitrogen, A13199). Apoptotic and necrotic cell death was analyzed with the help of double staining with Annexin V and propidium iodide (PI). Annexin V binds to the exposed phosphatidylserine on the apoptotic cells, while PI labels the cells with damaged membrane. Staining was carried out according to the manufacturer's instructions as described previously [16]. Percent of apoptotic and necrotic cells was determined with the help of flow cytometry using "FITC" and "PerCP-cy5-5 A" channels in the LSR Fortessa flow cytometer.

Determination of gene expression. Culture medium was removed from the wells after treatment of HepG2 with hormones, total RNA was isolated from the cells using TRIzol (Invitrogen). Synthesis of cDNA was carried out with the help of a ImProm-II™ Reverse Transcription System, (Promega, USA) according to manufacturer's instruction. Real-time PCR was preformed with a LightCycler 96 (Roche, Germany) using reagents containing an intercalating dye SYBR Green I (Sintol, Russia). Sequences of specific primers used for PCR are presented in the Table S1 (see Online Resource 1). Primers were designed with consideration of exon-intron structure of the genes to prevent formation amplicons on the genomic DNA under selected PCR conditions. In the case of the gene with only one exon, RNA preparation prior to the synthesis of cDNA was treated with a RQ1 RNase-Free DNase (Promega). The following amplification protocol was used: incubation at 95°C – 5 min; next 40 cycles: 95°C – 10 s, 58°C – 15 s, 72°C – 20 s; melting 72-95°C. Each sample was analyzed in triplicate. Levels of expression of mRNA of the investigated genes were normalized to the level of mRNA of the references gene encoding glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and next to the control sample to analyze relative changes in the gene expression with the ΔΔCt method [16].

Immunoblotting. For analysis of receptors, HepG2 cells were lysed with the help of a CelLytic™ M reagent (Sigma-Aldrich) with addition of a MS-SAFE Protease and Phosphatase inhibitor (Sigma-Aldrich). Proteins were heated in a buffer with SDS and separated in a 13.5% PAAG followed by transfer onto membranes (GE Healthcare Life Sciences, USA, and Amersham, Germany), which were next treated for 60 min with a PBS-T solution (PBS and 0.1% (v/v) Tween 20) containing 5% of an ECL Advance Blocking Agent (GE Healthcare Life Sciences). After that membranes were incubated overnight with antibodies against mPRα (ab75508), mPRβ (ab123693), and mPRγ (ab79517) (Abcam, USA)

diluted 500-, 100-, and 500-fold, respectively. Next day membranes were washed and treated (for 60 min) with secondary goat antibodies against rabbit IgG (H + L) diluted 10,000-fold followed by washing with PBS-T. Chemiluminescence was measured with a ChemiDoc MP system (Bio-Rad, USA) using an ECL SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific). For the repeated use membranes were treated according to the Western blot membrane stripping for restaining protocol (Abcam) and incubated with anti-GAPDH antibodies G9545 (Sigma-Aldrich) following the same protocol as described above.

For determination of activating phosphorylation of kinases under the action of hormones, cells were exposed to the action of progesterone and selective ligands of mPRs at concentration of 20 μM for 40 min. Next, they were lysed using the reagent described above. Prior to electrophoresis in a 13.5% PAAG proteins were heated at 50°C for 15 min followed by separation with electrophoresis and transfer to membranes. After treatment with a blocking solution containing 5% of blocking agent in TBST [physiological solution based on Tris-buffer containing 0.1% (v/v) Tween 20] membranes were treated with primary antibodies against either activated JNK isoforms [monoclonal anti-JNK, activated (diphosphorylated JNK), J4750], against all JNK isoforms (Anti-JNK antibody, SAB4200176), or against phospho-p38 MAPK [Phospho-p38 MAPK (Thr180, Tyr182), S.417.1 (Invitrogen), or against p38 MAPK (Anti-p38 MAPK antibody, SAB4500490)] at 4°C overnight. Next membranes were washed with TBST, incubated (60 min) with secondary goat antibodies against rabbit or mouse IgG (H + L) at 10,000-fold dilution, washed with TBST, and chemiluminescence was measured with a ChemiDoc MP system.

Statistical processing of the results. Statistical processing of the data was carried out with the GraphPad Prism 6.01 program (GraphPad Software, USA). The results in histograms are presented as a mean ± standard deviation. Evaluation of differences was carried out using one-way ANOVA with Dunnett correction for multiple data groups. Differences were considered significant at $p < 0.05$.

RESULTS

Expression of progesterone receptors in the HepG2 cells of human hepatocellular carcinoma. Expression of the *PGR* genes of nuclear progesterone receptors and of *PAQR 7*, *PAQR 8*, and *PAQR 5* genes of three membrane progesterone receptors (mPRα, mPRβ, mPRγ) was investigated in the HepG2 cells. The results of experiments are presented in Fig. 1a. Average levels of *PAQR 7* mRNA (mPRα) were 23% of the level of the reference *GAPDH* gene mRNA, of *PAQR 8* mRNA (mPRβ) – 579%, of *PAQR 5* mRNA (mPRγ) – 17%, *PGR* mRNA (nPR) was practically not detected. Hence, the mPRβ subtype is predominantly expressed in the HepG2 cells β. The results of immunoblotting presented in Fig. 1b confirm this conclusion.

