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Comparative Evaluation of Cardiotonic Steroid Action on the Viability of Differentiated and Undifferentiated Human Neuroblastoma SH-SY5Y Cell Culture

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Abstract—Neuroblastoma (NB) is one of the most common childhood cancers. While it affects adults far less frequently than children, the prognosis for adults is significantly poorer. CTS, which are specific inhibitors of the Na^+,K^+-ATPase, are known to be cytotoxic to cancer cells. However, the mechanisms behind their differential effect on cancerous and mature cells are poorly understood. We used the SH-SY5Y NB culture to investigate these effects. We showed that the CTS ouabain, digoxin, and bufalin are toxic for both undifferentiated and differentiated SH-SY5Y neuroblastoma in the concentration range of 10–100 nM. Digoxin at a concentration of 100 nM, bufalin at a concentration of 10 nM. However, ouabain was an order of magnitude more toxic for the undifferentiated (10 nM) culture than for the differentiated (100 nM) culture. It was also shown that differentiation of SH-SY5Y neuroblastoma using retinoic acid causes a manifold increase in dopamine secreted by the culture into the culture medium. At the same time, ouabain causes a decrease in the amount of dopamine secreted by both undifferentiated and differentiated SH-SY5Y neuroblastoma. The toxic effect of ouabain in undifferentiated culture is associated with a decrease in the amount of anti-apoptotic protein Bcl-2. It was also shown that the toxic effect of ouabain on the undifferentiated culture of SH-SY5Y neuroblastoma is associated with PKC activity, since the addition of the PKC inhibitor chelerythrine neutralized the toxic effect of 10 nM ouabain. In all, our data suggests that CTS are important for future investigation of the differential effect of anticancer compounds on dividing cells and mature neurons.

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INTRODUCTION

Neuroblastoma (NB) is one of the most common childhood cancers [1], arising from defects in neural crest cell migration, differentiation, and maturation [2]. While low and medium-risk patients have a high (>90%) treatment success rate, in high-risk cases survival rates are below 50% [1]. Furthermore, in high-risk NB cases approximately 60% of surviving patients will experience a relapse, making high-risk NB a chronic disease with recurrent episodes that is poorly treatable [1]. While this cancer is extracranial, meaning it does not affect the central nervous system (CNS), metastatic spread affecting the brain leads to neurodevelopmental issues. The outcome for adult instances of NB appears to be worse than for children [3], in part because it is rare and poorly understood.

Several distinct NB cell lines exist, the most widely used being the SH-SY5Y cell line. They are capable of unlimited proliferation in vitro, and retain the ability to differentiate into mature neuronal cell types, displaying both electrophysiological and mediator activity [4]. Furthermore, it has been shown that while undifferentiated SH-SY5Y cells have significantly larger K^+ currents, which are known to be characteristic of cancer cells, differentiated SH-SY5Y cells have more pronounced Na^+ currents [5]. The differentiated SH-SY5Y neuroblastoma cells are characterized by an extensive growth of neurites that are morphologically similar to those of living neurons in the human brain. Depending on the conditions of SH-SY5Y differentiation, cells with adrenergic, dopaminergic or cholinergic phenotypes can be obtained [6]. This makes NB cells an excellent model for studying the effects of various pharmacological compounds on cancerous cells in comparison to mature non-malignant cells of the same origin [7], especially so for studying NB.

Some of the compounds undergoing research as a potential anticancer agents are cardiotonic steroids (CTS). CTS are specific inhibitors of the Na^+,K^+-ATPase, abnormal expression of which is closely correlated with cancer initiation, progression, and metastasis [8]. Their binding affects both the ion pumping

function of the enzyme, and the receptor function, which facilitates the triggering of a number of intracellular signaling pathways. Ouabain, along with other CTS, are conjectured to be endogenous regulators of various signaling processes in mammals. Ouabain binding to the Na^+,K^+-ATPase induces the activation of ERK1/2 through the activation of Src and the MEK1/2 pathway [9], and can indirectly act on a number of receptors [10]. It was also shown that ouabain is capable of causing the activation of Akt downstream of PI3K recruitment [11, 12].

