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In vitro and *in vivo* Anticancer Activity of Nanosize Zinc Oxide Composites of Doxorubicin

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***Abstract*—**The nanotechnology offers some exciting possibilities in cancer treatment, including the possibility of destroying tumors with minimal damage to healthy tissue and organs by targeted drug delivery systems. Considerable achievements in investigations aimed at the use of ZnO nanoparticles and nanocontainers in diagnostics and antitumor therapy were described. However, there are substantial obstacles to the purposes to be achieved by the use of zinc oxide nanosize materials in antitumor therapy. Among the serious problems are the techniques of obtaining ZnO nanosize materials. The article presents a new vector delivery system for the known antitumor drug, doxorubicin in the form of polymeric (PEO, starch-NaCMC) hydrogels, in which nanosize ZnO film of a certain thickness are deposited directly on the drug surface on glass substrate by DC-magnetron sputtering of a zinc target. Anticancer activity *in vitro* and *in vivo* of those nanosize zinc oxide composites is shown.

***Keywords*—**Anticancer activity, cancer specificity, doxorubicin, zinc oxide.

I. Introduction

THE current focus in the development of cancer therapies is targeted drug delivery to provide therapeutic concentrations of anticancer agents at the site of action, reduce off-target organ toxicities, and facilitate cellular uptake of therapeutics. The experience in technologies such as nanotechnology, advanced polymer chemistry, electronic engineering, and advances in our knowledge of molecular biology of cancer are being brought together in developing novel methods of drug delivery [1]–[4].

Numerous different polymer compositions have been synthesized and studied for using in cancer therapy. One of the simplest polymer is poly(ethylene glycol) (PEG) or polyethylene oxide (PEO) [5]. Its hydrophilic property minimizes the opsonization and prolongs the circulation half-life [6]. Another one of the most promising natural polymers is a polysaccharide starch [7], [8]. Among the hydrophilicity profiles polysaccharides possess many recognition functions, allowing e.g. mucoadhesion or specific receptor recognition [9], as well as providing neutral coatings with low surface energy, preventing non-specific protein adsorption [10]. However first and foremost, the major advantage of using PEO and starch as polymers in cancer therapy is a biocompatibility and biodegradability of those compounds [11]–[13].

Considerable achievements in the investigations aimed at the use of ZnO nanoparticles and nanocontainers in diagnostics and antitumor therapy have been achieved [14]–[16]. However, there are substantial obstacles to the purposes to be achieved by the use of nanosize zinc oxide materials in antitumor therapy. Among the serious problems are the techniques of obtaining nanosize ZnO materials that are very important in view of providing special purity of medical preparations, occurrence of impurities in them, as well as long-term stability and reproducibility of physical and chemical characteristics of the nanomaterials. In general, non-reproducibility of physical and chemical characteristics of the nanosize materials results in non-reproducibility of their biological activity [17]. Earlier we presented a new drug delivery system based on the thin film technology for obtaining zinc oxide composite drugs alternative to the traditional nanotubes, nanoparticles. The method of obtaining nanosize zinc oxide composites by magnetron deposition of nanosize ZnO film on the anticancer drugs surfaces at relatively low temperatures holds a unique position [18], [19].

The article presents a new vector delivery system for the known antitumor agent, doxorubicin (Dox), in which nanosize ZnO film of a certain thickness is deposited directly on the drug surface by DC-magnetron sputtering of a zinc target. The purpose of the presented work was the study of anticancer activity *in vitro* and *in vivo* of those zinc oxide composites in the form of polymeric (PEO, starch+NaCMC) hydrogels.

II. Materials and Methods

A. Nanosize Zinc Oxide Composites

Commercial doxorubicin preparation ("Belmedpreparaty" Company, Belorussia) in the form of lyophilized powder containing Dox and mannitol in the ratio of 1:4 was used.