Effect of progesterone and selective ligands of mPRs on viability of HepG2 cells in XTT-test. HepG2 cells were treated with progesterone and selective ligands for 24, 48, and 72 h. In the experiments with 24- and 48-h incubation no reliable effect of the hormones was

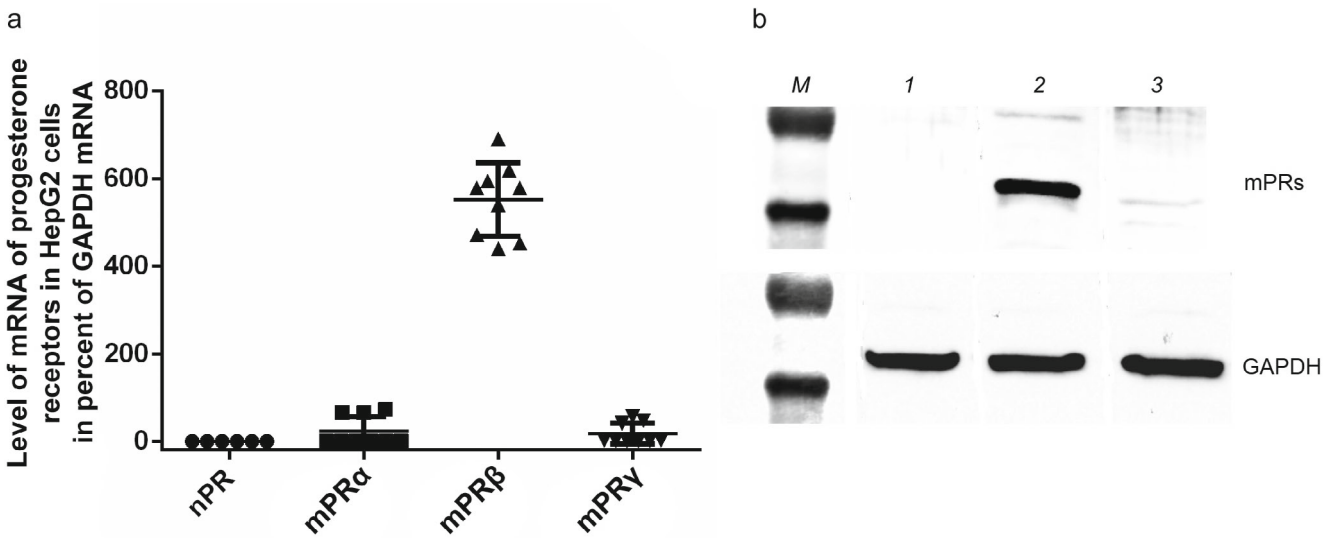

Fig. 1. Investigation of expression of the genes of progesterone receptors and content of respective proteins in HepG2 cells. a) Content of *PGR* mRNA (nPR) and *PAQR 7*, *PAQR 8*, and *PAQR 5* mRNAs (mPRα, mPRβ, mPRγ) in HepG2 cells in percent of the *GAPDH* mRNA. b) Immunoblotting of HepG2 cells lysates after incubation; lanes: *1*) with primary antibodies (ABs) against mPRα, *2*) with primary ABs against mPRβ, *3*) with primary ABs against mPRγ and ABs against the reference protein (GAPDH). mPRs – 40 kDA; GAPDH – 36 kDa; *M*) molecular weight markers 50 kDa and 34 kDa.

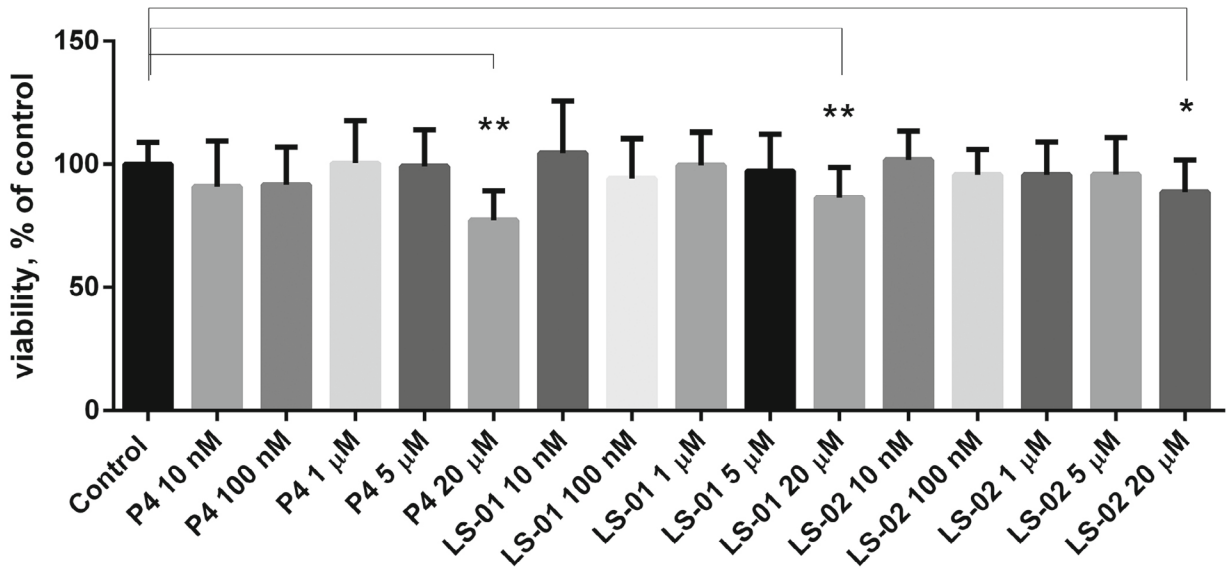

Fig. 2. Effect of different concentration of progesterone (P4) and selective ligands LS-01 and LS-02 on viability of HepG2 evaluated with the help of XTT test. Measurement results are presented as a mean ± standard deviation. Significant differences form the control values are marked: * $p = 0.0011$; ** $p < 0.0001$.

revealed (data not presented). The results of statistical processing of the results of six independent XTT-tests are presented in Fig. 2: progesterone (P4) reduced viability of HepG2 cells by 23% ($p < 0.0001$), LS-01 – by 14% ($p < 0.0001$), LS-02 – by 12% ($p = 0.0011$) at concentration 20 μM after 72-h incubation with hormones.

