ORIGINAL CONTRIBUTIONS

MOLECULAR PROFILE AND CELL CYCLE IN MCF-7 CELLS RESISTANT TO CISPLATIN AND DOXORUBICIN

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Aim: To compare ultrastructure, phenotypic profile and cell cycle progression of MCF-7 human breast cancer cells and MCF7 sublines resistant to cisplatin (MCF-7/DDP) and doxorubicin (MCF-7/DOX). *Methods:* MTT-test, immunocytochemistry, flow cytometry, electron microscopy. *Results:* The development of drug resistance to cisplatin and doxorubicin in MCF-7 cells upon the culturing of the initial cells with the raising concentrations of cytostatics was accompanied by the increase in cells adhesion, the increasing differentiation grade and the loss of steroid hormone receptors. Besides, it was shown that antiapoptotic mechanisms (decrease of Bcl-2 expression) and intracellular glutathione detoxifying system are involved in the process of cisplatin resistance development in MCF-7 cells. At the same time, P-glycoprotein overexpression in cells resistant to doxorubicin suggests MDR-dependent mechanism. Both doxorubicin- and cisplatin-resistant cells are characterized by the changes in the expression of several cell cycle regulators — Ki-67, cyclin D1, pRb and p21). *Conclusion:* The long-time culture of MCF-7 cells with cytostatic drugs results in the decreased cyclin D1, pRb, and Ki-67 expression and increased p21 expression with the increasing differentiation grade of the resistant cells. The underlying mechanisms of resistance to cisplatin and doxorubicin in MCF-7 cells may be different.
Key Words: drug resistance, cisplatin, doxorubicin, human breast cancer, MCF-7 cells, immunocytochemistry, cell cycle.

Tumor drug resistance is one of the most important problems in cancer treatment in general as well as breast cancer treatment [1–3]. Many chemotherapeutic schedules used in breast cancer treatment include anthracyclines and platinum derivatives [2, 3]. Cisplatin belongs to the group of alkylating agents. It binds to DNA bases causing crosslinks and breaks in DNA strands interfering with DNA replication [4]. The antitumor effects of doxorubicin are associated with DNA intercalation and degradation of the tubular apparatus caused by the active free radicals [5]. The mechanisms of drug resistance development for these two drugs are different [4–7]. It is believed that cisplatin resistance is caused by an expression of proteins involved in glutathione-mediating detoxifying pathways such as glutathione-S-transferase, glutathione-reductase and glutathione itself [8, 9]. DOX resistance, as believed, results from the overexpression of 170 kD Pgp glycopeptide, which is an energy-dependent pump that effluxes xenobiotics away from cells [10, 11]. It is also known that the development of drug resistance phenotype could be accompanied by changes in morphological structure, proliferative potential and adhesion properties of cells as well as the changes in expression of proteins involved in apoptosis and cell cycle control [12–15]. Studies on mechanisms of antitumor drug resistance development are of paramount importance for further understanding of fundamental processes in formation of drug resistance phenotype in tumors with the aim of searching the ways for overcoming such resistance.

The aim of the study was to compare the ultrastructure, phenotypic profile and cell cycle of MCF-7 human breast cancer cells and sublines resistant to cytotoxic effects of cisplatin (MCF-7/DDP) and doxorubicin (MCF-7/DOX).

MATERIALS AND METHODS

Cell lines and drug treatment. For our studies we used human breast cancer cell line MCF-7 and its sublines resistant to cytotoxic effects of cisplatin (MCF-7/DDP) and doxorubicin (MCF-7/DOX). The cells of the initial MCF-7 line were cultivated in modified Dulbecco's medium ISCOV ("Sigma", Germany) with addition of 10% of fetal calf serum ("Sangva", Ukraine) at the temperature of 37 °C and CO_2 concentration of 5%. Cells were reseeded twice a week at the density $2–4 \times 10^4$ cells/cm^2, when cell layer covered about half of the flask surface.