The glass substrate with Dox circular coating of a certain diameter (1.81cm, 0.3mg Dox for 5 mice at a dose 0.06 mg/mouse), obtained by drying in air Dox paste with DMSO was placed in a modernized UVN-71P3 device, a DC magnetron for ion-plasma sputtering of metal (zinc) targets. The device was equipped with a system of measurement and control of working gas flow (Ar + O_2) consisting of PR4000F power-supply and indication unit and two MFC 1179 gas flow regulators.

Technological parameters of deposition of nanosize ZnO films (time, current, target-to-substrate distance) were selected

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so as to deposit 180nm thick ZnO film on Dox surface. After deposition of ZnO Dox+ZnO composites were dissolved in physiological (saline) solution, containing 0.3 weight % of Starch+NaCMC (sodium carboxymethyl cellulose) or PEO.

B. Cell Lines and Cell Culture

The human cell lines used were HeLa (cervical carcinoma cells) and MRC5 (normal lung fibroblast cells) kindly provided by Prof. J. Masters (Institute of Urology and Nephrology, UCL, UK), and KCL-22 (chronic myeloid leukemia in blast crisis) generously provided by Dr. T. Liehr (Institute of Human Genetics and Anthropology, Germany). The cells were routinely maintained in the growth media DMEM (cell lines HeLa and MRC5, Sigma Aldrich, Germany) or RPMI-1640 (cell line KCL-22, Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (HyClone, UK), 2 mM L-glutamine (Sigma Aldrich, Germany), 100 IU/mL penicillin (Sigma Aldrich, Germany) and 100 µg/mL streptomycin (Sigma Aldrich, Germany) at 37^0C.

C. Estimation of Cytotoxicity and Cancer Specificity

MTT assay: the cytotoxicity of test compounds was analyzed against human cancer (HeLa) and normal (MRC5) cell lines using MTT colorimetric cell viability assay [20]. The cells were seeded at the density of 0.2-0.25x10^6 cell/mL into 96-well plates (Greiner, Germany, 125µl per well), incubated for 48 h, and then Dox and test compounds dissolved in distilled water were added to the cell cultures (12.5µl per well) at various concentrations of the test chemicals by diluting the stock solution with a constant factor covering a large range. The initial dilution series were log dilutions (e.g., 1:10, 1:100, 1:1000, etc.). After further incubation for 48 h, the sample solution in wells was flicked off and 50µl of MTT dye (Sigma Aldrich, Germany) was added to each well (0.5 mg/mL final concentration). The plates were gently shaken and incubated for 4 hours at 37°C. The supernatant was removed, 100 µl of DMSO (Sigma Aldrich, Germany) was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using ELISA plate reader (Human Reader HS, Germany) at a wavelength of 570nm.

Cell viability was expressed as a percentage of the negative control (cell cultures with no treatment). Doses inducing 50% inhibition of cell viability (the IC_{50} value) were determined and compared to reveal the cancer specificity of the test chemicals.

Trypan blue exclusion test: The IC_{50} value of selected compound was estimated (as it was mentioned above) for further cell cycle analysis in KCL22 cell line using vital dye (Trypan blue; Sigma-Aldrich, Germany) exclusion test [21]. Cells were seeded at a density of 0.5×10^6/mL into 15mL glass vials (1-2mL of cell suspension per vial), incubated for 48h, and then a test chemical dissolved in distilled water was added to the cell cultures at various concentrations as it was mentioned above. After further incubation for 48h, cells were stained with 0.4% Trypan blue solution for 5-15min and counted in a haemocytometer under a light microscope. The viable cell number was determined.

Selectivity index (SI): As an anticancer activity index the degree of selectivity of the synthetic compounds was estimated using following formula: SI = IC_{50} of the compound in a normal cell line / IC_{50} of the same compound in cancer cell line [22].