Effect of progesterone and selective mPRs ligands on DNA fragmentation in HepG2 cells determined with the TUNEL-test. Effect of three steroids on DNA fragmentation was investigated in the study. Histogram with the results of statistical processing of the data obtained in the experiments investigating DNA fragmentation in the cells using TUNEL-test is presented in Fig. 3. It can be seen that no reliable hormone effects were observed after 72-h incubation in comparison with the control (from the results of 7 independent experiments).

Effect of progesterone and selective ligands LS-01 and LS-02 on Annexin V binding by the HepG2 cells and permeability to propidium iodide. The 72-h incubation with progesterone resulted in significant increase of the annexin V binding to the HepG2 cells, but, at the same time, the number of cells permeable to PI decreased (Figs. 4 and 5). The selective ligands exhibited the same effect, but it was less pronounced (Figs. 5 and 6). In comparison with the control the number of cells binding Annexin V increased 2-fold in the presence of 5 μM of progesterone ($p = 0.044$), and 4.5-fold in the presence of 20 μM of progesterone ($p < 0.001$). LS-01 at concentration of 5 μM increased the number of such cells 1.3-fold and at concentration 20 μM – 2.2-fold ($p = 0.0136$). LS-02 at concentration 5 μM increased the number of such cells 1.2-fold, and at concentration 20 μM – 2.2-fold ($p < 0.0001$) (Fig. 5a). Among all the cells binding Annexin V under the action of progesterone, the number of cell impermeable to PI increased (Fig. 5b), at 5 μM – 2.7-fold and at 20 μM – 15-fold ($p < 0.0001$); and the number of cells that simultaneously bound Annexin V and were permeable to PI increased under the action of progesterone (Fig. 5c) 1.9-fold at progesterone concentration 5 μM ($p = 0.0005$) and 3.2-fold at concentration 20 μM ($p < 0.0001$). Under the action of LS-01 among all the cells binding Annexin V, the number of cell impermeable to PI increased (Fig. 5b)

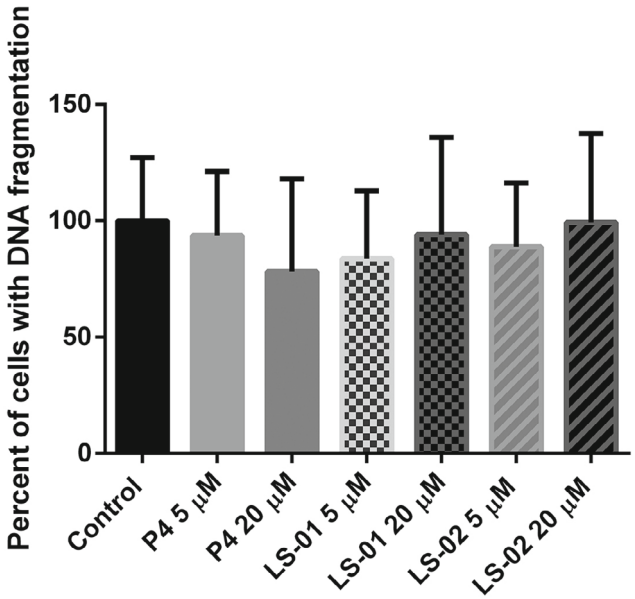

Fig. 3. Effect of progesterone (P4) and selective ligands LS-01 and LS-02 on DNA fragmentation in HepG2 cells assessed with the help of TUNEL-test. Measurement results are presented as a mean ± standard deviation.

