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ULTRASTRUCTURAL CHANGES IN TUMOR CELLS TREATED WITH LIPOSOMAL FORMS OF ANTICANCER DRUGS

O.V. Yurchenko, N.V. Rusetskya, L.A. Naleskina, V.F. Chekhun*
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kiev 03022, Ukraine

Aim: **To determine the main ultrastructural changes in MCF-7 sublines sensitive and resistant to cytotoxic action of anticancer drugs, resulting from the treatment with conventional and liposomal forms of cisplatin and doxorubicin.** ***Methods:*** **Electron microscopy, light microscopy, MTT-test.** ***Results:*** **It has been shown that the phenomenon of drug resistance is associated with complication of ultrastructural organization of cells and more high differentiation by the main cytomorphologic characteristics which promote their resistance to cytotoxic action of anticancer preparations. Cytoarchitectonics of all resistant cells possesses common patterns and doesn't depend on the particular drugs toward which the resistance has been developed. It has been shown that the cells of the parental form MCF-7 line are more sensitive to cytotoxic action of doxorubicin than to cisplatin. Liposomal forms of anticancer drugs used at the same concentrations that the conventional ones, especially that of doxorubicin, caused more expressed alterations in ultrastructural organization of cells of all studied sublines with dominance of apoptotic processes.** ***Conclusion:*** **Evaluating an effect of equal concentrations of cisplatin and doxorubicin in conventional and liposomal forms, one may conclude on higher cytotoxic action of doxorubicin *vs.* cisplatin that is expressed in a wider spectrum of ultrastructural changes of cell architectonics in different sublines of MCF-7 cells and higher rate of apoptosis.**
Key Words: **ultrastructure, tumor cell resistance, liposome, doxorubicin, cisplatin.**

Cancer chemotherapy occupies the leading position in treatment of cancer patients, but an absence of selectivity of action of the majority of anticancer drugs and often an expressed resistance of tumor cells to performed chemotherapy remains a major challenge for clinical oncology limiting successful therapy of cancer patients.

There are numerous approaches for elevation of the efficacy of medicinal preparation action, in particular, the development of new technologies for creation of systems for directed transport of cancer drugs. Liposomes, especially spatially stabilized ones, are considered optimal variants of carriers used for targeted delivery of medicinal preparations to tumor cells. Such liposomes acquire the property to enter easily solid tumors through the walls of newly developed during neoangiogenesis vasculature and, being accumulated there, to act as a system constantly releasing medicinal preparations [1–3], what is considered in the literature [4] as an effect of elevated vessel permeability (EPR-effect). In a number of experimental studies it has been demonstrated higher accumulation of liposomal anticancer drugs in cells compared to their free forms [5]. The use of liposomal forms of anticancer drugs in modern chemotherapy created an alternative approach that from one side, allows decrease significantly total toxic influence due to their selective accumulation in tumor tissue, and from other side, — to elevate antitumor effect. Protractedly circulating liposomes and other macromolecular carriers may increase drug depositing in tumors [6].

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*Correspondence: Fax: +38 (044) 258-16-56
Abbreviatons used: DDP – conventional forms of cisplatin; DDP-Lip – liposomal forms of cisplatin; Dox – conventional forms of doxorubicin; Dox-Lip – liposomal forms of doxorubicin; GER – granular endoplasmic reticulum; MDR – multiple drug resistance; P-gp – P-glycoprotein.

If anticancer drugs are included into liposomes, their distribution in body significantly decreases; while their concentration in tumor increases, thus unspecific toxicity is lower [7].