Apoptosis induction through the ERK1/2 pathway is known to occur in neurons under the influence of ouabain [13]. The cytotoxic, migration-inhibiting, and metastatic [14] effects which CTS exert towards cancer cells have been explained by a number of mechanisms [15]. Recently, ouabain has been shown to act through the AMPK and Src pathways, inhibiting mitochondrial oxidative phosphorylation (OXPHOS) and inducing other metabolic changes [9]. CTS lanatoside C caused apoptosis in human hepatocellular carcinoma cells through PKCδ activation [16]. However, the molecular mechanisms behind the differential effects of CTS on cancerous versus mature cells are poorly understood.

In this study, we set the goal of comparing the concentration-dependent toxicity of the CTS ouabain, digoxin, and bufalin in RA-differentiated and undifferentiated SH-SY5Y neuroblastoma cell culture. We also sought to compare the effects of ouabain on dopamine production of differentiated and undifferentiated SH-SY5Y neuroblastoma cell culture. Furthermore, we attempted to investigate the influence of ouabain concentrations toxic to the undifferentiated SH-SY5Y neuroblastoma cell culture on levels of Bcl-2 family apoptosis regulator proteins, and the role of PKC and ERK1/2 in facilitating the toxic effect of ouabain.

EXPERIMENTAL

Cell Culture

Human neuroblastoma cell culture SH-SY5Y (ATCC, USA) was cultivated as described earlier [17] in a 1:1 solution of MEM with Earl salts and glutamine (PanEco, Russia) and F-12 medium without glutamine (PanEco, Russia), with the addition of 100 U/mL penicillin-streptomycin (PanEco, Russia) and 10% fetal calf serum (Biosera, USA). The culture was kept in a cell incubator at 37°C, 90% humidity, 5% CO_2 (SHEL LAB, USA). The medium was changed every 3 days. Differentiation of cells to dopaminergic type was induced by cultivation for 7 days in the presence of retinoic acid (MP Biomedicals, USA) at a final concentration of 10 μM and a decrease in serum concentration in the medium to 1%.

Experimental Procedures and Chemicals

The CTS Ouabain (Sigma, USA), Digoxin (Sigma, USA) and Bufalin (Cayman Chemical, USA), as well as inhibitors of PKC—Cheleritrin (Sigma, USA) and MEK1/2—PD0325901 (Sigma, USA) were dissolved to a final concentration in culture medium immediately before use, after which they were administered into wells containing plated cells—200 μL of inhibitor solution for 96-well plates, and 2 mL for 6-well plates. The cultures were then incubated for the necessary amount of time, depending on the experiment.

MTT Assay

Cell viability was evaluated using MTT assays. This method is based on the reduction of yellow 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by living cells to blue formazan. The procedure was carried out as described previously [13], using 96-well plates for culture plating. A Synergy H1 plate reader (BioTek, USA) was used to measure sample absorbance. Data is presented as a percentage of the control wells signal with intact cells. Data analysis was performed with GraphPad Prism 7 software using Shapiro–Wilk normality test and one-way ANOVA, *p* value was calculated using Sidak's multiple comparisons test.

Determination of Catecholamine Levels

All procedures were conducted in an ice water bath. Samples collected from the culture medium were centrifuged in a refrigerated centrifuge at 10.000g over the course of 15 min (t = 4°C). 1.0 mL of cultural medium was adjusted to pH = 8.7 with 400 mL tris buffer, and 10 mg Al_2O_3 (Sigma, USA) for the dopamine adsorption were added. The tube was shaken upside down and then centrifuged at 2000 g. The residue was washed three times, using 0.5 mL of a 2% tris buffer pH 8.7. For desorption of the dopamine from Al_2O_3, 1 mL of 0.1 mol/L $HCLIO_4$ was added to the residue and vigorously shaken and centrifuged at 2000 g for 10 mm. Supernatant was used to determine dopamine concentration. Dopamine content was determined in the supernatant using high performance liquid chromatography (ion-pair chromatography) with electrochemical detection (HPLC-ED) on a System Gold liquid chromatograph (Beckman Coulter, Inc., USA) equipped with a Rheodyne 7125 injector (USA) with a 20 μL sample loop. A reverse-phase column Nucleodur C18 Gravity, 4.6 × 250 mm, pore diameter of 5 μm (Mashery-Nagel GmbH & Co. KG, Germany) was used to separate the studied compounds. The mobile phase (0.1 M citrate-phosphate buffer (pH 3.0) containing 1.1 mM octanesulfonic acid, 0.1 mM EDTA and 9% acetonitrile) with a flow rate of 1 mL/min at a pressure of 200 atm was achieved using a System Gold 125 pump (Beckman Coulter, Inc., USA). An EC3000 electrochemical detector