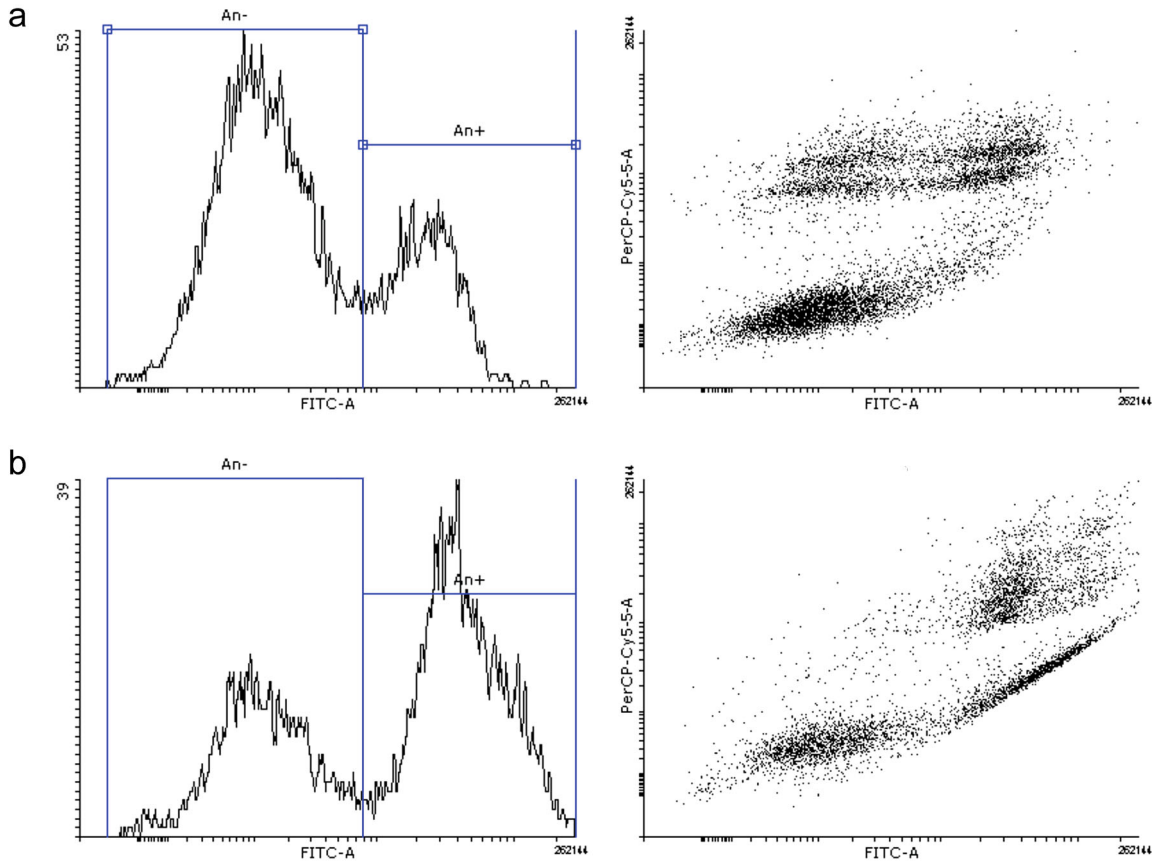

Fig. 4. Flow cytometry plots and dot diagrams of HepG2 cells distribution in the experiments with binding Annexin V and their staining with PI in the control sample (a) and after incubation with 20 μM progesterone for 72 h (b). An–, peak of the cells not binding Annexin V; An+, peak of the cells binding Annexin V.

1.9-fold at concentration 5 μM and 3.5-fold at concentration 20 μM ($p = 0.0302$); and the number of cells that simultaneously bound Annexin V and were permeable to PI (Fig. 5c) increased also 1.3-fold at concentration 5 μM and 2-fold at concentration 20 μM ($p = 0.0002$). And finally, under the action of LS-02, among all the cell binding Annexin V the number of cells impermeable to PI increased 1.4-fold at concentration 5 μM and 3.1-fold at concentration 20 μM ($p = 0.0839$); and the number of cells that bound Annexin V and were permeable to PI increased 1.2-fold at concentration 5 μM and 2.1-fold at concentration 20 μM ($p < 0.0001$) (Fig. 5, b and c). On the contrary, the number of all cells permeable to PI decreased by 22% under the action of 20 μM of progesterone ($p = 0.0004$), by 12% under the action of 5 μM of LS-01 ($p = 0.0358$), and by 12% under the action of 20 μM LS-01 ($p = 0.0403$) (Fig. 5d). This decrease occurred due to the cells, which did not bind Annexin V, but were permeable to PI. Under the action of 5 μM progesterone the number of such cells decreased by 23% ($p = 0,0226$), and under the action of 20 mM progesterone by 96% ($p < 0.0001$), under the action of 5 μM LS-01 – by 24% ($p = 0.0215$), under the action of 20 μM LS-01 – by 47% ($p = 0.0001$), and under the action of 5 μM LS-02 – by 17%, under the action of 20 μM LS-02 – by 38% ($p = 0.0005$) (Fig. 5e). As a result, the number of live cells unaffected by apoptosis and necrosis was reliably reduced only under the action of 20 μM progesterone by 23% ($p = 0.0003$) (Fig. 5f).

Determination of the changes in gene expression in the HepG2 cells under the action of progesterone, LS-01, and LS-02. Progesterone exhibits significant effect on expression of the genes of the selected markers of proliferation and apoptosis (Fig. 7). The only exception is expression of the gene encoding PCNA, which is reliably inhibited (by 18% in comparison with the control, $p = 0.0463$) only by 20 μM LS-01. Progesterone at two tested concentrations (5 and 20 μM) decreases the level of the Ki67 protein mRNA by 67% ($p < 0.0001$) and 96% ($p < 0.0001$), respectively, increases the level of the p21 protein mRNA by 52% ($p = 0.0002$) and 88% ($p < 0.0001$), respectively; progesterone at concentration 20 μM decreases the level of the cyclin D1 protein mRNA by 64% ($p < 0.0001$). Expression of the factors of apoptosis is mainly inhibited by progesterone with exception of the HRK (Harakiri) protein, expression of which increases 7-fold ($p < 0.0001$) in the presence of 20 μM of this steroid. At this concentration progesterone suppresses expression of proapoptotic

