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IN VITRO MODIFICATION OF CISPLATIN CYTOTOXICITY WITH MAGNETIC FLUID

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***Aim*: To study cytotoxicity of cisplatin conjugated with magnetic fluid (nanocomposite) upon exposure to magnetic field on sensitive and resistant to cisplatin MCF-7 human breast cancer cells. *Methods*: Cytotoxic activity was evaluated by MTT-test, intracellular iron accumulation was analyzed cytochemically, genotoxicity was studied by micronucleus test and DNA comet assay, ultrastructure was studied by electron microscopy techniques. *Results*: Nanocomposite of cisplatin was more toxic to MCF-7/S and MCF-7/CP cells compared to cisplatin in conventional pharmaceutical form. In nanocomposite-treated cells we observed more expressed signs of dystrophy (especially following application of magnetic field) and drastic alterations of nuclei ultrastructure with significant accumulation of iron nanoparticle clusters. The potent toxic action of nanocomposite is confirmed by electron microscopy and by marked genotoxicity, especially against MCF-7/CP cells. *Conclusion*: The enhancement of cyto- and genotoxicity of cisplatin nanocomposite combined with magnetic field in comparison with effect of convetntional cisplatin alone was demonstrated.**
***Key Words*: nanocomposite, magnetic fluid, cisplatin, MCF-7, electron microscopy, genotoxicity, static magnetic field.**

Recently the development of various nanotechnological platforms that may be useful in diagnosis and therapy has become the focus of interest. The nanomaterials for targeted drug delivery into cells and tissues are especially important in oncology [1, 2]. The development of targeted delivery of anticancer drugs is a topical problem taking into account their low selectivity as well as systemic toxicity. Up-to-date high-technological products based on biocompatible nanocomposites of "magnetite — biocompatible coating — anticancer drug" type are important for the development of novel pharmaceutical technologies allowing to enhance the therapeutic effects and decrease the doses providing in turn the improving safety of anticancer therapy [3].

The concept of using micro- or nanoparticles for targeted delivery of drugs has been put forward in late 70s by K.J. Widder et al., A.E. Sinyei et al. [4, 5]. The ways of targeted delivery of medicinal products provide optimal concentrations of the drugs in tumor with decreasing systemic toxic effects. Nanomaterials are of high interest since the basic properties of traditional materials change drastically upon their transition into ultradispersed state and even new patterns develop [6]. The principal reason of such alterations with decreasing particle size consists in relative increase in the percentage of "surface" atoms and higher contribution of surface energy in the overall chemical potential [7]. Unique properties of nanoparticles such as small size, high surface energy, biocompatibility, presence of magnetic properties open wide prospects for the use of nanomaterials in cancer therapy [8, 9].

The advantages of nanomaterials for clinical application are based not only on their physical properties, but also on the possibility of their conjugation with medicinal products providing for the release and targeted delivery of active ingredients upon the destruction of such complexes with minimal toxicity for the organism. It is also important that ferromagnetics, the compounds possessing spontaneous magnetization, are of significant interest as vectors for delivery of medicinal products for cancer treatment [10, 11].

The magnetic fluid representing a stable colloid dispersion of magnetic nanoparticles is the most customized nanotechnological product for targeted delivery of cytostatics [12]. The unique properties of magnetic fluids could be varied by changing the composition, coating, shape and sizes of nanoparticles [13, 14]. It is also important that the use of static magnetic field (SMF) could transform magnetic fluid in a unique vector system providing targeted delivery of nanoparticle composite to the malignant foci. SMF has been shown to affect membrane permeability resulting in elevated accumulation of nanocomposite (NC) containing anticancer drugs [15, 16]. This effect could be promising for overcoming drug resistance to different cytostatics, especially cisplatin (CP), which is one of the drugs of choice in breast cancer treatment.

Above-mentioned facts evidence on the necessity to study the mechanism of modification of cytotoxic activity of NC of CP with magnetic fluid and SMF as factors improving selectivity of the cytostatic agents and overcoming drug resistance.