(RECIPE Chemicals+Instruments GmbH, Germany) equipped with a ClinLab ECD cell, Model Sputnik, with a glassy carbon working electrode (+0.85V) and an Ag/AgCl reference electrode was used for measurements. MULTICHROM 1.5 software (AMPERSAND, Russia) was used for sample registration and chromatogram processing. All reagents used for the analysis were of analytical grade. The chromatograph was calibrated using a mixture of working standards of the detected substances at a concentration of 0.25 nmol/ml. Monoamine concentration in the test samples was determined using the internal standard method, by calculating the standard mixture to the test sample peak area ratio. Data analysis was performed with GraphPad Prism 7 software using Shapiro–Wilk normality test and two-way ANOVA, p value was calculated using Sidak's multiple comparisons test.

Western Blotting

After experimental procedures, the culture medium was removed, and the culture was rinsed twice with cool Hank's balanced salt solution (PanEco, Russia) and lysed in RIPA-buffer (Sigma, USA). The RIPA-buffer contained cocktails of protease and phosphatase inhibitors (Sigma, USA). Lysates were centrifuged for 10 minutes at 12000 g, the pellet was discarded. Protein concentration in samples was measured using the DC Protein Assay Kit (Bio-Rad, USA). Samples were subjected to Laemmli electrophoresis in a polyacrylamide gel, and transferred to a PVDF membrane. Then they were stained with SYPRO Ruby Protein Gel Stain (TheromoFisher, USA) and incubated with primary antibodies to Bcl-2, Bax, Bcl-xL and Bak (Santa Cruz Biotechnology, USA) and secondary antibodies—anti-rabbit IgG-HRP, antimouse IgG-HRP (Cell Signaling Technology, USA). The immunoreactive bands were visualized using Clarity Max ECL (Bio-Rad, USA) and detected with the ChemiDoc XRS+ (Bio-Rad, USA) system. Data analysis was performed with GraphPad Prism 7 software using Shapiro–Wilk normality test and one-way ANOVA, p value was calculated using Sidak's multiple comparisons test.

RESULTS AND DISCUSSION

Ouabain's Effect on the Viability of Differentiated and Undifferentiated SH-SY5Y Cultures

To compare CTS toxicity for undifferentiated and differentiated SH-SY5Y NB cells, cultures were incubated for 24 hours with 1 nM, 10 nM, 100 nM, and 1 μM ouabain, digoxin, and bufalin. Viability was evaluated using the MTT assay. From the three different CTS, one which possessed differential toxicity to undifferentiated and differentiated cells was chosen.

As seen in Fig. 1A, incubation with 1 nM ouabain did not affect undifferentiated SH-SY5Y culture viability. Incubation with 10 nM ouabain caused a 10% ($p < 0.001$) decrease in undifferentiated culture viability, 100 nM and 1 μM ouabain causing a 49% ($p < 0.001$) and 70% ($p < 0.001$) decrease, respectively (Fig. 1A). Viability was measured relative to the intact (control) culture post 24 hour incubation with the listed ouabain concentrations using the MTT assay. Incubation with 1 nM and 10 nM digoxin, according to the MTT assay, did not affect undifferentiated SH-SY5Y culture viability (Fig. 1A). On the other hand, incubation with 100 nM digoxin caused a 14% ($p < 0.001$) decrease in viability, while 1 μM digoxin caused a 22% ($p < 0.001$) decrease in viability relative to the intact (control) culture post 24 hour incubation (Fig. 1a). 1 nM Bufalin did not affect culture viability, while 10 nM bufalin caused a 17% ($p < 0.001$) decrease, 100 nm bufalin—a 36% ($p < 0.001$) decrease, and 1 μM—a 46% ($p < 0.001$) decrease relative to the intact (control) culture post 24 hours of incubation (according to the MTT assay) (Fig. 1A).