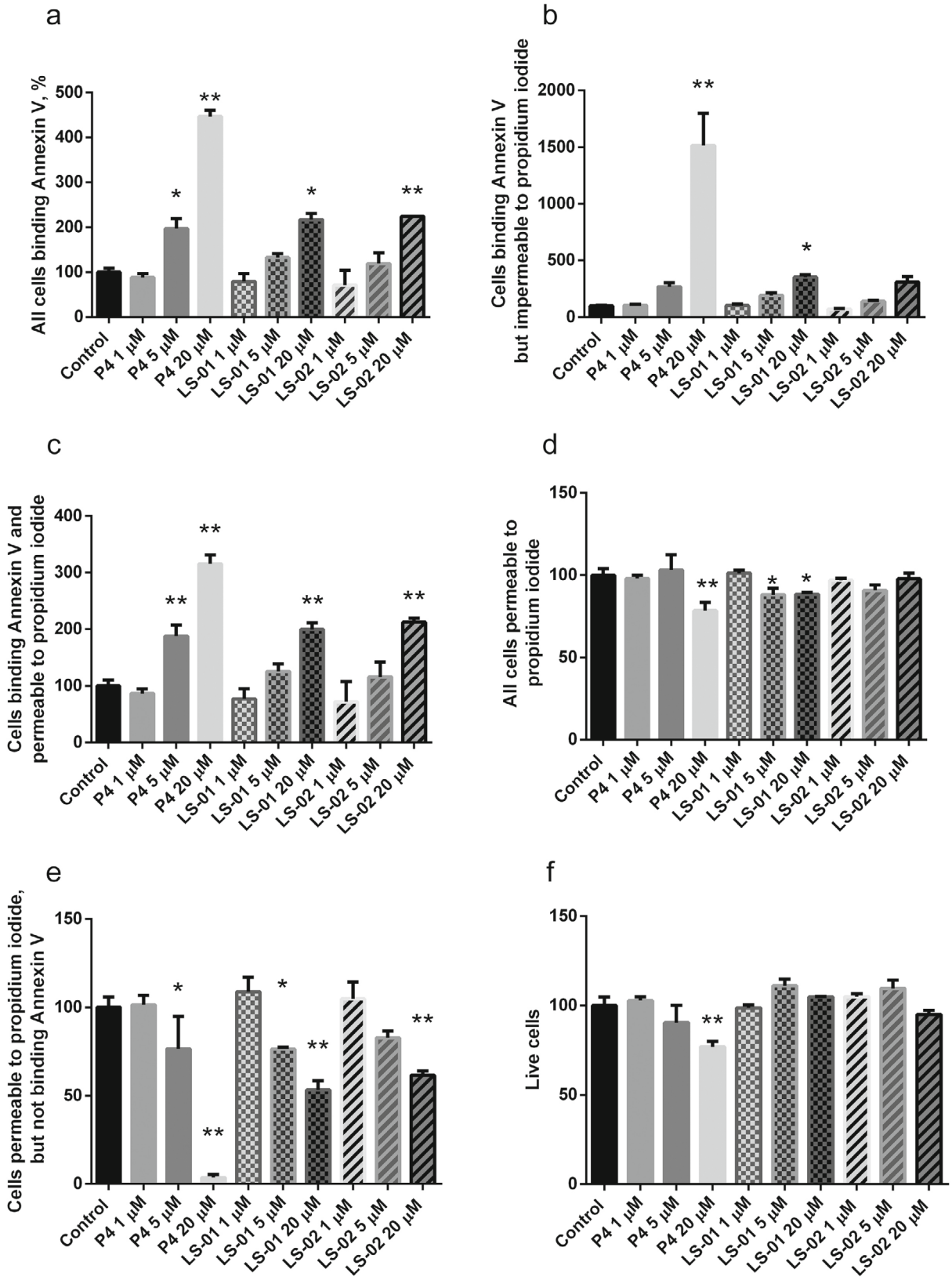

Fig. 5. Effect of different concentrations of progesterone (P4) and of selective ligands LS-01 and LS-02 on all the cells binding Annexin V (a); on the cells binding Annexin V and impermeable to PI (b); on the cells simultaneously binding Annexin V and permeable to PI (c); on all the cells permeable to PI (d); on the cells that do not bind Annexin V, but are permeable to PI (e); on the live cells unaffected by apoptosis and necrosis (f). Results are presented in percent of the number of corresponding cells in the control as a mean ± standard deviation. Significance of differences is marked: * $0.014 \leqslant p \leqslant 0.044$; ** $p \leqslant 0.0005$.