MATERIALS AND METHODS

***Object of study*.** The biological activity of NC and SMF was studied on sensitive and resistant to CP MCF-7 human breast cancer cells (MCF-7/S and MCF-7/CP). Cells were cultivated in modified Dulbecco ISCOV ("Sigma", Germany) culture medium supplemented with 10% fetal calf serum ("Sangva", Ukraine) at 37 °C in 5% CO_2 atmosphere. Cells were reseeded twice a week at seeding density of 2–4x10^4 cells/cm^2 when the cells reached 50% of confluence.

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Abbreviations used: CP – cisplatin; NC – nanocomposite; SMF – static magnetic field.

Resistance to CP in MCF-7 cell line was developed by cultivation of MCF-7 cells in medium with rising concentrations of CP. Each two months cell resistance was analyzed by MTT-test. Index of resistance to CP in MCF-7/CP cells was 4. CP IC_{50} for MCF-7/S and MCF-7/CP cells were equal to 3 and 6 μg/ml respectively.

NC of magnetic fluid with CP were prepared and kindly provided for the study by the scientists of the A.A. Chuyko Institute of Surface Chemistry, NAS of Ukraine.

SMF (150 mT) was generated by two parallel magnets. Induction of SMF was measured at the center of the system.

Study design. CP in conventional pharmaceutical form ("Ebewe", Austria) and NC were added into culture medium for 24 h. 150 mT SMF was applied for 1 h. The contents of iron and CP in NC were 3 mg/ml, and 350 μg/ml respectively. The doses for MTT test were calculated based on CP content in the range of 0.5–7.5 μg/ml. For further experiments the IC_{30} NC concentration of 1.5 μg/ml was chosen for MCF-7/S cells and 3.0 μg/ml for MCF-7/CP. At these doses the drug did not cause the increase of the number of dead cells. Nevertheless, the alterations in cell ultrastructure due to their toxic effects are evident.

Determination of cytotoxic activity. Cytotoxicity of CP and NC after 24-h exposure on MCF-7/S and MCF-7/CP cells was estimated by standard MTT-test with 3-[4,5-dimethylthiazol-2–1]-2,5-diphenyltetrasolium bromide ("Sigma", Germany).

The study of accumulation of NC by MCF-7 cells. NC accumulation in MCF-7/S and MCF/CP human breast cancer cells was studied by identification of magnetic fluid nanoparticles by modified Lilli method [17].

Electron microscopy. Cells were fixed in 1.6% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.3) for 1 h, and then washed in 0.1 M cacodylate buffer for 16–18 h. To achieve optimal isotonicity of fixing and washing buffers, cacodylate buffer was supplemented with saccharose (50 mg/ml). Postfixation of the cells was done in 2% osmium tetroxide, with following dehydration in alcohols, and the preparations were embedded in epoxide by standard technique. Ultrathin slides prepared on LKB-8800 ultratome were contrasted with uranyl acetate and plumbous citrate and studied in electron microscope JEM-100B ("JEOL", Japan) at accelerated voltage of 80 kV.

DNA Comet Assay. DNA comet assay was performed according to the classic alkaline single-cell electrophoresis protocol [18]. Samples were stained with SYBR Green I ("Sigma", Germany) and analyzed by CometScore 1.5 software. Percent of DNA in comet tails was considered as the marker of genotoxic effect.

Micronucleus test. Micronucleus test (MN-test) was performed according to Hayashi et al. [19] with staining by 3% acridine orange. We counted number of MN per 1000 cells using luminescent microscopy.

Statistical analysis. Statistical analysis was performed using STATISTICA 6.0 software. Differences between obtained values were studied using Student's t-test. Differences were considered significant in case of $p < 0,05$.