The resistant variants MCF-7/DOX and MCF-7/DDP were originated by growing initial MCF-7 cells with raising concentrations of cisplatin (from 0.01 to 6 µg/ml) or doxorubicin (from 0.1 to 32 µg/ml), respectively. Cisplatin and doxorubicin were added twice a week after reseeding. Every two months, cell survival was analyzed by MTT assay. IC50 values for MCF-7 and MCF-7/DDP cells were 0.25 and 1 µg/ml of cisplatin, respectively, and for MCF-7 and MCF-7/DOX cells — 0.5 and 8 µg/ml of doxorubicin, respectively. Therefore, MCF-7/DDP were 4 times as much resistant to the cytotoxic effect of cisplatin and MCF-7/DOX cells were 16 times as much resistant to the cytotoxic effect doxorubicin as compared with the initial MCF-7 cells.

MTT assay. Sensitivity to antitumor drugs (cisplatin and doxorubicin) was measured every two months using standard MTT-colorimetric test with 3-[4,5,dimethylthiasol-2-1]-2,5-diphenyltetrasolium bromide ("Sigma", Germany) [16].

Immunocytochemistry. Expression of surface and intracellular antigens was studied immunocytochemi-

cally using mouse monoclonal antibodies to P-glyco-protein (Pgp), glutathione-S-transferase (GST), RE, PR, p53, Bcl-2, E-cadherin, Ki-67, cyclin D1, pRb, c-myc, p21 ("Dako Cytomation", Denmark).

Flow cytometry. For cell cycle analysis of MCF-7 cells and its sublines, resistant to cisplatin (MCF-7/DDP) and doxorubicin (MCF-7/DOX), the cell suspension (10^6 cells per 200 µl of saline) was washed, 2 ml of cold 70% ethanol was added, and suspension was fixed on ice for at least 30 min. Specimens were centrifuged for 5 min at 300 g with further super-natant decantation. After resuspending the sample in 400 µl of saline, 50 µl of RNAse (25 mg/ml) and 10 µl of propidium iodide (0.5 mg/ml) were added. Samples were analyzed on PAS "Partec" flow cyto-meter (Germany) using red filter [17].

Electron microscopy. The cells were fixed in 1.6% glutharaldehyde solution in 0.1 M cacodylate buffer (pH 7.3) for 1 h followed by washing in 0.1 M cacody-late buffer solution for 16–18 h. To achieve the isotonic state, sucrose (50 mg/ml) was added. Cell postfixa-tion was done in 2% osmium tetraoxide with further dehydratation in alcohols and embedding in araldite as described elsewhere. The ultrathin sections pre-pared on LKB-8800 ultratome and contrasted by uranil acetate and lead citrate were examined in JEM-100B electron microscope with 60 kV acceleration voltage with further specimen photography [18].

Statistical analysis. Statistical analysis was done using STATISTICA 6.0 software (StatSoft Inc., USA) with acquisition of mean values and standard deviation (SD). Student's t-test was used to evaluate the signifi-cance of the differences between groups. $p > 0.05$ was considered as the significant difference.

RESULTS AND DISCUSSION

Morphological features of sensitive and re-sistant to cisplatin and doxorubicin MCF-7 cells. It is known, that the development of drug resistance phenotype is accompanied by the changes in different biological features of malignant cells, including mor-phological ones. In contrast to spindle-shaped MCF-7 and MCF-7/DOX cells, MCF-7/DDP cells were more spherical with high nucleus/cytoplasm ratio. In all three lines, the nuclei were rounded with 2–4 nucleoli. Cells resistant to doxorubicin and cisplatin were larger than initial MCF-7 cells and were characterized by stronger adhesion to the underlying surface.