As seen from Fig. 1B, ouabain at a concentration of 1 nM and 10 nM does not affect the viability of the differentiated culture of SH-SY5Y neuroblastoma. 100 nM ouabain caused a decrease in culture viability by 27% ($p < 0.001$), and 1 μM ouabain—by 41% ($p < 0.001$) relative to an intact culture after 24 hours of incubation according to the MTT assay (Fig. 1B). Digoxin at a concentration of 1 nM and 10 nM does not affect the viability of the differentiated culture of SH-SY5Y neuroblastoma (Fig. 1B). 100 nM digoxin causes a decrease in culture viability by 35% ($p < 0.001$), and 1 μM digoxin—by 66% ($p < 0.001$) relative to an intact culture after 24 hours of incubation according to the MTT assay (Fig. 1B). Bufalin at a concentration of 1 nM does not affect the viability of the differentiated culture of SH-SY5Y neuroblastoma (Fig. 1b). 10 nM bufalin causes a decrease in culture viability by 10% ($p < 0.01$), 100 nM bufalin—by 41% ($p < 0.001$), and 1 μM—by 63% ($p < 0.001$) relative to an intact culture after 24 hours of incubation according to MTT assay (Fig. 1B).

As such, it can be concluded that the minimum toxic ouabain concentration for undifferentiated SH-SY5Y culture is 10 nM, and 100 nM for the differentiated culture. In other words, ouabain is 10 times more toxic for the undifferentiated culture than for the differentiated culture. At the same time, digoxin and bufalin do not display such specificity. Digoxin is toxic to both undifferentiated and differentiated cultures in concentrations from 100 nM, while bufalin starts being toxic for both at a concentration of 10 nM.

Unfortunately, there is a distinct lack of literature data on the toxicity of different CTS concentrations towards undifferentiated and differentiated SH-SY5Y NB cultures. As such, we believe that we are the first to present a breakdown of ouabain, digoxin, and bufalin toxicity (and their effect on viability) towards the above mentioned cultures. However, there are a