RESULTS AND DISCUSSION

Cytotoxicity of CP and NC on MCF-7 cells. We have found that NC demonstrated higher cytotoxic effect than that of the conventional form of CP (Fig. 1). IC_{50} of conventional form of CP for MCF-7/S cells was 3 μg/ml, while IC_{50} of NC was 2 μg/ml.

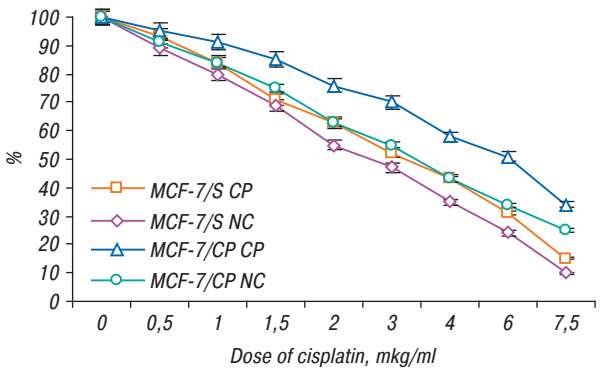

Fig. 1. Cytotoxic influence of CP and NC on MCF-7/S and MCF-7/CP cells

Analysis of NC accumulation in MCF-7 human breast cancer cells by visualization of nanoparticles using modified Lilli method showed several differences between sensitive and resistant cell lines. Total number of MCF-7/CP cells with NC nanoparticles and average number of nanoparticles per cell, their density and dimensions were greater compared to those in MCF-7/S cells. Also we found that resistant MCF-7 cells, which over-accumulated NC, were characterized by much higher NC activity: generation of "therapeutic giants" and cell death due to necrobiosis (Fig. 2).

We found several features of cytotoxic action of NC on ultrastructural organization of MCF-7/S and MCF-7/CP cells. MCF-7/S cells showed dystrophic changes, necrobiosis and cell death due to necrosis. Myelin figures and big comlex lysosomes with cell detritus and NC particles appeared in cytoplasm.

Also, sensitive cells after NC treatment showed nuclear membrane invaginations and loss of nucleoli. Cell cytoplasm was characterized by rough endoplasmatic reticulum (ER) enlargement, presented by round channels and cavities situated close to mitochondria, appearance of multilocus Golgi apparatus and elevation of number of phagosome with NC nanoparticles (Fig. 3).

Localization of NC nanoparticles in nucleus caused elevation of euchromatin and reduction of heterochromatin fractions.

These changes might be a result of small size of NC particles what allowed them to penetrate not only to cytoplasm but also into cell nuclei. After penetration into cells by endocytosis, nanoparticles concentrate in cytoplasm in endocytosis vesicles adjacent to lysosomes. Cells ultrastructure complication helps further internalization of nanoparticles into cells.

It should be mentioned that combined action of NC and SMF caused elevation of MCF-7/S dead cells