Electron microscopy demonstrated more com-plicated ultrastructural organization and increased differentiation grade upon formation of drug resis-tance both to cisplatin and to doxorubicin (Fig. 1, *a*). The number of the microtubules increased and the fibers comprising the microfilaments of varying width were evident. An active Golgi apparatus consisting of 2–3 loci with many multivesicular bodies was also re-vealed in the resistant cells (Fig. 1, *b*, *c*). The formation of a lot of multivesicular bodies and their positioning near the plasmatic membrane may be indicative of the increased efflux of the foreign substances through cell

membrane, which decreases the amounts of cisplatin and doxorubicin in cytoplasm of resistant cells and their cytotoxic activity. In MCF-7/DDP cells, the system of actin filaments located in the cortical layer of cyto-plasm was activated (see Fig. 1, *b*), while in MCF-7/DOX cells, the intermediate filaments were activated in the central area of cytoplasm (see Fig. 1, *c*). Therefore, the development of resistance to cisplatin and doxorubicin in MCF-7 cells was accompanied by the significant changes in ultrastructural organization suggestive of the increasing differentiation grade.
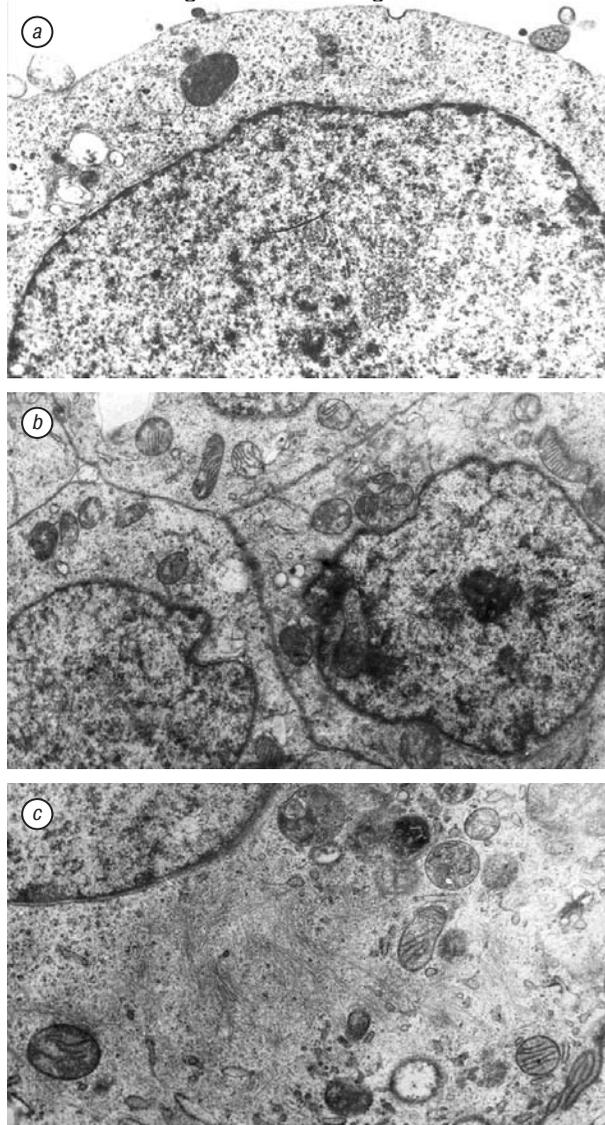

Fig. 1. Electron microscopy of MCF-7 cells (*a*) and subclones with drug resistance to cisplatin MCF-7/DDP (*b*) and doxorubicin MCF-7/DOX (*c*)