D. Flow Cytometric Analysis of Cell Cycle

Cultures of the KCL-22 cell line 48h after seeding were incubated with test compound at the concentrations IC_{50} and IC_{60} for 48h. About 10^6 cells were collected by centrifugation and treated on ice with 1mL cold 70% ethanol added drop wise on a vortex to prevent cell aggregation. Then cells were fixed in ethanol at 4ºC overnight and stored at -20°C for few days (up to a week) until the analysis performance. For the cell cycle analysis the cells were carefully washed twice with PBS and treated with PI-staining buffer containing 0.1 mg/mL RNase (Sigma-Aldrich, Germany) and 0.05 mg/mL propidium iodide (PI) (Fluka, Switzerland) for 30min. The DNA content was determined using a FACS Calibur flow cytometer (Beckton & Dickinson, San Jose). Cell doublets and aggregates were excluded based on forward and side scatter parameters. Gated events considered to be single particles were analyzed with FlowJo 7.6 software cell cycle analysis module using Dean-Jett-Fox model, and were presented as the number of cells versus the amount of DNA as it indicated by the intensity of fluorescence [23].

E. Estimation of Anticancer Activity in vivo

The study was carried out in toxicology and chemotherapy laboratory of Scientific Technological Centre of Organic and Pharmaceutical Chemistry of the National Academy of Sciences of the Republic of Armenia under conditions close to GLP principles and European standards. 8-10 animals (white nondescript mice, 6-7 weeks old with an average weight of 20 to 25g) were used for each experimental group. Ehrlich's ascitic carcinoma was inoculated intraperitoneally (1x10^6 tumor cells per animal). Tested compounds were introduced to the animals intraperitoneally in the gel form 24 hours after the tumor subinoculation. In contrast to therapeutic doses (5 mg/kg) the study was carried out with low doxorubicin doses (0.3 mg/kg) in the zinc oxide composites taking into account vector character of the doxorubicin zinc oxide composite transport to tumor tissue. The life span increase (IL) was estimated as an anticancer activity index after the test compounds' exposure at these doses.

F. Statistical Analysis

All experiments were repeated at least three times. At least quadruplicate cultures were scored for an experimental point. All values were expressed as means ± S.E.M. The Student's one tail t-test was applied for statistical treatment of the results; $p < 0.05$ were considered as the statistically significant value.

III. Results and Discussion

A. The Cytotoxicity and Cancer Specificity in vitro of Tested Compound

Table I presents the results of cytotoxic activity study of the tested compounds. It is seen from the table that the concentration range of cytotoxicity of Starch+NaCMC+Dox for tumor (IC_{50} = 7 ± 2.4 μg/mL) overlap with that for normal cells (IC_{50} = 5 ± 1.2 μg/mL), and the difference between IC_{50} values is not statistically significant. Since Starch + NaCMC is a biocompatible polymer mixture and doesn't show any toxic activity *in vitro* throughout the investigated concentration range, it can be assumed that a high degree of cytotoxicity of Starch+NaCMC+Dox is due to the presence of Dox in the compound. However Starch+NaCMC+Dox doesn't provide the desired selectivity at the cellular level since the same dose-dependent death of normal and tumor cells has been observed (Fig. 1 (a)).

After ZnO deposition the cytotoxic activity of Starch+ NaCMC+Dox+ZnO against tumor cells was increased 1.5 times (IC_{50} = 5 ± 0.5 μg/mL), whereas the selective resistance was shown for normal cells (IC_{50}>>20 μg/mL) (Fig. 1 (b)). It should be noted that free Dox also demonstrated *in vitro* selectivity for the examined tumor cells, and the profile of normal cell survival was similar to that of Starch + NaCMC + Dox + ZnO, except for the highest used concentration (20 μg/mL) at which, in the case of Starch+NaCMC+Dox+ZnO 85% survival of normal cells was observed, while free Dox resulted in 80% of cell death (Fig. 1 (b)).