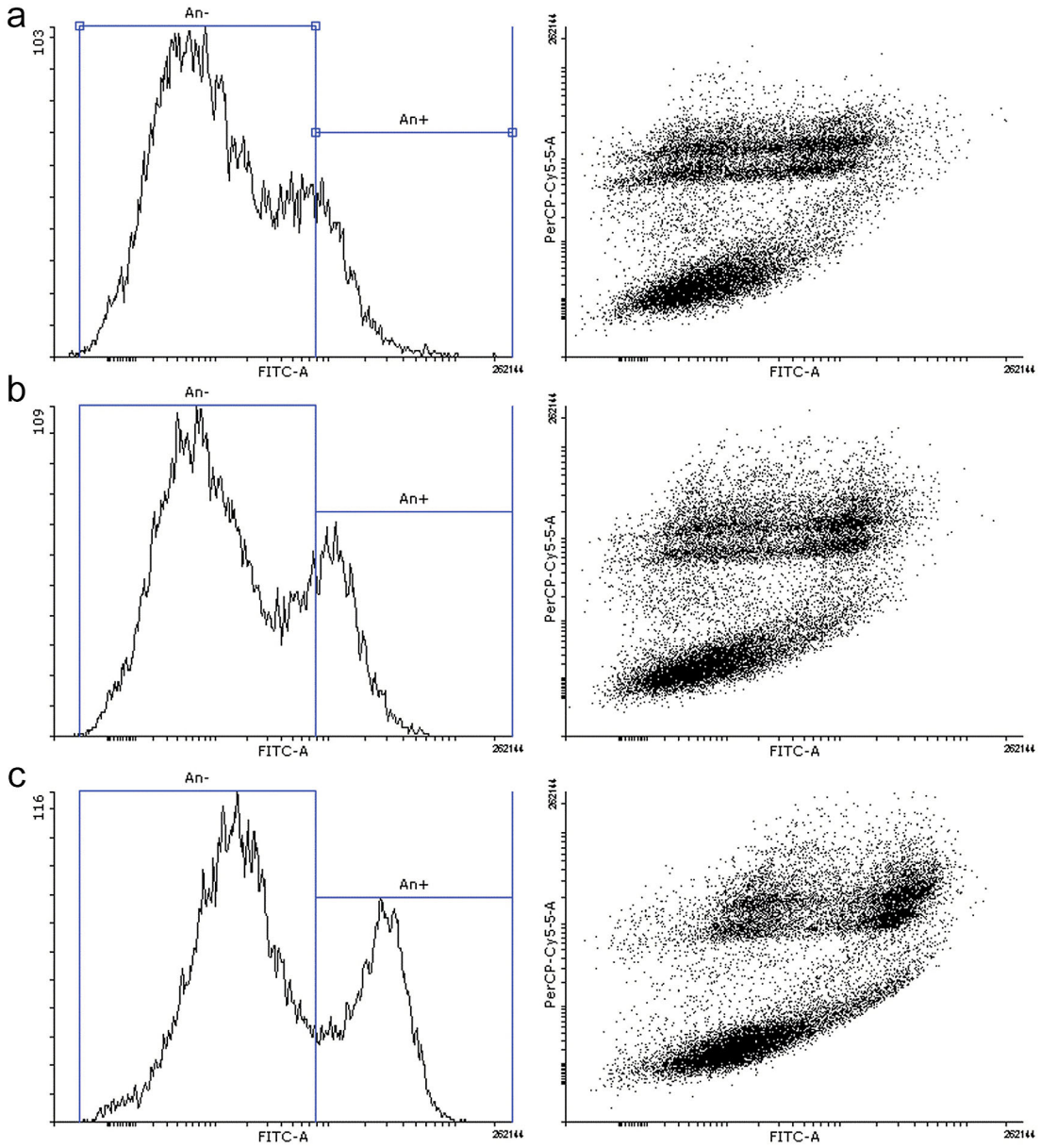

Fig. 6. Flow cytometry plots and dot diagrams of the HepG2 cells in the experiments on binding labeled Annexin V and their staining with PI in the control (a), after incubation with 5 μM (b) and 20 μM (c) of LS-01 for 72 h. An–, peak of the cells not interacting with Annexin V; An+, peak of the cells binding Annexin V.

factors: caspase 9 – by 33% ($p = 0.0164$), BAX – by 62% ($p < 0.0001$), BAD – by 31% ($p = 0.0001$). Reduction of expression of the proapoptotic factor DAPK (by 33%) has been observed already at the progesterone concentration of 5 μM ($p = 0.0013$), and by 73% at concentration 20 μM ($p < 0.0001$). The selective ligands did not exhibit any significant effect on the expression of the genes of apoptotic factors. Expression of the gene of the Bcl2A1 factor was also investigated in this study. All three investigated steroids did not affect the level of mRNA of this antiapoptotic factor (data not shown).

Effects of progesterone and selective ligands of mPRs on activating phosphorylation of p38 MAPK and JNK. Representative data of one of the three experiments investigating activating phosphorylation of two kinases under the action of 20 μM progesterone, LS-01, and LS-02 are presented in Fig. 8. Based on the results of densitometry the ratio phospho-p38/p38 MAPK in the control (Fig. 8, lane *1*) and under the action of all three steroids (Fig. 8, lanes *2-4*) practically did not change. The ratio phospho-JNK/JNK increased for the first isoform under the action of progesterone 1.9-fold (*2*), under the action of LS-01 – 1.5-fold (*3*), and under the action of LS-02 – 1.7-fold; for the sum of the second and third isoforms the increase was 2-fold (*2*), 1.7-fold (*3*), and 2.2-fold (*4*), respectively. Hence, all tested compounds activated all JNK isoforms in the HepG2 cells, but did not affect activity of p38 MAPK.

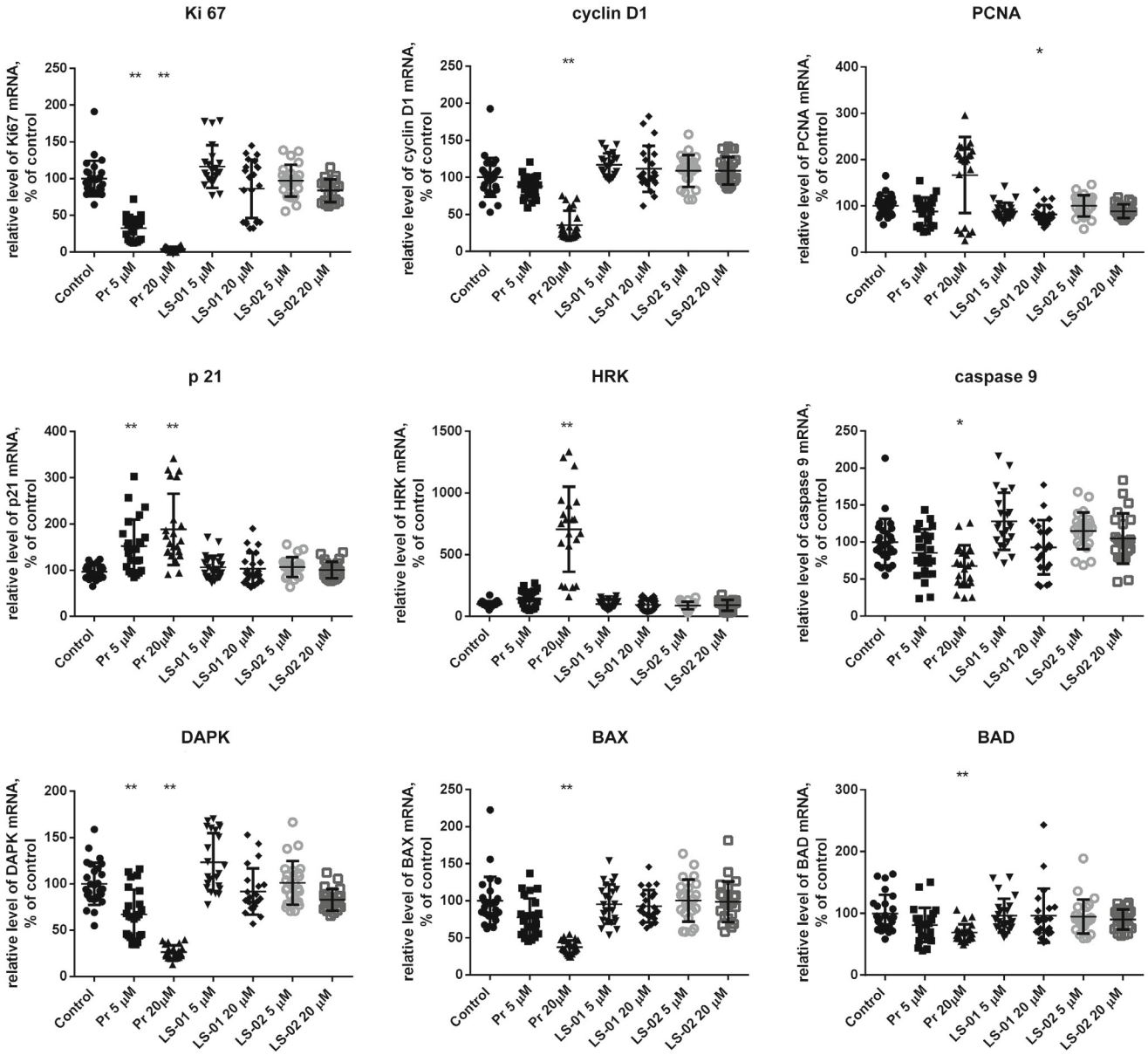

Fig. 7. Regulation of the level of expression of the genes encoding factors associated with proliferation and apoptosis in the HepG2 cells by progesterone (P4) and compounds LS-01 and LS-02. Contents of mRNA were determined using reverse-transcription quantitative PCR; data are presented as a mean ± standard deviation. Significant differences from the control values are marked: * $0.013 \leqslant p \leqslant 0.046$; ** $p \leqslant 0.0002$.