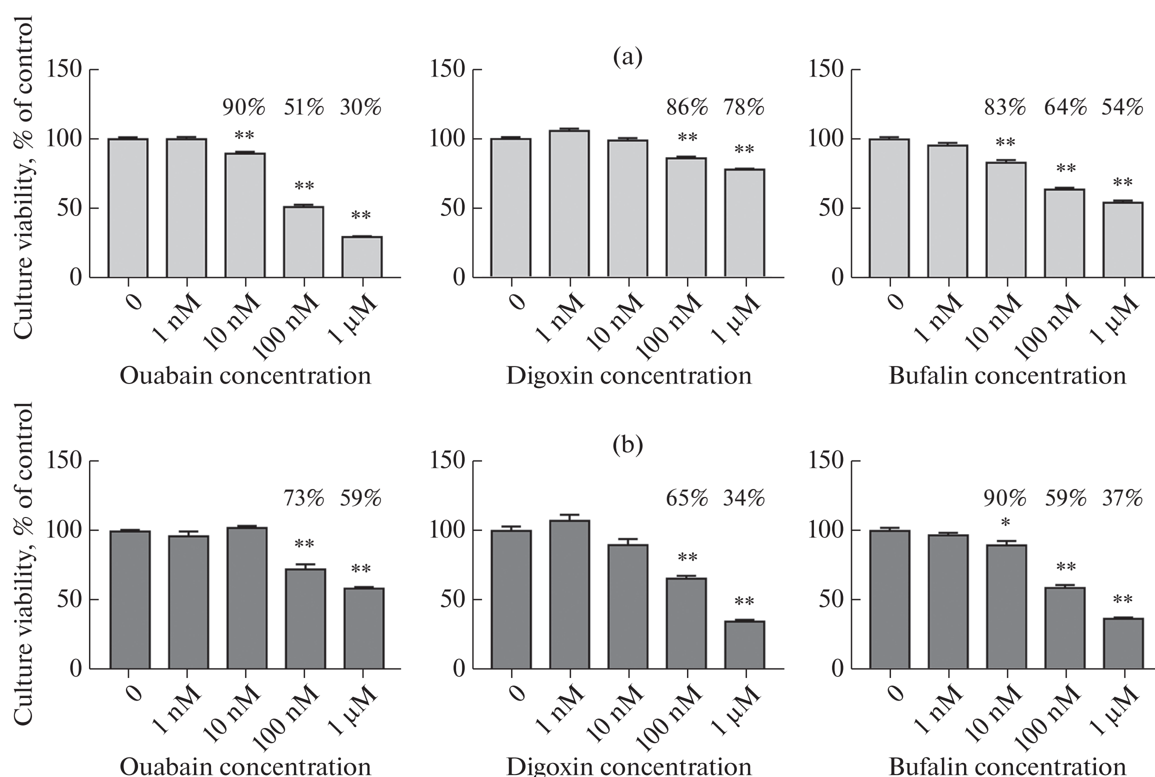

Fig. 1. The influence of 24 h incubation with the CTS ouabain, digoxin and bufalin in concentrations of 1 nM, 10 nM, 100 nM and 1 μM on the viability of undifferentiated (a) and differentiated (b) cultures of SH-SY5Y neuroblastoma according to the MTT assay. Data is presented as mean ± SEM, $N = 12$, *—$p < 0.01$, **—$p < 0.001$.

number of studies investigating the effects of CTS on cancer cells. Notably, a study on SH-SY5Y NB cultures showed the toxic effect of two ouabain concentrations towards undifferentiated cells. Incubation with 1 μM ouabain for 180 min caused approximately a 75% reduction in cell number, while incubation with 10 nM ouabain caused a cell number reduction by about 20% [18], which is similar to our results. A different study using three different NB types, including SH-SY5Y xenografted to mice which were then fed with ouabain reports that tumor growth was slowed by over 50% [19]. Taking into account the lesser affinity of the murine α1 Na^+,K^+-ATPase subunit to CTS in comparison with humans [20, 21], it should be noted that while the strategy of administering high doses of ouabain to mice in this manner may be effective, it is inadvisable to perform similar manipulations with humans. Digoxin has been shown to inhibit the development of SH-SY5Y cells grafted to mice by 44%, and the authors report a responsiveness to digoxin in plated cultures, but at a much higher dose (53 ng/ml) [22]. Unfortunately, considering the side effects of digoxin when used as a cardiac stimulant in a low dose [23], further investigation of its applicability as an anti-cancer agent in neuroblastoma seems futile. The CTS lanatoside C also displayed anti-glioma activity in primary GBM cells dissociated from tumor sections of different GBM patients [24]. A549 lung cancer and MCF7 breast cancer cells treated with 25 nM ouabain demonstrate rapid activation of AMPK (blocking mTOR activation) and Src/ERK1/2 signaling pathways, and a 2-fold decrease in intracellular ATP content over the course of 24 hours [9]. However, 1 nM and 10 nM ouabain promoted proliferation of both Jhhan and M07e cells [25], so it seems that comparisons between CTS mechanisms in different cancerous cell types should be drawn with extreme caution. In all, our data, showing a 10-fold difference in sensitivity of differentiated and undifferentiated SH-SY5Y NB cultures to ouabain, suggests that ouabain may have serious potential as an anticancer agent for treating this specific pathology.

Comparison of the Effect of Ouabain on the Amount of Dopamine Released by the Undifferentiated and Differentiated Culture of SH-SY5Y Neuroblastoma

In order to understand how ouabain affects the amount of dopamine produced by the undifferentiated and differentiated culture of SH-SY5Y neuroblastoma, cultures were incubated for 1 hour with 10 nM and 100 nM ouabain. Then the amount of dopamine in the incubation medium was measured, normalizing to the amount of protein in the culture.

As seen in Fig. 2, RA differentiation causes a 2.6-fold increase in dopamine levels in the incubation medium ($p < 0.001$) relative to the undifferentiated control. In undifferentiated NB medium, 1 hour incubation with 10 nM ouabain causes a 6.1-fold decrease in dopamine levels ($p < 0.05$), while 100 nM causes a 3.4-fold decrease ($p > 0.001$) relative to control values. In the differentiated SH-SY5Y culture, incubation with 10 nM ouabain caused a 4.8-fold decrease in dopamine levels ($p < 0.001$), while 100 nM ouabain caused a 2.8-fold decrease ($p < 0.001$) relative to control values.

Thus, our data confirms that RA causes dopaminergic differentiation of SH-SY5Y NB, which has previously been shown for other NB cultures, such as SK-N-SH [12]. We also noted that ouabain appears to decrease the amount of dopamine in the extracellular medium, affecting the undifferentiated culture more than the differentiated in this regard, which may be due to its effect on viability. This decrease in dopamine level in the medium is unexpected, since literature data points towards ouabain causing a short-term increase in dopamine release [26], and decreased uptake speed [27]. We conjecture that the observed effect may be linked to either dopamine synthesis disruption or changes in metabolization rate, leading to less dopamine being released after the initial increase. Thus, by the time we measured it, the dopamine from the initial release may have already degraded.