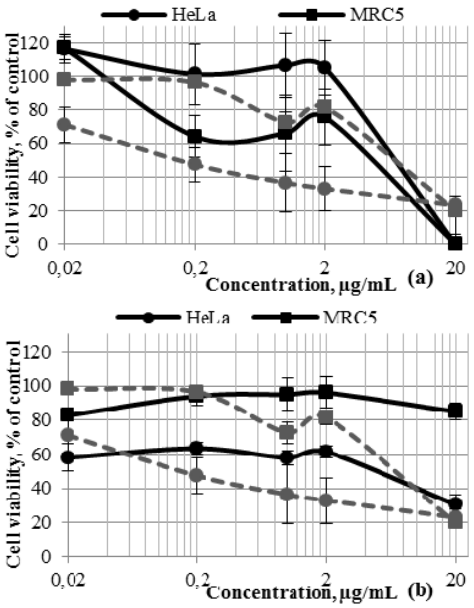

Fig. 1 The cytotoxicity of Starch+NaCMC+Dox (a) and Starch+ NaCMC+Dox+ZnO (b) in HeLa (human cancer cells) and MRC5 (human normal cells) cell lines. The dotted lines represent the cytotoxicity of free DOX in the same cell lines

In the case of PEO(gel)+Dox, IC_{50} value was found neither for tumor nor for normal cells even at the highest concentration tested (20 μg/mL), although there was dose-dependent cell death to a similar extent (Fig. 2 (a)). The lack of tumor specificity of PEO(gel)+Dox in the investigated concentration range was confirmed by earlier studies, which showed that the PEO cell uptake is energy-dependent but not a receptor-mediated endocytosis process [24].

It was also shown that the level of cytotoxicity of PEO (gel) + Dox decreases compared with free Dox from 10 (normal cells) to 100 times (tumor cells) (Table I). This phenomenon has been described previously for other Dox conjugates using various linear and branched PEO polymers; however, it has been shown that this phenomenon is associated with the release rate of Dox [25].

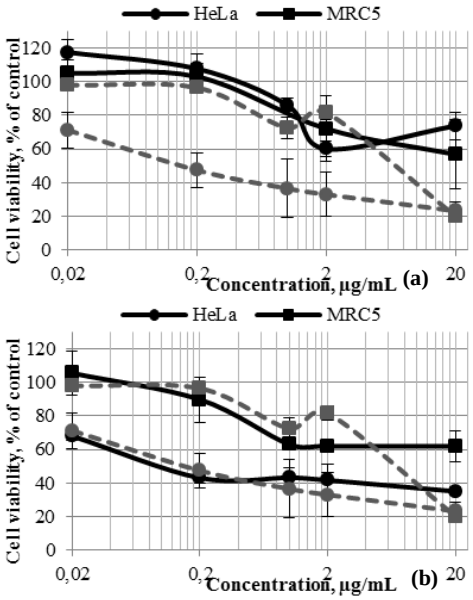

Fig. 2 The cytotoxicity of PEO(gel) +Dox (a) and PEO(gel)+Dox+ ZnO (b) in HeLa (human cancer cells) and MRC5 (human normal cells) cell lines. The dotted lines represent the cytotoxicity of free Dox in the same cell lines

As a result of ZnO deposition, the sharp increase in cytotoxic activity against tumor cells was shown for PEO (gel) +Dox+ZnO (IC_{50} = 0.09 ± 0.02 μg/mL). At minimum concentrations (0.02-0.2 μg/mL) of PEO(gel)+Dox+ZnO the tumor cell death was observed from 40% to 60%, while at the same concentrations of PEO(gel)+Dox cell survival remained at 100% (Figs. 2 (a), (b)). In comparison with free Dox, the cytotoxic activity of PEO(gel)+Dox+ZnO against tumor cells increased more than 2 times. At the same time no change was observed in sensitivity of normal cells with respect to PEO(gel)+Dox+ZnO compared to PEO(gel)+Dox. The 60% survival of normal cells at the maximum tested concentration (20 μg/mL) was shown in both cases (Figs. 2 (a), (b)), while free Dox at the same concentration resulted in 80% death of normal cells.

The result of calculating the selectivity index (SI) showed that the addition of ZnO to Starch+NaCMC+Dox and PEO (gel)+Dox systems provided tumor specificity of the

compounds at the cellular level. Comparative analysis showed that PEO(gel)+Dox+ZnO (SI >> 200) has a higher selective cytotoxic activity against tumor cells (more than 4 times) than Starch+ NaCMC+Dox+ZnO (SI >> 4) and free DOX (SI = 56).