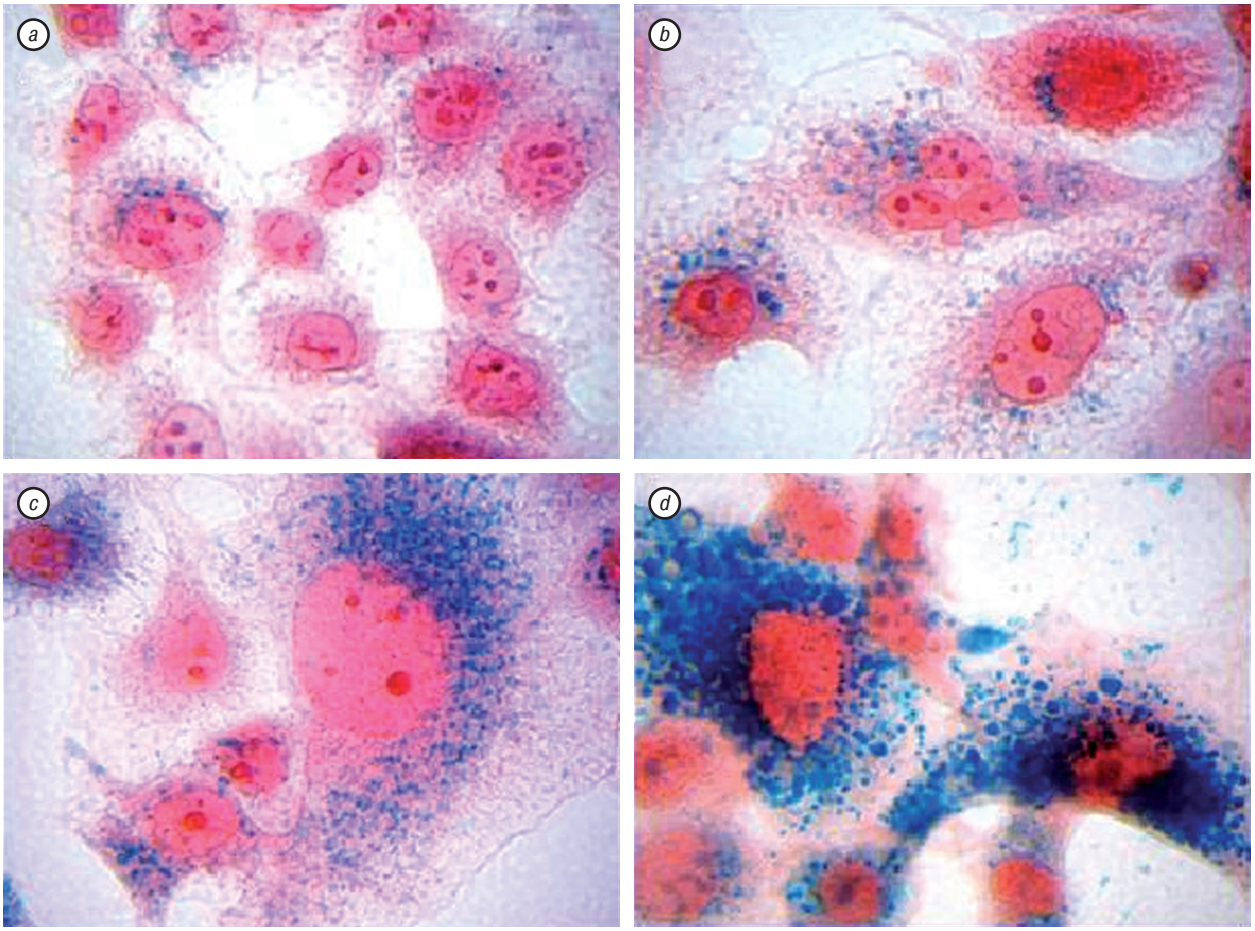

Fig. 2. Histochemical visualization of NC particles in MCF-7 cells by modified Lilli method, x100, oil immersion. *a* — Several NC nanoparticles found in MCF-7/S cells cytoplasm. *b* — MCF-7/S cells with separate and enlarged granules of NC particles. *c* — MCF-7/CP cells with big number of NC nanogranules, "therapeutic giant" with many small dense granules. d — MCF-7/CP cells in necrobiotic death due to overaccumulation of large dense granules of NC

number pointing on amplifying effect of SMF. While most part of cells died due to necrosis, we found cells with ultrastuctural features of apoptosis and apoptotic bodies.

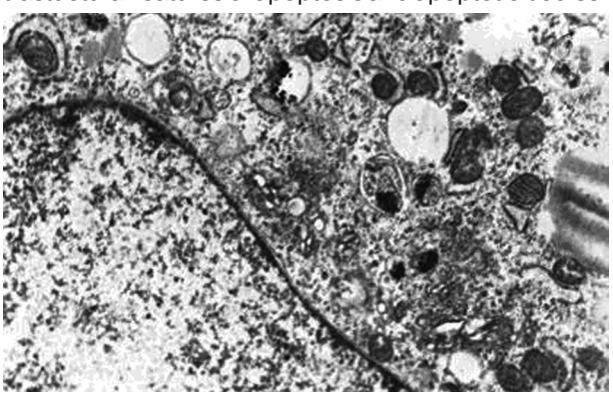

Fig. 3. Changes in MCF-7/S cells after NC treatment. Enlarged rough ER is presented by round channels and cavities situated close to mitochondria. Electron microscopy. X 10 000