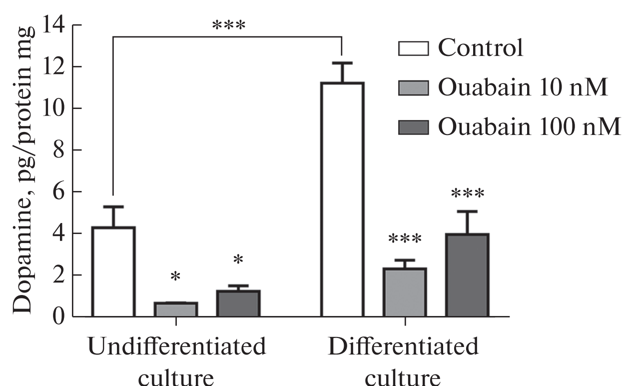

Fig. 2. Comparison of the effect of 1 hour incubation with 10 and 100 nM ouabain on the amount of dopamine released by the undifferentiated and differentiated culture of SH-SY5Y neuroblastoma. Data are presented as mean ± SEM of the amount of dopamine (pg) normalized to the amount of protein in culture cells (mg), $N = 5$, *—$p < 0.05$, ***—$p < 0.001$.

Effect of Ouabain on the Amount of Bcl-2 Family Proteins in the Undifferentiated Culture of SH-SY5Y Neuroblastoma

Overexpression of anti-apoptotic proteins of the Bcl-2 family is one of the characteristic properties of neuroblastoma cells [28]. We assessed the amount of anti-apoptotic proteins Bcl-2 and Bcl-xL and proapoptotic proteins Bax and Bak in the undifferentiated SH-SY5Y NB culture after 3 hours of exposure to 10 nM and 100 nM ouabain.

After 3 hours of incubation with 10 nM ouabain, the amount of Bcl-2 in the undifferentiated culture of SH-SY5Y neuroblastoma does not change, while after 3 hours of incubation with 100 nM ouabain, it decreases by 65% ($p < 0.05$) (Fig. 3a, 3e). At the same time, 3 hour incubation with either 10 or 100 nM ouabain did not change the amount of anti-apoptotic protein Bcl-xL and pro-apoptotic proteins Bax and Bak (Fig. 3b–3e). Thus, it can be concluded that the effect of ouabain on apoptosis regulation associated with the Bcl-2 proteins family is mediated via causing a decrease in Bcl-2 amount. A similar Bcl-2 downregulation has been reported previously [18] on undifferentiated SH-SY5Y NB cultures using a higher (1 μM) concentration of ouabain. Ouabain also seems to be capable of inducing a decrease in Bcl-xL concentration [18], which we likely do not observe due to the significantly lower concentration used in our study.

The Involvement of the PKC and MEK1/2 Kinases in Ouabain's Toxicity to Undifferentiated Culture of SH-SY5Y Neuroblastoma

In order to elucidate the role of PKC and MEK1/2-ERK1/2 signaling pathway activation in ouabain toxicity, we performed an inhibitory analysis. To assess the role of PKC in the realization of the toxicity of ouabain during 24 hour incubation, cultures of undifferentiated SH-SY5Y neuroblastoma were supplemented with 10 nM and 100 nM ouabain without the PKC inhibitor chelerythrine and with 2 μM chelerythrine and compared with the intact culture using the MTT assay. To assess the role of activation of the MEK1/2-ERK1/2 signaling pathway in realizing the toxicity of ouabain during 24 hour incubation, cultures of undifferentiated SH-SY5Y neuroblastoma were supplemented with 10 nM and 100 nM ouabain without the MEK1/2 inhibitor PD0325901 and with 1 μM PD0325901 and compared with intact culture using the MTT assay.

As seen from Fig. 4a, after 24 h of incubation, 10 nM ouabain caused a 10% decrease in culture viability ($p < 0.01$), while incubation with 10 nM ouabain and 2 μM chelerythrine did not differ from that of the intact culture and significantly differed from that for the incubated culture. with only 10 nM ouabain ($p < 0.05$). 100 nM ouabain caused a 32% decrease in culture viability ($p < 0.001$), while 2 μM chelerythrine did not cause changes in culture viability against the background of 100 nM ouabain. 2 μM chelerythrine alone did not induce changes in culture viability (Fig. 4a).