TABLE I
THE CYTOTOXICITY (IC_{50} VALUE) AND SI (SELECTIVITY INDEX) OF TESTED COMPOUNDS IN HELA (HUMAN CANCER CELLS) AND MRC5 (HUMAN NORMAL CELLS) CELL LINES

Tested compound	IC_{50} (μg/mL) ± S.E.M.		SI
	HeLa	MRC5	
Starch + NaCMC + Dox	7 ± 2.4	5 ± 1.2	0.7^{ns}
Starch + NaCMC + Dox + ZnO	5 ± 0.5	>>20	>>4
PEO(gel) + Dox	>>20	>>20	0
PEO(gel) + Dox + ZnO	0.09 ± 0.02	>>20	>>200
DOX	0.2±0.02	11.2±0.56	56

[ns]Not statistically significant difference between two sample groups, p>0.05

B. Cell Cycle Delay Caused by PEO(gel)+Dox+ZnO

The results of flow cytometric analysis of KCL-22 cells treated with various concentrations of PEO(gel)+Dox+ZnO are shown in Fig. 3. The first large peak in each plot represents the cells in G0/G1-phase (DNA content 2c) of the cell cycle; the following plateau (between 2c and 4c) represents the S-phase cells. The second peak shows the cells in G2/M-phase (4c). The number of events forming the S-plateau is higher in treated cultures in comparison with the untreated control; the G1 peaks reduce and the G2 peaks are not changed.

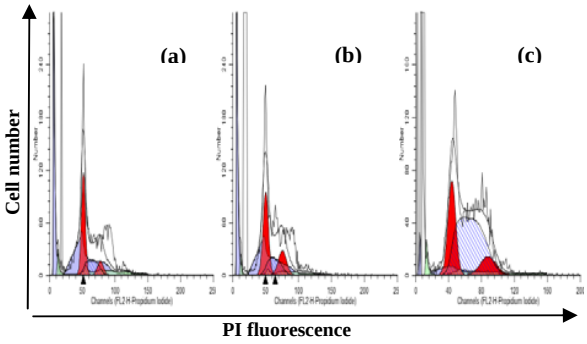

Fig. 3 DNA content-frequency histogram of KCL-22 cells untreated (a) and treated with IC_{50} (b) and IC_{60} (c) concentrations of PEO(gel) + Dox + ZnO

Analysis of cell distribution in the cell cycle (Table II) also shows that the treatment with PEO(gel)+Dox+ZnO significantly increases the S-phase cell number. The statistically significant reduction of cells in G1 phase is also evident in concentration of IC_{50} and IC_{60}. At the same time the number of cells in G2 phase is not changed. The results (Fig. 3 and Table II) suggest that treatment of KCL-22 cells with PEO(gel)+Dox+ZnO interferes with the cell cycle, inducing the arrest of a portion of cells at S phase.

TABLE II
CELL CYCLE DISTRIBUTION IN KCL-22 CELL LINE TREATED WITH PEO(GEL)+DOX+ZNO

Concentration of PEO(gel)+Dox+ZnO	G0/G1 (%)	S (%)	G2/M (%)
0 (control)	56 ± 1.3	31 ± 0.2	8 ± 1.4
IC_{50}	42 ± 1.3*	48 ± 0.6*	5 ± 1.4
IC_{60}	33 ± 1.3*	59 ± 0.6*	6 ± 1.4

*Statistically significant difference between two sample groups, p<0.05

These results are consistent with the cytotoxicity data of PEO(gel)+Dox+ZnO, where the overall efficacy of the cytotoxicity *in vitro* reaches a plateau and doesn't improve at high drug concentrations. This plateau is typical for drugs that kill in a cell cycle dependent manner or that requires entry in a specific phase of the cell cycle [26].