Immunocytochemical characteristics of MCF-7 cells, sensitive and resistant to cisplatin and doxorubicin. Mdr1 gene amplification followed by P-glycoprotein overexpression is one of the mecha-nisms involved in the development of drug resistance. An alternative mechanism is connected with GST isoenzymes expression and increased metallothionein expression [19–21]. Glutathione-S-transferases are responsible for conjugating glutathione with different xenobiotics. The detoxification of anticancer drugs by metallothioneins is related to their linkage with

electrophilic antitumor drugs from cisplatin group, because free metallothioneins are nucleophilic compounds [21]. In our experiments, P-gp expression was absent both in cisplatin-sensitive and resistant cells (Table 1, Fig. 2, *a*) suggesting mdr1-independent way of drug resistance. In contrast, in cytoplasm of most doxorubicin-resistant cells (MCF-7/DOX), P-gp was overexpressed (see Table 1; Fig. 2, *c*). Also we found out the significant differences in GST expression among the sublines studied. In cisplatin-resistant cells, the increase in GST content (Fig. 2, *b*) along with the decrease in the percentage of MT-positive cells (see Table 1) was evident.

According to current knowledge, receptor status of breast tumors is a prognostic factor that is connected with their sensitivity to neoadjuvant therapy and radiotherapy. We have reported earlier that the drug resistance in MCF-7 human breast cancer cells developed upon cell culture in raising cisplatin and doxorubicin concentrations was accompanied by the changes in hormone receptor expression [22]. Par-

ticularly, estrogen and progesterone receptors were expressed in 20% and 40% respectively of the initial cells, while in MCF-7/DDP and MCF-7/DOX cells these receptors were absent (see Table 1). According to the clinical experience, in a third of breast cancer patients the worse prognosis is associated with the absence of steroid hormone receptors.

It is known that cytotoxic activity of many antitumor drugs is linked to their ability to induce apoptosis in target cells [23–25]. We have attempted to compare the content of several apoptosis-related proteins in MCF-7 sublines with acquired drug resistance and in the initial MCF-7 cells. The acquisition of resistance to cisplatin or doxorubicin was not associated with changes of p53 expression, which was detected at a low level in all cell lines studied (see Table 1). Antiapoptotic Bcl-2 protein was present in cytoplasm of almost all sensitive and doxorubicin-resistant cells (Fig. 2, *d*, *f*) while the long-time culture of MCF-7 cells in the presence of cisplatin facilitated reduction of Bcl-2-positive cell percentage from 80 to 10% (see Table 1; Fig. 2, *e*). Therefore, the

Table 1. Phenotypic features of breast cancer MCF-7 cells and sublines with induced resistance to cisplatin and doxorubicin

Cell line	Cells, positive for studied proteins, %												
	RE	PR	Pgp	GST	MT	p53	Bcl2	Ki-67	E-cad	C-myc	p21	pRb	Cyclin D
MCF-7	20[2,3]	40[2,3]	0[2]	0[3]	70[2,3]	60[2,3]	60[2,3]	40[2,3]	90[2,3]	15	10[3]	60[2,3]	40[2,3]
MCF-7/DOX/16	0[1]	0[1]	70[1,3]	0[3]	10[1]	50[1]	15[1]	10[1]	10[1]	10	20	40[1]	20[1]
MCF7/DDP/4	0[1]	0[1]	0[2]	70[1,2]	15[2]	50[1]	10[1]	20[1]	20[1]	10	20[1]	30[1]	20[1]