In the experiment with inhibitory analysis using PD0325901, 10 nM ouabain at 24 h incubation caused a 6% decrease in culture viability ($p < 0.01$), and 100 nM ouabain—37%. 1 μM PD0325901 did not affect the toxic effect of either 10 nM ouabain or

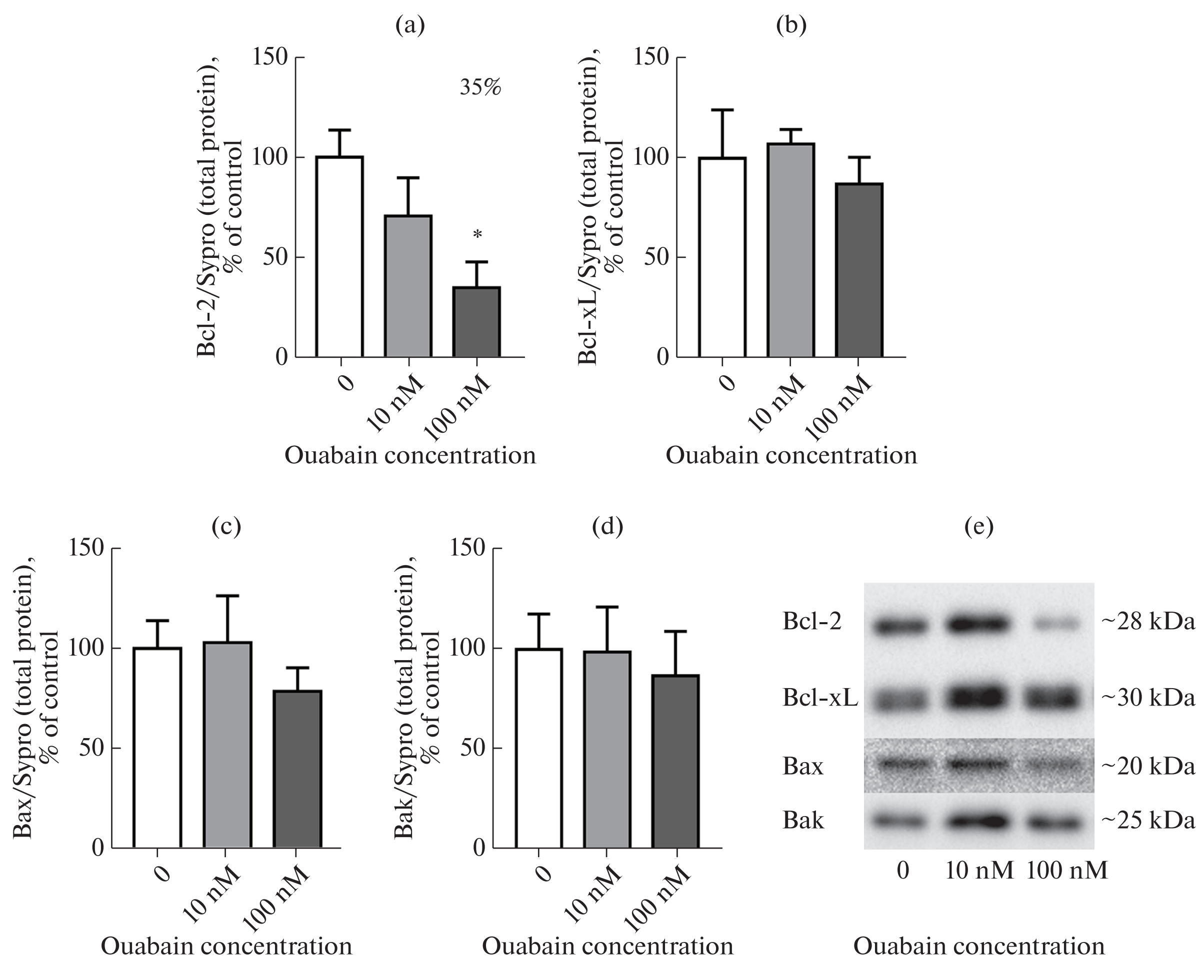

Fig. 3. Effect of 3 hour incubation of an undifferentiated SH-SY5Y NB culture with 10 nM and 100 nM ouabain on the amount of proteins Bcl-2 (a), Bcl-xL (b), Bax (c), Bak (d) normalized to the total amount of protein per membrane track (Sypro stain). (e) Image of representative immunoreactive bands on the membrane. Data is presented as mean ± SEM, $N = 5$, *—$p < 0.05$.