There are the data showing that liposomal systems of drug delivery may overcome an activity of transport proteins of multiple drug resistance (MDR) even in highly resistant tumors. By endocytosis, liposomes deliver the preparation in cytoplasm and its direct interaction with P-glycoprotein (P-gp) located in plasma membrane is decreased. According to observations of Michieli et al. [8], incubation of cells with MDR patterns and P-gp hyperexpression with liposomal daunorubicin inserted into membrane composed from phosphotidylcholine/cholesterol, led to its significant intracellular accumulation compared with its free form and to 4–5-fold increase of cytotoxic effect. The mechanism of such action is not studied completely yet. However, experimental data evidence that MDR of cells modulated by P-gp, may be altered significantly by optimally designed liposome composition and inserted cancer drugs [9, 10]. The results of *in vitro* studies and clinical studies with the use of liposomal doxorubicin have shown that drug resistance may be partially reversed if one would use liposomal form of doxorubicin or other cancer grugs [11–13].

Cisplatin and doxorubicin are widely used for therapy of cancer patients, but differ in their mechanism of action. It has been demonstrated that the use of liposomal forms of the drugs is beneficial to their conventional analogs. Despite multiplicity of fundamental studies directed on the research of peculiarities of interaction between liposomal forms of anticancer drugs with cells, presently many questions remain unanswered. There is no data on the character of alterations of cells sensitive to cytotoxic action of anticancer drugs, after their treatment with liposomal analogs *versus* that of the cells resistant to antican-

ment of drug resistance, the cells became somewhat larger, and on their surface one could see an appearance of expressed protrusions of plasma membrane and increased number of microvilli of different length. MCF-7/Dox cells possess more spread shape what may be observed during their examination by light microscopy. With the use of electron microscopy it has been detected that cell nuclei are characterized by appearance of notable regions of heterochromatin located close to inner side of nuclear membrane, and by altered shape. The majority of cells possess rugged shape of nucleus due to appearance of nuclear membrane invaginations that respectively increase its area (Fig. 1, *b*). Such patterns were not observed in drug-sensitive cells. In nucleus one or sometimes two nucleoli located on periphery are detected. Cells of MCF-7/DDP and MCF-7/Dox sublines are characterized by higher density what is related to a large number of free ribosomes that tend to aggregate with the formation of polysomes. High electronic density is also related to big quantity of glycogen that forms characteristic rosettes all around cytoplasm. One may detect the increase of the number of all cell organelles. Mitochondria possessing the structure analogous to that of original MCF-7 cells, are located throughout cell perimeter, sometimes forming small aggregations close to nuclear invaginations. During development of drug resistance GER also underwent alterations and occupies large area in cells and is presented by long channels with significant number of ribosomes on its outer membranes. One should point on a partial vacuolization of channels tubules in some cells that may be related to increased protein synthesis [18].

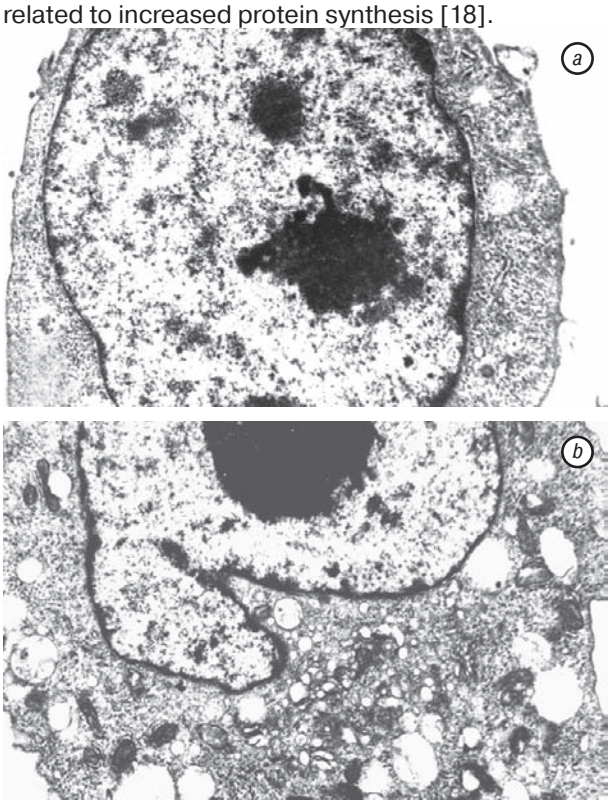

Fig. 1. Ultrastructure of MCF-7 cells: (*a*) parental cells, which are sensitive to cytotoxic action of antitumor drugs, x 6900; (*b*) variant with acquired resistance to doxorubicin, x 8400