It is well known that free Dox binds to DNA by intercalation and this fact results in protein-concealed DNA strand breaks as well as cell cycle arrest, which causes apoptotic cell death pathway targeted in cancer therapy [27], [28]. Since cell cycle arrest is doxorubicin concentration and exposure time dependent with continuous exposure to high concentrations inducing delayed S phase transit [29], [30], higher S phase arrest by half dose of PEO(gel)+Dox+ZnO may serve the same purpose as done by a double amount of free Dox. Earlier it was shown that PEO is capable of penetrating cell membrane as well as nuclear membrane [24]. Thus, considerable lower IC_{50} value of PEO(gel)+Dox+ZnO estimated for cancer cells (Table I) and higher S phase arrest might be due to the enhanced cellular uptake causing inhibition of macromolecular DNA biosynthesis.

C. Antitumor Activity in vivo of the Tested Composites

0.3 mg/kg was used as a therapeutic dose for Starch+NaCMC+Dox+ZnO, PEO(gel)+Dox+ZnO and 5 mg/kg for free Dox (control test). The significant life span increase (IL) was evident in all exposure cases at therapeutic doses compared to control. The anticancer activity of Starch+Dox+ZnO (384%) was higher than that of PEO(gel)+Dox+ZnO (332.6%). The lowest IL was estimated for free Dox (165%).

The unique coincidence was revealed by comparison of *in vitro* (IC_{50}) and *in vivo* (LI) data of PEO(gel)+ Dox+ZnO, which was 2 times more active than free DOX in both cases. In contrast with *in vitro* data of cancer specificity the Starch+ NaCMC+Dox+ZnO demonstrated highest anticancer activity *in vivo*. So, the pharmacokinetic properties of Starch-based composites are better than those of PEO(gel)-based, since the only variable in tested composites is the polymer. The lack of correlation between *in vitro* and *in vivo* studies is a common problem during the risk assessment of new compounds. There are a lot of studies that addressed the issue of the *in vivo* relevance of *in vitro* assays for evaluating the nanocomposites activity [31], [32]. All of them found poor correlation and were directed to choose the rationale endpoints for each of them [33], [34].

It can be concluded that the doxorubicin based polymeric nanosize ZnO composites PEO(gel)+Dox+ZnO and Starch+ NaCMC+Dox+ZnO have excellent physicochemical

properties for drug delivery. It is obvious that only the sufficient size, surface properties, drug release kinetics (and other key physicochemical characteristics) of tested compounds could allow to avoid the composites uptake by mononuclear phagocytic system (MPS), rapid renal excretion, hepatic clearance, *etc.* and increase the circulation half-life, which insure drug delivery into the tumor tissue revealed for PEO(gel)+Dox+ZnO and Starch+NaCMC+Dox+ZnO [35].

IV. Conclusions

Novel nanosize zinc oxide composites of doxorubicin obtained by deposition of 180 nm thick zinc oxide film on the drug surface using DC-magnetron sputtering of a zinc target in the form of gels (PEO+Dox+ZnO and Starch+NaCMC+Dox+ZnO) were studied for drug delivery applications. The cancer specificity was revealed both *in vitro* and *in vivo* models. It was shown *in vitro* that the zinc oxide nanosize film deposition on the drug surface led to the selective anticancer activity of composites at the cellular level with the selectivity index from 4 (Starch+NaCMC+Dox+ZnO) to 200 (PEO(gel)+Dox+ZnO). The significant increase in antitumor activity (by a factor of 2-2.5) and decrease of general toxicity of zinc oxide compositions of doxorubicin on the model of Ehrlich's ascitic carcinoma were shown compared to free doxorubicin. Mechanistic studies of anticancer activity revealed the cytostatic effect based on the high level of DNA biosynthesis inhibition at considerable low concentrations of zinc oxide compositions of doxorubicin. The results of studies of PEO(gel)+Dox+ZnO and Starch+NaCMC+Dox+ZnO composites' *in vitro* and *in vivo* behavior confirmed the high potential of nanosize zinc oxide composites obtained by DC-magnetron deposition as a vector delivery system for future applications in cancer chemotherapy.

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