100 nM ouabain. At the same time, 1 μM PD0325901 itself did not affect the viability of the culture (Fig. 4b).

Thus, we found that PKC activity, but not ERK1/2, is associated with the toxic effect of 10 nM ouabain on the culture of undifferentiated SH-SY5Y neuroblastoma. While PKC is generally associated with an increase in cell survival [29, 30], several studies on epithelial cells [31, 32] show that PKC may be involved in reactive oxygen species formation through TNF-α. Taking into account that ouabain is known to cause oxidative stress [33], and that a different CTS, lanatoside C, has been shown to induce apoptosis of hepatocellular carcinoma cells through PKCδ activation [16], we can conclude that our results are in accordance with literature data.

CONCLUSIONS

In this study, it was shown that the CTS ouabain, digoxin, and bufalin are toxic to both undifferentiated and differentiated SH-SY5Y NB in the investigated concentration spectrum. However, ouabain was found to be 10-fold more toxic to the undifferentiated culture, versus the differentiated culture. This is promising for developing a future ouabain-based therapy of this cancer. It was also shown that differentiation of SH-SY5Y NB culture using RA causes a significant increase in dopamine production by the culture, as seen in the extracellular medium. At the same time, ouabain causes a decrease in dopamine content in the medium of both the undifferentiated and the differentiated SH-SY5Y NB. The toxic effect of ouabain in the undifferentiated culture is associated with a decrease in Bcl-2 protein amount. Furthermore, it was shown that the toxic effect of ouabain in the undifferentiated culture of SH-SY5Y NB is associated with increased PKC activity.

As such, the acquired data suggests that ouabain and CTS as a whole are important for future investigation of the differential effect of anticancer compounds on dividing cells and mature neurons, and may be used

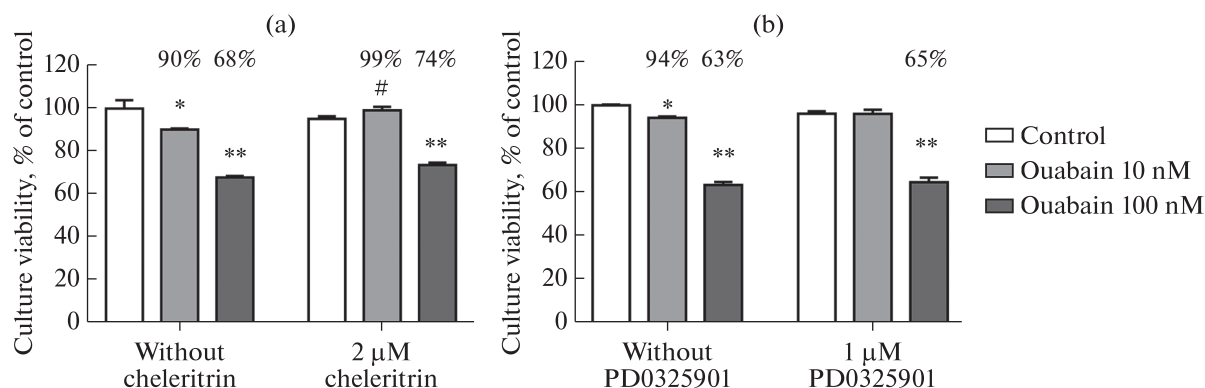

Fig. 4. Effect of 2 μM chelerythrine on the toxicity of 10 nM and 100 nM ouabain (a); the effect of 1 μM PD0325901 on the toxicity of 10 nM and 100 nM ouabain (b) at 24 h incubation according to the MTT test. Data are presented in% of intact culture mean ± SEM, N = 12, *—$p < 0.01$, **—$p < 0.001$, differences from control for the group; #—$p < 0.05$, differences from the corresponding subgroup of another group.

to continue investigating the mechanisms of CTS regulation of neuronal cell viability.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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