Golgi complex in the majority of cells is composed from 2–3 loci placed in one region of cytoplasm, not far from nucleus. Each locus has a large number of microvesicles that from one side, are bound to few flat cisterns, and from other side — with large enough vacuoles. The presence of well developed numerous structural components of the complex in resistant cells vs sensitive MCF-7 cells evidences on functional loading of this cell organelle [19]. Using immunocytochemical analysis of resistant cells, Molinari et al. [21] have been shown that P-gp was present not only on the surface of plasma membrane, but was also located in cytoplasm, in particular on vesicles of Golgi complex and lyzosomal system components [20], but not on mitochondrial membranes.

In cytoplasm of MCF-7/DDP and MCF-7/Dox cells, one may detect also a big number of lyzosome-like formations, electron-transparent vesicles of different size, and lipid inclusions in some cells. It's necessary to note the presence of some cell polymorphism that was observed in parental MCF-7 cell line as well.

So, ultrastructure of resistant cells evidences on elevation of synthetic processes in the cells (increased number of free ribosomes and their aggregation in polysomes, higher numbers of GER components and elements of Golgi complex). The data point on alteration of cell metabolism that causes modification of their architecture [22, 23].

Ultrastructural changes in MCF-7 cells caused by action of conventional and liposomal forms of cisplatin and doxorubicin. Alterations of parental variant of MCF-7 cells caused by treatment with conventional forms of cisplatin (DDP) and doxorubicin (Dox) were of similar character but differ by the degree of expression of toxic manifestations. Our study has shown that parental MCF-7 cells are more sensitive to Dox that causes more expressed cytotoxic alterations than DDP does.

In the majority of cells dystrophic changes were detected that were expressed in significant vacuolization of cell cytoplasm and decrease of the number of cytoplasmic organelles. Some cells were in a state of necrobiosis, with significant nuclear alterations, in particular, with loosen euchromatin, while cytoplasm contained a big number of vacuoles of various sizes (Fig. 2, *a*). There were found the cells in necrotic state where integrity of cell membrane was disturbed, and cytoplasm components were disorganized. The number of cells with significant toxic manifestations was higher in the case of Dox treatment than that of DDP treatment. In the majority of studied cells nuclei with low electronic density and cytoplasm with large number of vacuoles and dense single mitochondria were observed, while clear detection of other organelles was problematic (Fig. 2, *b*).

Apoptotic cell death upon the action of conventional forms of the preparations was more characteristic for cells treated with doxorubicin, where all stages of apoptosis (from initial cytoplasm and nuclei condensation to formation of apoptotic bodies) have been observed. DDP Action also led to apoptosis of small part of sensitive

cells, but here the quantitative domination of irreversibly altered cells and cells in necrobisis state have been detected. Potential mechanism of cell death upon DDP influence is thought to be apoptosis that develops due to interaction of DDP with cell DNA; however, in some cases at early stages of apoptosis initiation, damage of cytoplasmic proteins occurs as well leading to necrotic damage of cells [24]. Despite the fact that apoptosis and necrosis are the forms of cell death differing by morphological and biochemical patterns, both their types may be present simultaneously in tissues and cultured cells upon the action of DDP [25]. The degree of intracellular damage caused by DDP may affect the form of cell death as a result of necrosis or not fulfilled apoptotic program, i. e. cell death induced by DDP not always responds to "classic" apoptosis, where an important role is played by preparation dose and cell cycle phase at the moment of DDP treatment [26].

Action of Dox in the majority of cases leads to development of apoptosis which efficacy depends on the dose of the preparation and incubation period.