In Table 1 and 2: [1]significantly ($p < 0.05$) different from MCF-7; [2]significantly ($p < 0.05$) different from MCF-7/DOX/16; [3]significantly ($p < 0.05$) different from MCF-7/DDP/4.
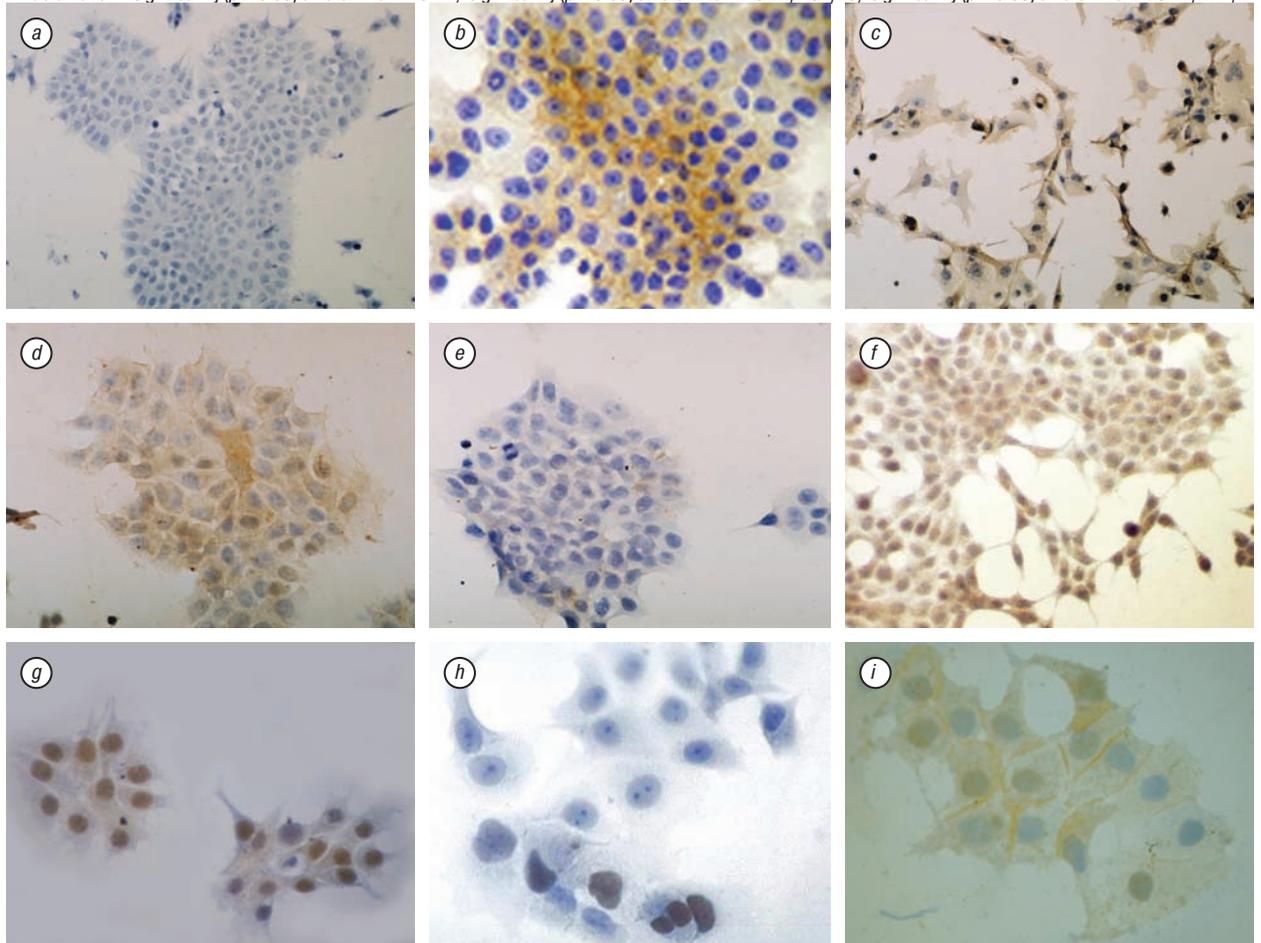
Fig. 2. Imunocytochemical features of MCF-7 cells with cisplatin and doxorubicin drug resistance phenotype: *a* — loss of Pgp expression in sensitive MCF-7 cells; *b* — expression of GST in MCF-7/DDP cells; *c* — Pgp expression in MCF-7/DOX cells; *d, e, f* — Bcl-2 expression in MCF-7, MCF-7/DDP and MCF-7/DOX, respectively; *g, h* — Ki-67 expression in sensitive MCF-7 cells and MCF-7/DDP cells; *i* — p21 expression in MCF-7/DOX cells

development of cisplatin resistance in breast cancer cells is associated with the lowered Bcl-2 expression. These data agree with both experimental and clinical findings demonstrating that high Bcl-2 expression predicts chemosensitivity in breast and lung cancer.