MCF-7/CP cells showed more significant changes in their ultrastructure compared to sensitive ones. We mentioned that nuclei of these cells were electron-transparent as a result of significant qualitative and quantitative changes of chromatin structure with loss of heterochromatin and elevation of euchromatin regions (Fig. 4).

Significant changes in nuclei of resistant cells might be a result of NC accumulation, which could be a target for SMF that caused chromatin disorganisation and, further, cell death. We also found significant changes in cytoplasm ultrastructure of resistant cells: multiple vacuolization of cytoplasm on cell organelles, especially mitochondria (Fig. 5).

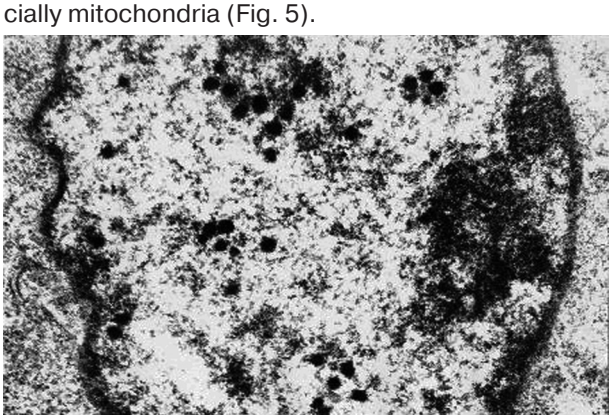

Fig. 4. Changes in nuclear chromatin ultrastructure in MCF-7/CP cells with high accumulation of NC particles. Electron microscopy. X 15 000

SMF enforced cytotoxic effects in MCF-7/CP cells compared to sensitive ones because of elevated accumulation of NC nanoparticles in cells. This caused elevation of number of dystrophically changed and dying cells. 34% of cells were dead after combined action of NC and SMF, and the most part of them (almost 25%) were dead due to apoptosis (Fig. 6).

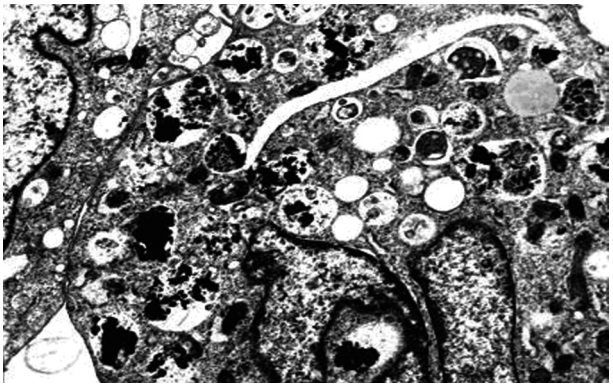

Fig. 5. Significant vacuole-hydropic dystrophy in the cytoplasm of MCF-7/CP cells after combined effect of NC particles accumulation and SMF. Electron microscopy. X 10 000

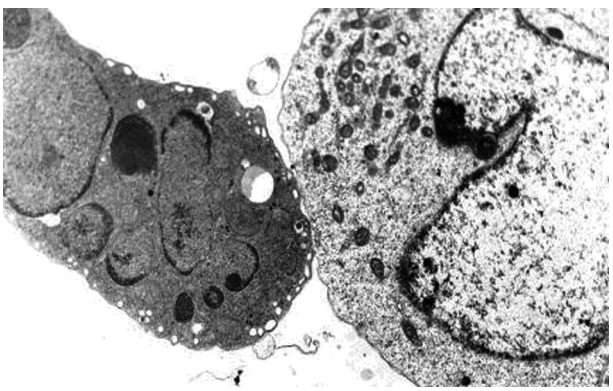

Fig. 6. Death of MCF-7/CP cell due to apoptosis as a result of NC and SMF combined effect. Electron microscopy. X 13 300

Studies of genotoxic effect of NC and its combined action with SMF was performed by MN-test and DNA comet assay (Fig. 7).