Action of liposomal drugs on parental MCF-7 cells also led to death of a part of the cells. A number of cells that died upon the action of liposomal forms of the preparations, has increased at average by 3–5%, and also in the majority of cells significant cytotoxic alterations that manifest themselves in dystrophy of different degree, have appeared. It's necessary to note that cells became bigger, possibly, because of expressed vacuolarization of cytoplasm (Fig. 2, *c*). Dystrophic cell changes are also expressed in damaged structure of mitochondria which number decreased. Cristae deformation and their lower numbers are related to lower activity of mitochondria. Apart from this, vacuolization of the part of mitochondria was detected, pointing on cell autolysis. GER is presented by widened cisterns, on which surface there is a moderate number of ribosomes. A part of cells died due to necrosis. Upon the action of DDP-Lip there was observed a characteristic dominance of expressed dystrophic processes that finally lead to cell death, while apoptotic cells are rarely observed. For Dox-Lip there was detected a characteristic dominance of cells with morphologic signs of apoptosis (Fig. 2, *d*).

Thus, upon the action of liposomal forms of the anticancer drugs which concentration is equal to that of conventional forms, on drug-sensitive MCF-7 cells, one may detect more expressed cytotoxic damage of cell components, elevated apoptosis rate, especially in the case of Dox-Lip, compared to conventional forms. Such effect may be related to higher accumulation of liposomal drugs compared to conventional forms as well as their different intracellular distribution [6].

Ultrastructural changes of resistant MCF-7 cells caused by action of conventional and liposomal forms of cisplatin and doxorubicin. Alteration of ultrastructure of MCF-7/DDP cells upon the action of DDP manifested themselves by the presence of dystrophy signs: one could detect a large number of cells with electron-transparent vacuoles (Fig. 3, *a*). In a part of cells significant changes related to disorganization of all cell components and cytoplasmic matrix evidencing on cytotoxic damage, have been detected. Cell surface was bigger via formation of multiple cytoplasmic bubbles bound to membrane. The number of apoptotic cells was insignificant similarly to that of sensitive MCF-7 cells. Homotypic changes of resistant and sensitive cells treated with conventional form of the preparation may be explained by its concentration (IC_{10} for all studied cells).