We have also found decreased proliferative activity of MCF-7 cells with resistance to cisplatin and doxorubicin (Fig. 2, *g*, *h*) with three-fold reduction of proliferative potential in MCF-7/DDP cells and two-fold reduction in MCF-7/DOX cells (see Table 1). The decrease in the proliferative potential may be correlated with differentiation of the resistant cells. In fact, both resistant variants (MCF-7/DOX and MCF-7/DDP) were characterized by strong expression of E-cadherin as compared to low expression of E-cadherin limited to the tight intercellular contact only in the initial MCF-7 cells (see Table 1).

The decreased proliferative activity in cells acquiring drug-resistant phenotype is believed to be associated with changes in the expression of some cell cycle-controlling proteins [26]. In our study, all three MCF-7 sublines expressed a low level of c-myc. The development of cisplatin and doxorubicin resistance in human breast cancer cells was accompanied by the reduction of cyclin D1, pRb expression and the overexpression of p21 (Fig. 2, *i*). These findings correspond with the available data of other authors [22, 26].

Therefore, the formation of drug resistance to cisplatin and doxorubicin in human breast cancer MCF-7 cells is characterized by changes in expression of proteins involved in control of apoptosis, cell cycle, proliferation, and adhesion.

Analysis of cell cycle in sensitive MCF-7 cells and cells with resistance to cisplatin and doxorubicin. The abnormal regulation of cell cycle is known as one of the characteristic features of the malignant cells. We have shown that the patterns of cell cycle distribution in the initial MCF-7 cells are the same as in cisplatin and doxorubicin-resistant sublines (Table 2, Fig. 3). The mechanisms of cytotoxicity upon cell exposure to cisplatin and doxorubicin are different. It is believed that cisplatin is not a phase-specific drug because it causes disorders in DNA transcription and replication, which lead to cell cycle arrest and apoptosis. In contrast to cisplatin, doxorubicin is a phase-specific drug, affecting predominantly S and G_2 phases of the cell cycle. It was of interest to compare cell cycle traverse in the initial and resistant MCF-7 cells upon cell exposure to cisplatin and doxorubicin. We have shown that in the initial MCF-7 cells, incubation with cisplatin at a dose of IC_{10} for 24 h resulted in the significant decrease of S phase percentage (from 28.52% to 19.55%) with G_2/M arrest (from 16.37% to 33.4%). Meanwhile, doxorubicin treatment resulted in the accumulation of MCF-7 cells in G_0/G_1 phase with the G_0/G_1 cell percentage increasing from 55.11% to 76.8%. In contrast, exposure to doxorubicin in MCF-7/DOX cells and exposure to cisplatine in MCF-7/DDP cells had no effect on cell cycle traverse (Fig. 4).

Table 2. Cell cycle distribution of MCF-7 cells and sublines with induced resistance to cisplatin and doxorubicin