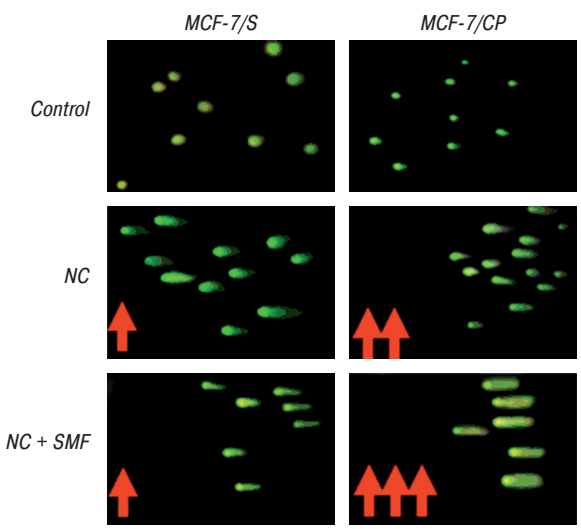

Fig. 7. Genotoxic effect of NC and its combination with SMF against MCF-7/S and MCF-7/Dox cells. DNA comet assay

Significant elevation of tested parameters (number of micronuclei, % of DNA in comet tails) in MCF-7/S cells after NC and NC+SMF treatment compared to control was observed (Table).

Studies of genotoxic effects of NC and its combined action with SMF on MCF-7/CP cells showed that number of MN and percent of DNA in comet tails after both agents were higher compared to MCF-7/S corresponding values (Table). It should be mentioned that control values for both parameters in resistant cells were significantly higher compared to those for MCF-7/S cells. So, presence of NC in cultivation medium caused significant elevation of genotoxic effects not only in sensitive but also in resistant to CP MCF-7 cells. Combination with SMF slightly increased the observed effects.

Table. Percent of DNA in comet tails after impact of studied factors

Cell line	Control		NC		NC+SMF	
	MN, ‰	of DNA in comet tails, %	MN, ‰	of DNA in comet tails, %	MN, ‰	of DNA in comet tails, %
MCF-7/S	4.8±0.2	3.2±0.2	19.2±1.4*	5.7±0.6*	21.2±1.2*	6.8±0.8*
MCF-7/CP	10.8±1.1**	3.0±0.1	25.2±1.3**	7.9±0.5**	27.2±1.4**	10.1±0.6**

Notes: *significantly higher ($p<0.05$) compared to control; **significantly higher compared to control and MCF-7/S cells with corresponding impact.

The obtained data show that NC incorporation into MCF-7/CP cells compared to MCF-7/S cells is much more active. It is proved by results of histochemical staining of magnetic fluid nanoparticles which were the part of NC and microscopy studies of nanoparticles in MCF-7 cells. NC had higher cytotoxic effect compared to CP against both MCF-7/S and MCF-7/CP cells. IC_{50} for CP against MCF-7/S cells is 3,5 μg/ml, while IC_{50} for NC was 2,5 μg/ml.

Cytomorphology and electron microscopy of resistant cells with high levels of accumulated NC showed its higher cytotoxic effects in particular dystrophy, necrobiosis, necrosis and apoptosis. External SMF caused elevation of NC cytotoxicity especially against MCF-7/CP cells. NC and its combination with SMF caused significant genotoxic effects both in MCF-7/S and especially in MCF-7/CP cells.

REFERENCES

1. Chekhun VF. Nanotechnologies in oncology: perspectives of development and unforseen difficulties. Lancet Oncol (Ukr Ed) 2010; **4**: 2–3 (In Russian).