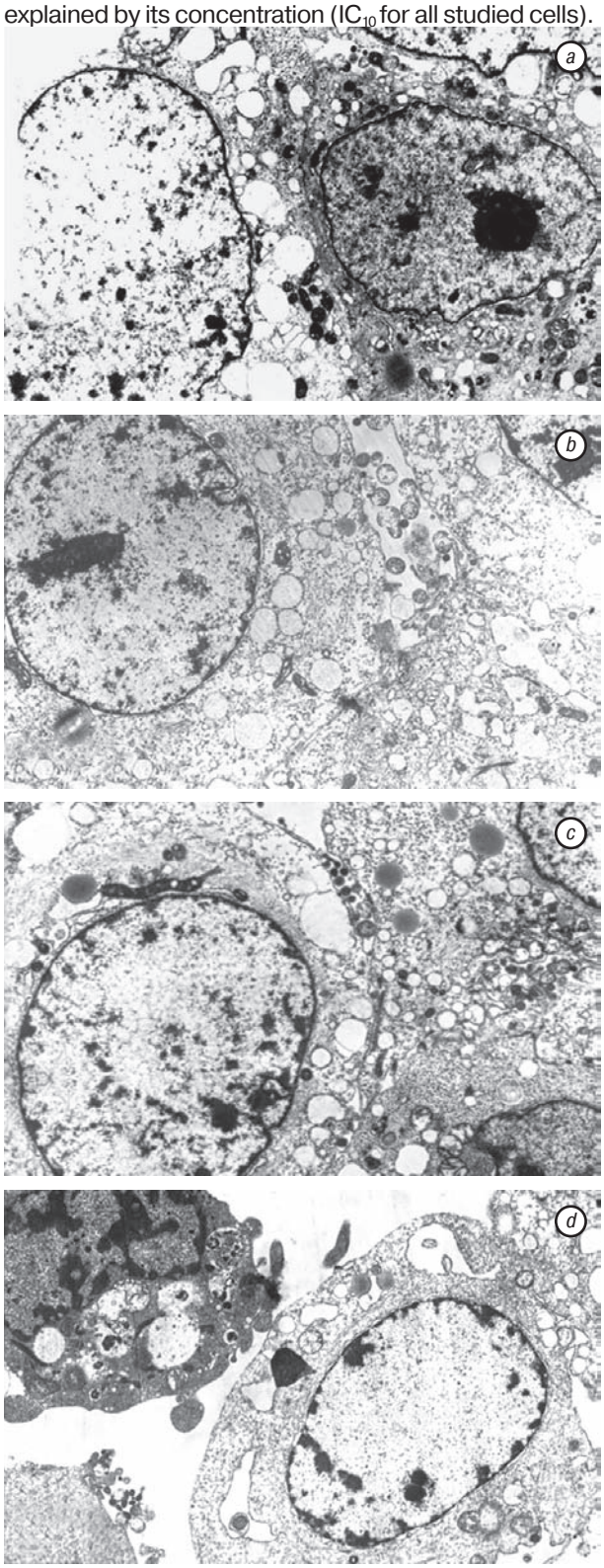

Fig. 2. Changes in ultrastructure of MCF-7 cells, which are sensitive to the cytotoxic action of antitumor drugs: (*a*) influence of DDP, x 5000; (*b*) influence of Dox, x 3300; (*c*) influence of DDP-Lip, x 5000; (*d*) influence of Dox-Lip, x 3300