DISCUSSION

Effects of progesterone and its derivatives is determined mainly by the composition of progesterone receptors as well as by the phenotype of the particular cells. In this study we investigated effect of progesterone and selective ligands of mPRs (LS-01 and LS-02) in the cells of human hepatocellular carcinoma HepG2. This effect is mediated predominantly through the mPRβ subtype of the receptor, because the level of mPRβ mRNA in these cells was 579% of the mRNA of the reference gene, while for other membrane receptors it did not exceed 23%, and the level of nPR mRNA was zero (Fig. 1a). It must be mentioned that such high level of expression of the *PAQR8* gene was not observed in any of the 16 cell lines examined in our previous study; the maximum observed levels of expression were 154% (in the LN229 cells) and 112% (in the U87MG cells), in other cell lines mRNA level was 10-50% of the reference gene mRNA [13]. This receptor protein was also identified with immunoblotting, while mPRα and mPRγ protein were practically not detected (Fig. 1b).

Affinity of LS-01 and LS-02 to the membrane receptors was investigated using human pancreatic adenocarcinoma cells BxPC3 with predominant expression of mPRα and mPRγ (270% and 283% of the level of the reference gene mRNA, respectively) and absence of nPRs. It was lower than the affinity of progesterone

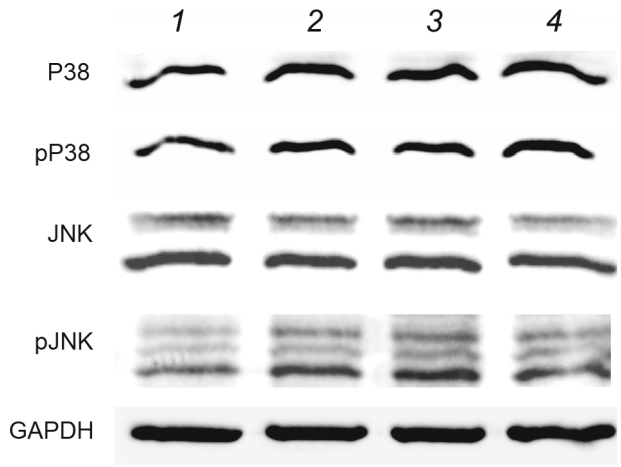

Fig. 8. Effects of progesterone, LS-01, and LS-02 on activating phosphorylation of p38 MAPK and JNK in the HepG2 cells. Lanes: *1*) control, *2*) effect of 20 μM progesterone, *3*) effect of 20 μM LS-01, *4*) effect of 20 μM LS-02.

to these receptors [14]. As was show in this work, content of mPRα and mPRγ in the HepG2 cell line was very low, and nPRs mRNA was not detected. Affinity of the selective ligands to mPRβ was not studied. It was important to find out whether the selective ligands are capable to regulate cell functions through mPRβ. HepG2 cells were found to be the most suitable for this purpose. It was revealed based on the effects of these compounds on activation of JNK, stimulation of the labeled Annexin V binding to the cells, and suppression of necrotic changes leading to permeabilization of the membrane that there are interactions of LS-01 and LS-02 with this subtype of mPRs. Considering that the effects of progesterone are stronger than the effects of the selective ligands, it could be assumed that affinity of progesterone to the mPRβ subtype is higher than affinity of its derivatives. The investigated steroids exhibited anti-proliferative and proapoptotic effects in the HepG2 cells. The tested compounds suppressed viability of the cells (Fig. 2) and stimulated antiapoptotic changes in their membranes (Figs. 4-6). Action of all three steroids resulted in activation of all JNK isoforms, moreover, no activating phosphorylation of p38 MAPK was observed in the process (Fig. 8).

It was of interest to compare the effects of investigated steroids in the HepG2 cells that predominantly express mPRβ with their effects in the BxPC3 cells, where predominant expression of mPRα and mPRγ was observed. In both cell types nPR mRNA was not found. As a result of our investigation, we demonstrated that the effects of investigated compounds are different in the cell lines HepG2 and BxPC3, but have the same direction [16]. Reliable decrease of viability was demonstrated for the HepG2 cells in the presence of maximum used concentration of all steroids, while viability of the BxPC3 was significantly reduced in the presence of progesterone and LS-01, while LS-02 did not affect viability of the BxPC3 cells according to the results of XTT-test. Availability of one subtype of mPRs results in more universal action of the investigated progestins. In the HepG2 cell line there was no such diversity in the action of the three steroids as was observed in the BxPC3 cells. In these cells the effects of LS-02 were significantly different than the effects of progesterone and LS-01 compound, likely due to the very high expression of the two subtypes of mPRs, which could have different affinity towards the three investigated steroids.

The effect of progesterone and selective ligands of mPRs on fragmentation of the nuclear DNA was different in the HepG2 and BxPC3 cell lines. In the BxPC3 cells progesterone and LS-01 reliably and significantly increased (3-3.5-fold) the number of cells with fragmented nuclear DNA, and LS-02 increased this number 2.2-fold. The main mediator of this effect was p38 MAPK. In the HepG2 cells we found neither reliable change of this parameter, not activation of p38 MAPK under the action of the investigated compounds.