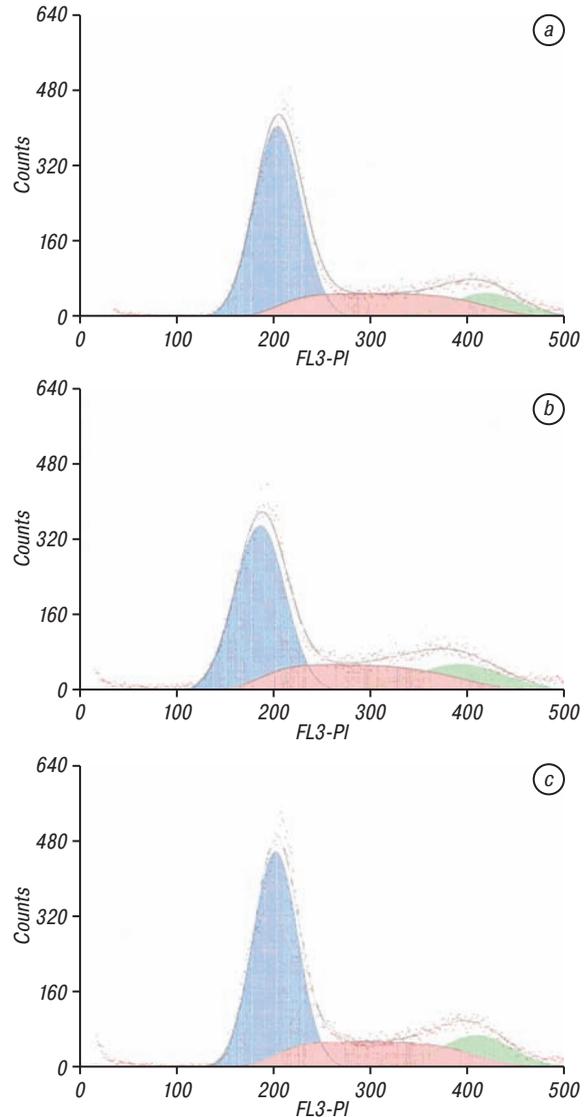| Cell line	Percentage of cells in a phase of the cell cycle		
	G_0/G_1	S	G_2/M
MCF-7	55.11	28.52	16.37
MCF-7/DOX/16	61.05	27.25	11.73
MCF7/DDP/4	59.42	26.20	14.38

Fig. 3. Distribution of MCF-7, MCF-7/DDP, MCF-7/DOX cells between cell cycle phases
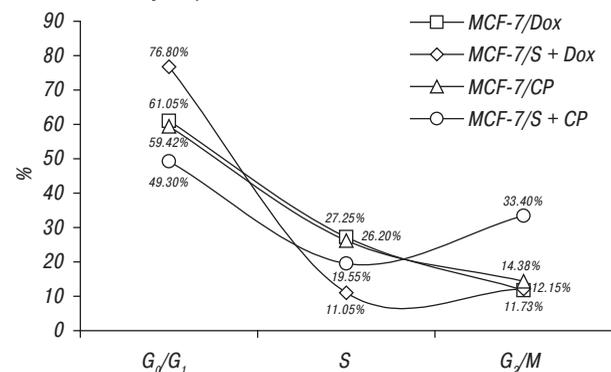
Fig. 4. Changes in cell cycle as result of impact of studied antitumor drugs

To sum up, the long-time culture of human breast cancer MCF-7 cells in the presence of cisplatin or doxorubicin *in vitro* is accompanied by the pronounced

changes in molecular-biological properties of the cells with both shared and drug-specific molecular mechanisms of the formation of the resistant phenotype. In both doxorubicin-resistant and cisplatin-resistant cells, the steroid hormone receptors have been lost. The development of cisplatin resistance involves the antiapoptotic mechanisms (decreased Bcl-2 expression) and intracellular glutathione detoxifying system while the development of doxorubicin resistance seems to follow MDR-dependent mechanism suggested by P-glycoprotein overexpression. In cells resistant to either cisplatin or doxorubicin, the adhesive properties are enhanced and the ultrastructural organization is characterized of more complicated patterns implying the increased differentiation grade. At the same time, in resistant cells cyclin D1, pRb, and Ki-67 expression decreased while p21 expression increased. The changed expression pattern suggests the decreased proliferative potential of the cells with drug resistant phenotype. Therefore, the resistant MCF-7 cells differ from the initial cell line by the expression of proteins associated with drug resistance as well as proteins involved in control of apoptosis, proliferation and cell adhesion. Our data may be advantageous for developing the novel schedules of anticancer treatment accounting for the molecular-biological characteristics of drug-resistant cells.

ACKNOWLEDGMENTS

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