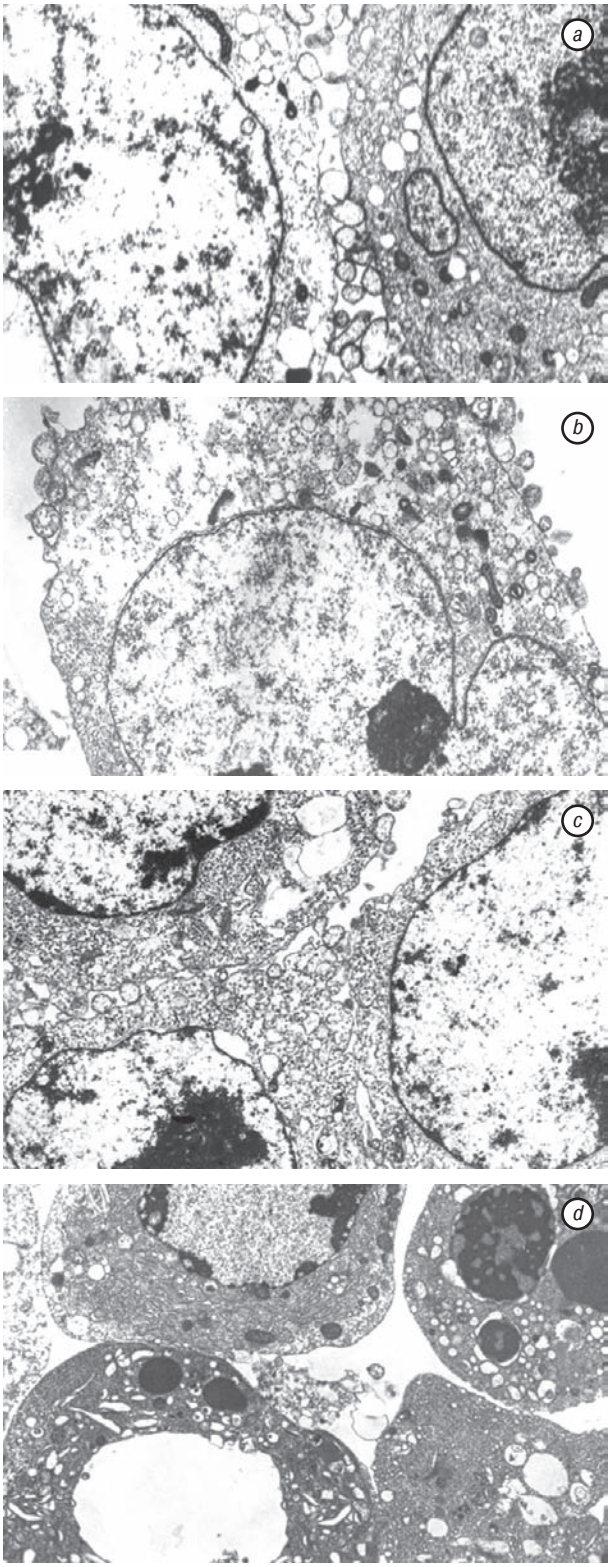

Fig. 3. Changes in ultrastructure of MCF-7/DDP and MCF-7/Dox cells upon the influence of antitumor drugs: (*a*) influence of free DDP on MCF-7/DDP cells, x 5000; (*b*) influence of DDP-Lip on MCF-7/DDP cells, x 5000; (*c*) influence of Dox on MCF-7/Dox cells, x 15 000; (*d*) influence of Dox-Lip on MCF-7/Dox cells, x 3000

Upon the influence of DDP-Lip toxic patterns are related not only to cytoplasm, but to nuclear ultrastructure where loosen euchromatin could be detected. In nucleoli, granular and dense fibrillar components presented by RNA-containing ribosome subunits, were dominating. In cytoplasm of the majority of cells disorganization of cell organelles occurred, electron-transparent area in cytoplasm appeared, vacuolization of cytoplasm and mitochondria were detected (Fig. 3, *b*). So, treatment of resistant cells with DDP-Lip led to more expressive toxic effect than that with conventional form of the drugs at equal concentration (IC_{10}) did.

Changes of doxorubicin-resistant cells upon the influence of Dox evidence on the presence of dystrophy of various degrees — cytoplasm disorganization, altered density of cytoplasmic matrix, vacuolization of cytoplasm, mitochondria, and Goldgi complex (Fig. 3, *c*). Electron-microscopic changes of nuclei were characterized by altered chromatin structure that was dispersed in nucleus as small granules without formation of characteristic aggregation along nuclear membrane. Experimental studies performed *in vitro* by other authors with the use of confocal microscopy, have shown that in resistant ovarian cancer cells of SKVLB line and MCF-7/ADR cells, doxorubicin is localized and transported mainly by cytoplasmic vesicles. The fact that in sensitive cells the preparation may have in part nuclear localization, what possibly determines its higher cell toxicity, is very important. Intraliposomal preservation of doxorubicin alters its distribution in resistant cells with partial transfer to nuclear compartments. So, liposomal doxorubicin may enter the cells with MDR phenotype bypassing vesicular transport and increasing its anticancer activity [27].

An influence of Dox-Lip on doxorubicin-resistant cells led to elevation of its toxic effect resulting in acceleration of dystrophic processes in cells, and increased number of cells in necrobiosis state with the tendency to necrotic death. It's necessary to note that among the majority of dying cells, all stages of apoptosis have been detected (Fig. 3, *d*).

The performed electron-microscopic study has shown that common cytomorphological manifestations evidencing on toxic effect of studied cancer drugs, account dystrophic alterations in cytoplasm, nucleus, and cell organelles, and also death of the part of cells by necrosis and apoptosis, what is more notable in the case of treatment with liposomal pharmaceutical forms, especially Dox-Lip.

In total, evaluating an effect of action of equal concentrations of cisplatin and doxorubicin in conventional and liposomal forms, one may conclude on higher cytotoxic action of doxorubicin *vs* cisplatin that is expressed in a wider spectrum of ultrastructural changes of cell architectonics in MCF-7 cells of different sublines and apoptotic death of higher numbers of cells.

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