On the contrary, the effect of progesterone and selective ligands on the early stages of apoptosis revealed by binding of the labeled Annexin V was pronounced in the HepG2 cells. No reliable changes in the number of apoptotic cells and cells with signs of necrosis were observed in the BxPC3 cells after 72-h incubation with the hormones in comparison with the control. And *vice versa*, in the HepG2 cell line all three steroids increased the number of cells with phosphatidylserine on the outer membrane that binds Annexin V, moreover, progesterone was shown to be the strongest effector. At the same time, all three steroids decreased the number of necrotic cells that have membranes permeable to propidium iodide, and again progesterone demonstrated the strongest effect. The cells of the HepG2 cell line grow in clusters not forming a monolayer, unlike the cells of the BxPC3 cell line. Cultivation of the HepG2 cells in the steroid-free medium (DFBS) for 72 h resulted in appearance of a significant population of cell with signs of necrotic death (i.e., cells with membranes permeable to PI). It could be, probably, explained by the fact that the cells grow into clusters, which reduce access to nutrients and/or oxygen in the lower cell layers. In the presence of investigated steroids, the rate of cells growth slowed down significantly, especially in the presence of progesterone. After 72-h incubation the number of cells in the wells with hormones was significantly lower than in the control wells. Hence, the lower cell layers in this case did not experience nutrient deficiency or hypoxia in such extent, and manifestations of necrosis were not so pronounced. At the same time steroids initiated the process of apoptotic death in the cells, with early stages of this process becoming visible after 72-h incubation through binding with labelled Annexin V.

Genes of proliferation markers and of apoptosis factors were selected for investigation of the gene expression profile under the action of progesterone and selective ligands, regulation of expression of these genes at the transcription level was observed in our study of the BxPC3 cells. In the HepG2 cell line progesterone suppressed expression of the genes of proliferation marker proteins Ki67 and cyclin D1, and enhanced expression of the inhibitor 1A of the cyclin-dependent kinase p21. Similar effects of progesterone were observed in the BxPC3 cells. However, no effects of LS-01 and LS-02 were observed. Possibly, activation of mPRβ under the action of ligands that exhibit lower affinity was insufficient for the effect. The observed effects of progesterone on expression of the genes of the factors associated with apoptotic processes in the cells were unanticipated. In the HepG2 cells this hormone enhanced expression of the proapoptotic protein HRK (Harakiri), which interacts with Bcl2, but decreased expression of the genes of other proapoptotic factors – BAX, BAD, caspase 9, and DAPK (Death-associated protein kinase 1). As a result, combined effect on the process of apoptosis was stimulating. It can be suggested that the effect of progesterone at high concentration could be mediated through activation of nuclear receptors of glucocorticoids. Glucocorticoids inhibit apoptosis in human and rat hepatocytes and facilitate their survival [22, 23]. Progesterone could affect expression of the genes sensitive to these hormones through glucocorticoid receptors. Selective ligands do not interact with the nuclear receptors of steroid hormones, because they do not have 3-keto/3-hydroxy group in the structure of A ring, which is required for such interaction [9, 24, 25]. The resulting effect of selective ligands and progesterone itself on viability and apoptosis in HepG2 cells revealed from exposure of phosphatidylserine on the outer membrane of the cells is opposite to the action of glucocorticoids on hepatocytes. It can be suggested that in this case main effect of these compounds is not the change of gene transcription, but post-transcriptional activating modification of the proteins themselves (apoptosis-inducing factors), which has been described in the literature [26-29]. It is likely that the effect at post-transcriptional level supersedes the side effect of progesterone-mediated activation of other steroid receptors.

It can be concluded based on the obtained results that in the cells of human hepatocellular carcinoma, HepG2, with high level of mPRβ, progesterone and selective ligands activate the JNK associated signaling pathway, which results in the suppression of cell proliferation and stimulation of the exposure of phosphatidylserine on the outer membrane of the HepG2, which is manifestation of the early stages of apoptotic changes. This is accompanied by slowing down of the processes of necrotic changes in the investigated cells due to, likely, suppression of proliferation and decrease of pressure on the lower layers of upper cells in the formed clusters. No effects of LS-01, LS-02, and progesterone on the later stages of apoptosis were observed over the investigated period of time. Considering that the indicated effects were caused not only by progesterone, but also by the selective ligands of mPRs, it can be concluded that the observed effects are realized through activation of mPRβ. All tested steroids acted in a similar way in the investigated HepG2 cells, unlike in the case of the BxPC3 cells, which also confirm their interaction only with one type of the receptors. Effect of progestins on the HepG2 cells is, likely, specific for this particular phenotype, because in the primary culture of myometrium, for example, action of the hormones realized through mPRα and mPRβ was accompanied by activation of p38 MAPK and resulted in phosphorylation of the myosin light chains and contractile activity in myometrium [18].

Hence, we showed in this study that interaction of mPRβ in the cells of hepatocellular human carcinoma with the ligands activates the JNK signaling cascade, which results in the decrease of proliferative activity and stimulation of apoptotic processes in these cells. The compounds LS-01 and LS-02 regulate functions of these cells, but in order to enhance this effect, search for more effective ligands with affinity to these receptors comparable with the affinity of progesterone, but, at the same time, without any affinity to nuclear receptors of progesterone and of other steroid is required.

Contributions. T.A.S. concept and supervision of the study; T.A.S., I.A.M., A.I.G. conducting experiments; I.S.L., Yu.V.K., I.V.Z. chemical synthesis of compounds LS-01 and LS-02; T.A.S. I.S.L., I.A.M., P.M.R., O.V.S. discussion of the results of the study; T.A.S. writing text of the paper; T.A.S. I.S.L., I.A.M., P.M.R., O.V.S. editing text of the paper.

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