2. Chekhun VF. Development of new medicinal forms on the basis of nanocomposite materials for solvation of modern problems in oncology. Collected scientific works "Nanosystems, nanomaterials, nanotechnologies" 2011; **9**: 261–74 (In Russian).

3. Chekhun VF, Sukhodub LF, Movchan BO, ***et al.*** Development of standartization methods for nanocomposite materials on the basis of magnetite for oncology. Collected scientific works "Nanosystems, nanomaterials, nanotechnologies" 2011; **9**: 247–59 (In Ukrainian).

4. Widder KJ, Sinyei AE, Scarpelli GD. Magnetic microspheres: a model system of site specific drug delivery *in vivo*. Proc Soc Exp Biol Med 1978; **158**: 141–6.

5. Sinyei A, Widder K, Czerlinski C. Magnetic guidance of drug carring microspheres. J Appl Phys 1978; **49**: 3578–83.

6. Gusev AI, Rempel AA. Nanocristal materials. M.: Fismatlit 2001; 224 p (In Russian).

7. Gubin SP, Koksharov YuA, Khomutov GB, ***et al.*** Magnetic nanoparticles: methods of production, structure and properties. Uspekhi Khimii 2005; **74**: 539–70 (In Russian).

8. Chekhun VF, Todor IN, Lukyanova NYu, ***et al.*** The use of nanoferromagnetics to increase the cytotoxic effect of antitumor drugs. Exp oncol 2009; **31**: 163–7.

9. Naleskina LA, Boroday NV, Chekhun VF. Present state and perspectives of development of nanosystems for directed delivery of medicinal preparations to tumor cells. Onkologiya 2009; **11**: 166–73 (In Ukrainian).

10. Lubbe A, Bergemann C, Riess H, ***et al.*** Clinical experiences with magnetic drug targeting: a phase I study with

4'-epidoxorubicin in 14 patients with advanced solid tumors. Cancer Res 1996; **56**: 4686–96.

11. Matsumura Y, Kataoka K. Preclinical and clinical studies of anticancer agent-incorporating polymer micelles. Cancer Sci 2009; **100**: 572–9.

12. Lahaye T, Koch T, Fröhlich B. Strong dipolar effects in a quantum ferrofluid. Nature 2007; **448**: 672–5.

13. Rabias M, Fardis M, Devlin E, ***et al.*** No aging phenomena in ferrofluids: the influence of coating on interparticle interactions of maghemite nanoparticles. ACS Nano 2008; **2**: 977–82.

14. Rabias I, Pratsinis H, Drossopoulou G, ***et al.*** *In vitro* studies on ultrasmall superparamagnetic iron oxide nanoparticles coated with gummic acid for T2 MRI contrast agent. Biomicrofluidics 2007; **1**: 7–12.

15. Foy SP, Manthe RL, Foy ST, ***et al.*** Optical imaging and magnetic field targeting of magnetic nanoparticles in tumors. ACS Nano 2010; **4**: 5217–24.

16. Gorbik PP, Abramenko NV, Petronovskaya AL, ***et al***. Synthesis and properties of magnetic fluid on the basis of nanosized Fe3O4. Collected scientific works "Surface" 2011; **3**: 287–97 (In Russian).

17. Naleskina LA, Lukyanova NYu, Kunskaya LN, ***et al***. Visualization of the features of the distribution and accumulation of iron nanoparticles in human breast cancer cells sensitive and resistant to antitumor drugs after cultivation with liposomal ferromagnetic during various time intervals. Cytology Genetics 2011; **45**: 395–9.

18. Olive PL, Wlodek D, Banath JP. DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. Cancer Res 1991; **51**: 4671–6.

19. Hayashi M, Hashimoto S, Sakamoto Y, ***et al***. Statistical analysis of data in mutagenicity assays: rodent micronucleus assay. Environ Health Perspect 1994; **102**: 42–52.